The role of interleukin-6 classical and trans-signaling in allogeneic stem cell transplantation

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Thesis for the degree of Philosophiae Doctor (PhD)
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1. SCIENTIFIC ENVIRONMENT

The project was carried out at the Section for Hematology, Department of Medicine, Haukeland University Hospital and the Leukemia Research Group at the Department of Clinical Science, the University of Bergen. The main supervisor was Øystein Bruserud, with Annette Brenner as co-supervisor. Collaborating partners were Tobias Gedde-Dahl at Rikshospitalet, Oslo University Hospital, who provided access to clinical data and patient material, and Professor Stephan Rose-John at Christian-Albrechts-Universität in Kiel, who provided Hyper-IL-6. The PhD fellowship was financed by Helse-Vest and also received financial support for running costs from The Norwegian cancer society, the Family Blix Foundation and Øyvind Mølbach Petersens Foundation.

2. ACKNOWLEDGEMENTS

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Tor Henrik Anderson Tvedt
Bergen, September 2019

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4. ABBREVIATIONS.

ADAM	A disintegrin and metalloproteinase
aGVHD	Acute graft-versus-host disease
AKT	Protein kinase B
ALL	Acute lymphoblastic leukemia
AML	Acute myeloid leukemia
AP-1	Activator protein 1
APC	Antigen-presenting cells
ASCT	Allogeneic stem cell transplantation
ATG	Antithymocyte globulin
C/EBP-α	CCAAT/enhancer-binding protein alpha
CD126	Membrane-bound IL-6 receptor
cGVHD	Chronic graft-versus-host disease
CLC	Cardiotrophin-like cytokine
c-MAF	Musculoaponeurotic fibrosarcoma oncogene homolog
CNTF	Ciliary neurotrophic factor
CRP	C-reactive protein
CT-1	Cardiotrophin 1
DAMPS	Danger-associated molecular patterns
DLA	Dog leukocyte antigen
EBMT	European Group for Blood and Marrow Transplantation
ERK	Extracellular Receptor Kinase
Fas	Apoptosis antigen 1
G-CSF	Granulocyte colony-stimulating factor
GMP	Guanosine monophosphate
gp130	Glycoprotein 130
GVL	Graft-versus-leukemia
GWAS	Genome-wide association study
HLA	Human leukocyte antigen
HR	Hazard ratio
ICAM	Intercellular adhesion molecule
IL	Interleukin
IL-6	Interleukin-6
IPS	Idiopathic pneumonia syndrome
JAK	Janus kinase
LIF	Leukemia inhibitory factor
LIFr	Leukemia inhibitory factor receptor

MAC	Myeloablative conditioning
MAdCAM-1	Mucosal addressin cell adhesion molecule
MAP	Mitogen-activated protein
MDS	Nucleotide-binding oligomerization domain-like receptors
mIL-6R	Membrane-bound IL-6 receptor
MHC	Major histocompatibility complex
mi-RNA	microRNA
MKP-1	MAP kinase phosphatase 1
MMF	Mycophenolate mofetil
MPN	Myeloproliferative neoplasms
mTOR	mechanistic target of rapamycin
MTX	Methotrexate
MUD	Matched unrelated donor
NFAT	Nuclear factor of activated T cells
NF-κb	Nuclear factor kappa b
NOD-Like	Nucleotide-binding oligomerization domain-like receptors
NRM	Nonrelapse mortality
OSM	Oncostatin M
OSMR	Oncostatin M receptor
PBMC	Peripheral blood mononuclear cell
PBSC	Peripheral blood stem cells
PI3K	Phosphoinositide 3-kinase
RIC	Reduced-intensity conditioning
RORγT	Retinoic-acid-receptor-related orphan nuclear receptor gamma
sIL-6R	Soluble IL-6 receptor
SHP-2	Src homology region 2 domain-containing phosphatase-2
SHR	Subdistribution hazard ratio
SNP	Single nucleotide polymorphism
SOCS	suppressor of cytokine signaling
SR-aGVHD	Steroid-refractory aGVHD
STAT	Signal transducer and activator of transcription
TBI	Total body irradiation
TCR	T-cell receptor
TGF-β	Transforming growth factor beta
Th-cell	T-helper cell
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor alpha
Treg	Regulatory T cells

TYK	Tyrosine-protein kinase
TRM	Treatment-related mortality
UCB	Umbilical cord blood
VCAM	Vascular cell adhesion molecule

5. ABSTRACT

IL-6 family cytokines share structural similarities and utilize glycoprotein 130 (gp130) for signal transduction. IL-6 itself has both pro- and anti-inflammatory effects. IL-6 trans-signaling is mediated by the soluble IL-6 receptor (IL-6R) and is responsible for most of its proinflammatory effects, while the anti-inflammatory classical IL-6 signaling is mediated by membrane-bound IL-6R. Availability of soluble IL-6R regulates the balance between classical and trans-signaling. Dysregulation of this balance has been implicated in immune-mediated diseases, including graft-versus-host disease (GVHD) that occurs after allogeneic stem cell transplantation (ASCT) and can results in disabling and life-threating complications. The pathogenesis of GVHD is very complex, and IL-6 seems to contribute to this process. However, the role of classical and IL-6 trans-signaling in GVHD has not been investigated previously in either clinical studies or animal models. The aim of the thesis was, therefore, to investigate whether various forms of IL-6 signaling and various IL-6 family members influence outcomes after ASCT.

In the first study, we investigated effects of serum levels of IL-6 family cytokines on outcomes after ASCT in a population of 100 consecutive allotransplant recipients. C-reactive protein (CRP) levels partly reflect IL-6-trans-signaling. We show that pretransplant CRP and IL-6 levels showed significant correlation for allotransplant recipients, but only CRP levels were significantly associated with treatment-related mortality (TRM) in multivariate analyses. Of the other IL-6 family cytokines, only for high IL-31 could a significant association with clinical outcome (increased TRM) be observed.

In the second study we investigated how genetic variations in the IL-6R genes of donors and recipients influenced pretransplant level of IL-6 family cytokines, pretransplant CRP levels and posttransplant outcome. Ten single nucleotide polymorphisms (SNPs) with and without known association to immune-mediated diseases/biological effects were selected. Homozygosity for the major alleles of the IL-6R SNPs rs2228145 and rs4845618 was associated with high pre- and posttransplant CRP serum levels and decreased sIL-6R levels but did not influence transplant outcomes. Homozygosity for the minor allele of rs4379670 was associated with decreased

pretransplant CRP levels, whereas rs4845618 donor genotype was associated with aGVHD. Finally, the recipient genotype of the IL-6R SNP rs432950 was associated with the probability to wean of immunosuppression.

The effects of G-CSF administration on systemic levels of IL-6 family cytokines in healthy stem cells were investigated in the third study. G-CSF administration significantly increased the levels of both IL-6 and CRP, whereas the levels of the other IL-6 family cytokines were not significantly altered. G-CSF was also able to potentate IL-6 release from *in vitro* cultured monocytes, fibroblasts and mesenchymal stem cells stimulated by various Toll-like receptor agonists.

Finally, we investigated how various forms of IL-6 signaling influenced the activation of intracellular signaling pathways (i.e. mediator phosphorylation status) in resting and activated (CD3/CD28 receptor ligation) peripheral blood $CD4^+$ and $CD8^+$ T cells derived from allotransplant recipients 90 days posttransplant. We used the two designer cytokines hyper-IL-6 and sgp130-FC that allows for both isolated IL-6 trans-signaling stimulation and blockage. We observed that IL-6 signaling potentiated the phosphorylation/activation of STAT3, AKT and mTor; these effects were observed especially after activation of circulating $CD4^+$ cells derived from patients with previous acute GVHD (aGVHD).

Taken together, our results suggest that IL-6 family cytokines are important for the regulation of inflammation and immunity in allogeneic stem cell transplant recipients. However, the influence of IL-6 and IL-6 family cytokines is only one of several factors that contribute to the final clinical outcome after allotransplantation, and the heterogeneity among both donors and recipients with regard to IL-6 family levels/activity suggests that the impact of these cytokines differs between patients.

6. LIST OF PUBLICATIONS:

Article I

Tvedt THA, Lie SA, Reikvam H, Rye KP, Lindås R, Gedde-Dahl T, Ahmed AB, Bruserud Ø.

Pretransplant levels of CRP and interleukin-6 family cytokines; effects on outcome after allogeneic stem cell transplantation

Int J Mol Sci. 2016 Nov 1; 17(11)

Article II

Tvedt THA, Hovland R, Tsykunova G, Ahmed AB, Gedde-Dahl T, Bruserud Ø.

A pilot study of single nucleotide polymorphisms in the interleukin-6 receptor and their effects on pre- and post-transplant serum mediator level and outcome after allogeneic stem cell transplantation

Clin Exp Immunol. 2018 Jul; 193(1)

Article III

Tvedt THA, Melve GK, Tsykunova G, Ahmed AB, Brenner AK, Bruserud Ø.

Immunological heterogeneity of healthy peripheral blood stem cell donors-effects of granulocyte colony-stimulating factor on inflammatory responses

Int J Mol Sci. 2018 Sep 22; 19(10)

Article IV

Tvedt THA, Rose-John S, Tsykunova G, Ahmed AB, Gedde-Dahl T, Ersvær E, Bruserud Ø.

IL-6 responsiveness of $CD4^+$ and $CD8^+$ T cells after allogeneic stem cell transplantation differs between patients and is associated with previous acute graft-versus-host disease and pretransplant antithymocyte globulin therapy

Manuscript

7. INTRODUCTION

7.1 CYTOKINES AND THE INTERLEUKIN-6 FAMILY

7.1.1 Definition and classification of cytokines

Cytokines are a large group of diverse proteins that are involved in communication between cells, and the cytokine system plays a key role in the development and normal function of almost all tissues. There is no generally accepted definition of the term cytokine, but a cytokine usually has several of the following characteristics [1]. First, they are usually simple polypeptide glycoproteins that exert their functions through ligation of membrane-bound receptors. Second, their constitutive production is low but can be transiently upregulated through specific stimulation. Third, the main effects of cytokines are usually local (i.e. autocrine/paracrine) effects. Finally, cytokines exert biological effects through regulation of gene expression; these effects are diverse and can be detected in various tissues, but almost all cytokines have specific effects on immunocompetent and hematopoietic cells. Since the term cytokine refers to large groups of structurally and functionally heterogeneous proteins, no ideal classification system can be made. One commonly used classification of cytokines is based on protein structure homologies [1,2]; a brief overview of this system is given in Table 1.

7.1.2 The hematopoietic growth factor/Interferon-type cytokines

The interleukin-6 (IL-6) cytokine family is included among the hematopoietic growth factor/ Interferon type cytokines [1]; these cytokines usually rely on specific transmembrane receptors consisting of one protein responsible for ligation and another protein that initiates the intracellular signaling. The transmembrane protein responsible for signal transduction is often shared by different receptors and constitutes the basis for classification into subfamilies (Table 2). The extracellular binding of the ligands results in the formation of a molecular complex that allows binding of Janus-kinases (JAK molecules) with activation of their tyrosine kinase function, phosphorylation of the JAK molecules themselves as well as the ligand-specific receptor and finally recruitment and phosphorylation of signal transducer and activator of transcription (STAT) molecules. STATs are transcription factors, and their phosphorylation

leads to dimerization and translocation to the nucleus. Four JAK proteins and seven STAT molecules have been identified, and they have different affinities for the various receptors (Table 2) [1], but receptor activation may also initiate additional signaling through the MAP kinase and PI3K/AKT/mTOR pathways [3].

Table 1. *An overview of different cytokine families based on structural homologies [2].*

Key members	The families and their common characteristics
	TNF receptor superfamily [4,5]
TNF-α, TNF-β CD40-Ligand Fas Ligand	• These proteins share structural homology to TNF. • Each cytokine is a trimer that consists of three β-sheets. • A cluster of receptors is required for adequate signaling.
	IL-1 cytokine superfamily [6-9]
IL-1β, IL1-RA IL-36α IL-37	• This family is characterized by a conserved cytoplasmic Toll/IL-1R (TIR) domain and three extracellular immunoglobulin (Ig)-like domains in the receptors, and the cytokines adopt a conserved signature β-trefoil fold comprised of 12 anti-parallel β-strands. • This family is further divided into three subfamilies (IL-1, IL-18 and IL-36).
	The cysteine-knot growth factor superfamily [1,10]
TGF-β β-HCG PDGF-β	• These cytokines contain six cysteine residues that form a "cysteine-knot" conformation. • This class includes otherwise structurally unrelated subfamilies.
	IL-17 cytokine superfamily [11]
IL-17A-E	• Members of this cytokine family contain five spatially conserved cysteine residues at their C-terminal ends and form a cysteine-knot-fold structure that is critical for their function.
	Chemokines [1,12]
CCL1 CXCL1 CX3CL1	• Chemokines are small molecules (8-10 kDa) characterized by specific domains containing four cysteine residues that secure a common 3-dimensional structure. • Their cell surface receptors are linked to G-proteins. • Chemokines are divided into subgroups based on the spatial position of the cysteine residues.
	Type 1 and type 2 hematopoietin cytokines [1]
Type 1: IL-2, IL-3 and IL-6 subfamilies **Type 2:** Interferons IL-10 subfamily	• This family is divided into type I and type II hematopoietin based on the architecture of the extracellular segments. • Signal transduction occurs via JAK/STAT. • Type I cytokines have a typical four-α-helix bundle structure. • Receptors often consist of a ligand-specific binding protein and a signal-transducing protein shared with other family members. • Subclassification is based on the signal-transducing receptor chain.

Table 2. *An overview of the subfamilies of Type 1 hematopoietin cytokines. The table lists the main member of each subfamily together with proteins used for signal transduction, utilized tyrosine kinases and targeted transcription factors (adapted from [1]).*

Cytokines	Transmembrane signal transducer	Non-receptor tyrosine kinase	Transcription factor
	IL-2 cytokine family		
IL-2 IL-7 IL-9 IL-15	Common gamma chain (CD131/ IL-2RG)	JAK1, JAK2	STAT5
IL-4			STAT6
IL-21			STAT 1, STAT 3
	IL-6 cytokine family		
IL-6 IL-11 IL-27 LIF CNTF OCM	Glycoprotein 130 (CD130/ gp130)	JAK1	STAT1, STAT3, STAT5
	IL-12 cytokine family		
IL-12 IL-23 IL-35	IL-12Rβ1 or IL-12Rβ2 WSX1 or gp130	JAK1, JAK2	STAT1, STAT3, STAT4
	IL-3/ IL-5 cytokine family		
IL-3 IL-5 GM-CSF	IL-5 receptor-β	JAK2	STAT5

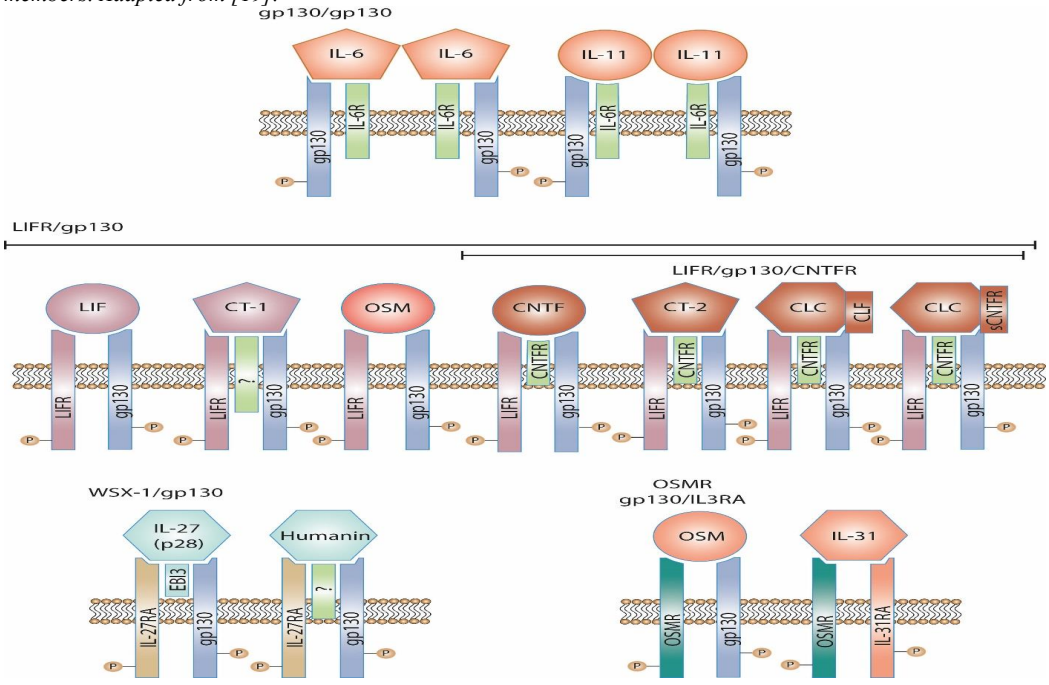
7.1.3 **The interleukin-6 family**

The IL-6 cytokine family encompasses the nine members IL-6, IL-11, IL-27, IL-31, Oncostatin M (OSM), Ciliary neutrophilic factor (CNTF), Leukemia inhibitory factor (LIF), Cardiotrophin 1 (CT-1) and Cardiotrophin-like cytokine (CLC) [13]. All members have a 4-helix structure, but they share only 10-20% sequence identity, and the positions of cysteine residues are not conserved [14,15]. A common characteristic is that they all utilize gp130 or a gp130-like protein (IL-31R) for intracellular signal transduction [13]. The extracellular domains of these two proteins share structural resemblance with the other receptor proteins of the hematopoietic growth factor/interferon family [16]; their encoding genes are located head-to head on chromosome 5q11.2 and share 28% sequence homology [17]. There is a structural and functional overlap between the IL-6 and theIL-12 cytokine families. The cytokines share the helix bundle structure, and IL-12 family receptor subunits share a modular homology with

gp130 and LIFR. Several of the IL-12 receptor complexes (e.g. IL-35) also utilize gp130 for signal transduction [18].

Most members of the IL-6 cytokine family bind to ligand-specific receptors; with the probable exceptions of CLC and CT-1 [13]. However, several of these cytokines have shared receptor components with cross-reactivity between different receptors and ligands. Some of the receptors have only short intracellular domains and are incapable of signal transduction (e.g. IL-6R and IL-11R), whereas others have intracellular domains that initiate signaling through cascades other than gp130 (e.g. LIFR and OSMR). Based on the different combinations of the utilized transmembrane proteins, the IL-6 cytokine family can be divided into different subgroups (Figure 1) [19].

Figure 1. *A brief overview of the nine IL-6 cytokine members and their receptor complexes. All the different receptor complexes utilize gp130 for signal transduction with the exception of the receptor for IL-31, which uses the gp130 homolog IL-31R. The different receptor complexes can be classified into five different groups based on the interaction of the different ligand-specific receptors with gp130 or IL-. The upper gp130/gp130 receptors are presented as dimers, the two lower parts show the various monomers that have been identified. CT-2 and humanin are mediators that can function as ligands even though they are not regarded as classical IL-6 family members. Adapted from [19].*



7.2 IL-6 AND IL-6 SIGNALING

7.2.1 The structure of IL-6 and the regulation of IL-6 release

IL-6 consists of 184 amino acids and is heavily glycosylated. The molecular weight is 23 to 28kDa, depending on the degree of glycosylation [20]. Similar to the other hematopoietic growth factor cytokines, IL-6 consists of four alpha helix proteins organized in a top-down-top-down topology [21,22]. It is present in all organs; the low molecular weight allows it to reach most extracellular compartments, and it can cross the blood-brain barrier by a specific saturable transport mechanism [23].

IL-6 is produced by a large variety of cells, but especially monocytes, macrophages, lymphocytes, fibroblasts, keratinocytes, endothelial cells, muscle and tumor cells [24]. Under normal conditions, local and systemic levels of IL-6 are low, but adequate stimulation can lead to a more than 100,000-fold increase in local or systemic levels [25]. Inflammatory stimuli are the most potent drivers of IL-6 production. Macrophages and monocytes are the main sources of IL-6 for acute inflammation, while T cells are the more prevalent source for chronic inflammation [26]. Increased IL-6 is also seen in non-inflammatory processes, such as during exercise when systemic IL-6 concentration increases 100-fold as IL-6 is released from contracting muscles [27,28]. During acute inflammation, the main transcription factors responsible for IL-6 productions are NF-κb, C/EBP-α, AP-1 and nuclear factor IL-6. These factors are activated through the Toll-like receptor pathways (TLR). However, TNFα, IL-1, and NOTCH, as well as IL-6 itself, promote the binding of these cis-regulatory factors at the 5′-flanking region on the IL-6 gene. Several miRNAs have also been shown to either repress IL-6 transcription or induce posttranscriptional downregulation of IL-6 expression [29,30]. Furthermore, several RNA-binding proteins control the stability of mRNA through binding to AU-rich elements in the 3′ untranslated region of mRNA, including Regenase-1 and Arid5a, which inhibit IL-6 production through degradation of IL-6 mRNA [31]. Humans with Regenase-1 deficiency show increased IL-6 levels and spontaneous autoimmune disorders [32]. Corticosteroids also directly suppress IL-6 production in several cell types, probably by reducing the stability of the IL-6 mRNA transcript [33]. Finally, the SNP rs1800795 (-174

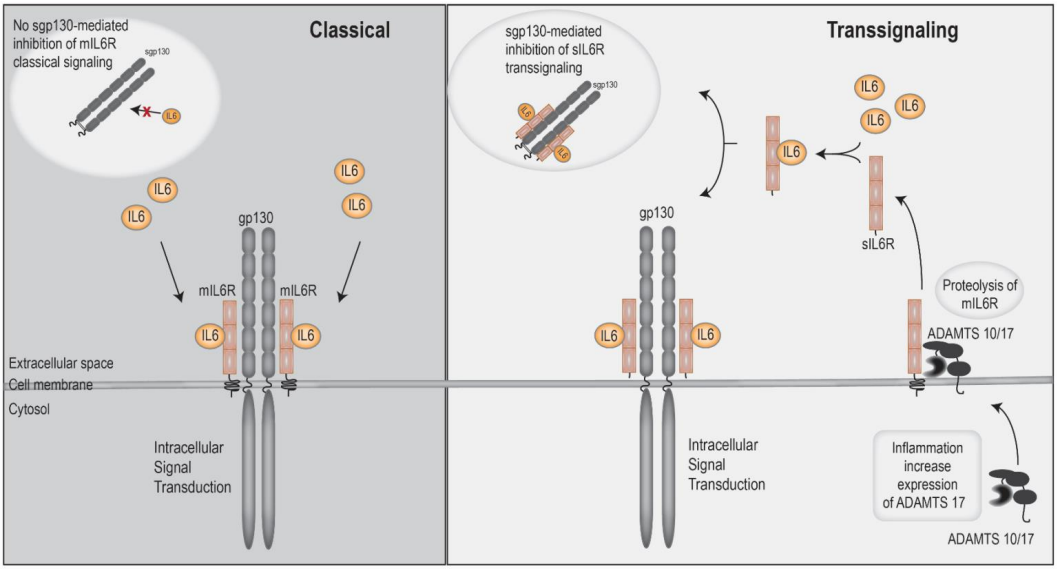
(G>C) is in complete disequilibrium with rs1800797 and is located in the proximal promotor of the IL-6 gene. The presence of the minor allele is associated with increased production of IL-6 by fibroblasts and, in some studies, with higher systemic IL-6 levels [34,35].

7.2.2 **Initiation of intracellular signaling by the IL-6 receptor complex**

The activated IL-6 receptor complex consists of two 80kDa type-1 cytokine receptor chains, named IL-6R or CD126, two chains of the IL-6 family-specific 130 kDa signal-transducing transmembrane glycoprotein gp130 and two IL-6 molecules (Figures 1, 2) [36]. This complex is stable only after IL-6 binding [37]. The IL-6R receptor alone cannot initiate intracellular signaling. Formation of the four-chain receptor complex only occurs after ligation [36]. CNTF and IL-30 can also utilize IL-6R for initiation of intracellular signal transduction but the significance of these interactions *in vivo* is not known [38,39].

Membrane-bound IL-6R is expressed only by certain cell types, such as hepatocytes, neutrophils, naive T cells, macrophages and a subset of intestinal epithelial cells [40-44]. In contrast, gp130 is expressed by most cells [36]. Classical IL-6 signaling then occurs in cells that express the membrane-bound IL-6R, and the complex of IL-6, IL-6R and gp130 then initiates intracellular signaling [36]. This classical signaling is often important for tissue regeneration and anti-inflammatory activity. The alternative IL-6 trans-signaling can also be initiated in cells that do not express IL-6R [36]. Unlike many other cytokine receptors, the soluble IL-6R receptor does have an antagonist through binding and inactivation of its receptor ligands; the soluble IL-6/IL-6R complex can instead bind to and activate gp130 that is expressed by most cells, thereby initiating IL-6 trans-signaling [36]. This signaling has been observed to play an important role , especially in relation to the proinflammatory effects of IL-6. An overview of classical and IL-6 trans-signaling is given in Figure 2. Finally, IL-6 cluster signaling (also termed trans-presentation) has been detected for dendritic cells. IL-6 is bound to IL-6R intracellularly before this complex is expressed on the cell surface and activates gp130 on neighboring cells through direct cell-cell contact [45]. Cluster signaling has only been detected for murine Th17 cells. Whether antibodies directed against IL-6 or IL-6R block cluster signaling is not known.

Figure 2. *An overview of classical IL-6 signaling and IL-6 trans-signaling. Classical IL-6 signaling takes place only in cells expressing the membrane-bound IL-6 receptor. IL-6 trans-signaling occurs on cells not expressing membrane-bound IL-6R by binding IL-6/soluble-IL-6R complex directly to gp130. The degree of IL-6 trans-signaling is regulated by proteolytic shedding of the IL-6R. Inflammatory stimuli upregulate shedding of the IL-6R (for additional details, see sections 7.2.3 through 7.2.5).*



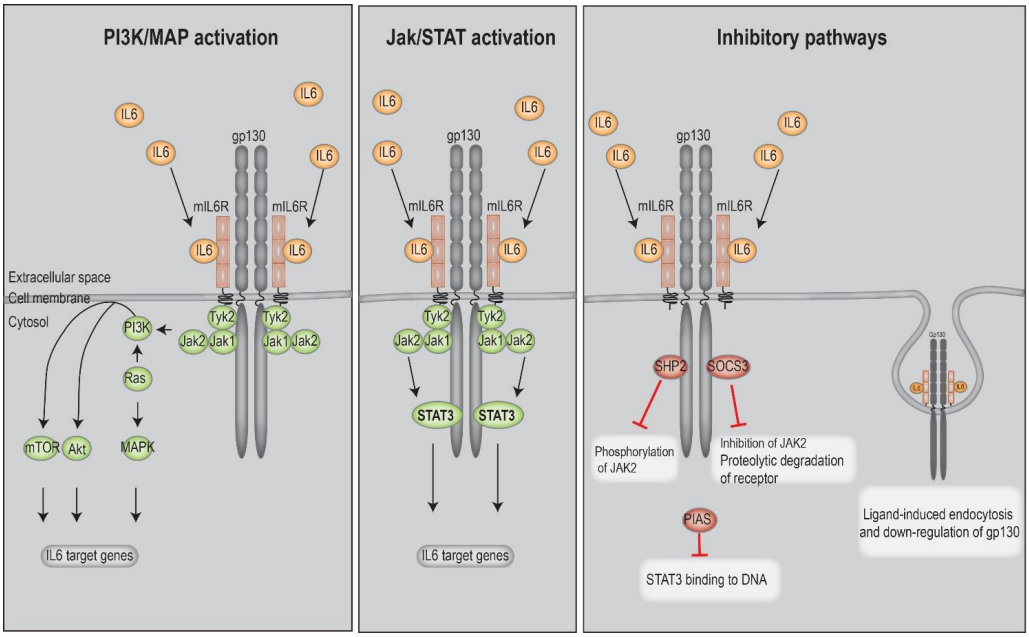
7.2.3 **Initiation and termination of intracellular IL-6 signaling**

As can be seen from Figure 2, the activated homodimeric IL-6R/gp130 complex binds non-covalently to kinases JAK1, JAK2 and TYK2, which phosphorylate gp130 and are also auto-phosphorylated [16,46]. This provides docking sites for the phosphorylation of STAT3 and, to a limited degree, STAT1 and the protein tyrosine phosphatase SHP-2 [46]. Phosphorylated STAT3 dimerizes and translocates to the nucleus where it acts as a transcription factor. SHP-2 activates the MAPK/ERK pathway, which eventually activates the RAS protooncogenes. Activation of gp130 also leads to activation of the PI3K/AKT/mTOR pathway (Figure 3) [3]. However, the IL-6 effect is mediated mainly by JAK-STAT3, since pharmacological inhibition of this pathway blocks most effects [47].

The IL-6 signal is terminated though several mechanisms [16,46,48]; the most prominent is probably internalization and degradation of the activated receptor complex. This leads to termination of the IL-6 signal and also limits the number of available receptors, thereby

blocking further IL-6 stimulation [48,49]. Furthermore, stimulation of cells by IL-1β and TNF-α leads to inhibition of gp130/mIL-6R internalization, thereby altering IL-6 sensitivity [50]. SOCS3 also inhibits IL-6 signaling by several mechanisms [51]. First, it binds to the phosphotyrosine 759 of gp130, thereby inhibiting co-location of STAT3, gp130 and JAK [52]. Second, the kinase inhibitory region (KIR) of SOCS3 directly inhibits the catalytic domain of JAK2 [53], and the negative feedback mechanism of SOCS3 targets gp130 and JAK2 for proteolytic degradation [53]. Third, SHP2, a phosphatase that uncovers its catalytic center upon binding to activated gp130, regulates STAT3 and gp130 in the absence of cytokine stimulation. It regulates the basal activity of gp130 in the absence of cytokine stimulation [54,55]. Finally, IL-6 signaling downstream to STAT3 by PIAS3 is also regulated preventing the binding of STAT3 to DNA [56]; IL-6 then acts to suppress E3 SUMO-protein ligase (PIAS3) by miR-18a induction (Figure 3) [57].

Figure 3. *An overview of the intracellular signaling cascade after IL-6 stimulation. IL-6 activates JAK/STAT, MAPK/ERK and the PI3K/AKT/mTOR pathways. However, most of the IL-6 effects are mediated through JAK2/STAT3 (for additional details see section 7.2.3.)*



7.2.4 **Extracellular regulation of IL-6 signaling**

The availability of soluble IL-6R is the best described and probably most important regulatory mechanism; IL-6 signaling is also regulated by the release of sIL-6R and the amount of soluble gp130 [36]. Soluble IL-6R (sIL-6R) is mainly produced by cleavage of membrane-bound IL-6R (mIL-6) [58]. Synthesis of the soluble form though alternate splicing also contributes to production levels [59]. ADAM (A disintegrin and metalloprotease) proteases are zinc dependent and membrane-bound; they are involved in the production of several soluble receptors [60]. mIL-6R is cleaved by ADAM10 and ADAM17, forming soluble receptors [36]. ADAM10 is responsible for a slow continuous release of IL-6R. Selective knockdown experiments of membrane-bound IL-6R have shown that approximate 30% of this release originates from the liver whereas approximately 60% originates from hematopoietic cells under physiological conditions [61,62].

Upregulation of ADAM17 results in an increased rate of proteolytic cleavage of IL-6R and is mainly observed during inflammation and apoptosis; IL-6 trans-signaling is thereby increased [36,63]. However, IL-6R shedding can also be caused by bacterial proteases, such as streptolysin O from *Serratia marcescens* and hemolysin from *Escherichia coli* [64,65]. This release is probably independent of ADAM17, and it is not known whether their cleavage products contribute to IL-6 trans-signaling.

7.2.5 **Soluble gp130 functions as an IL-6 buffer**

A soluble dimeric form of gp130 is present at relatively high serum concentrations, and is able to bind and inactivate IL-6 in complex with soluble IL-6R but not in complex with membrane-bound IL-6R. Hence, sgp130 blocks IL-6 trans-signaling leaving classical IL-6 signaling intact [36]. Under normal circumstances, sgp130 has a molar concentration corresponding to approximately twice the IL-6 level, and therefore acts as a physiological buffer that blunts IL-6 transactivation. Inflammatory stimuli upregulate ADAM17, causing a rapid increase in local sIL-6R levels [36]. Neutrophils express mIL-6R, and their influx to inflamed tissues, followed by rapid apoptosis, enhances IL-6 trans-signaling [63]. This probably means that the immunological effects of IL-6 differ during the various phases of inflammation; an altered

balance between sIL-6R and sgp130 has also been implicated in the pathogenesis of several autoimmune disorders.

7.2.6 Experiential models for examination of pleiotropic IL-6 effects

The development of the designer proteins Hyper-IL-6 and sgp130Fc made it possible to investigate IL-6 classical and trans-signaling separately [66-69]. Hyper-IL-6 is a designer cytokine consisting of IL-6 linked to IL-6R through a linker molecule; this complex mimics trans-signaling through binding to and thereby activating gp130 on cells that do not express IL-6R [68]. sgp130Fc consists of two monomeric sgp130 molecules coupled with the Fc-region of human immunoglobulin [66]. sgp130Fc has a 100-1,000-fold higher affinity to the IL-6/IL-6R complex than do natural sgp130 monomers; it thereby abolishes IL-6 trans-signaling completely but leaves classical IL-6 signaling intact. The use of these tools in selective gene knockout animal models has made it possible to characterize the pleiotropic effects of IL-6 [70-74]. sgp130Fc can also be used as a therapeutic tool for selective inhibition of IL-6 trans-signaling in a wide variety of inflammatory and malignant disorders (Table 3). However, to the best of our knowledge, the possible use of this strategy has not been investigated in animal models of GVHD.

7.3 IL-6 IN IMMUNOREGULATION

7.3.1 IL-6 in the acute phase response

Acute phase response is a physiological increase in systemic levels of specific proteins in response to inflammation. It is usually due to an increased production and release of these proteins by liver cells and is most notable for the C-reactive protein (CRP), serum amyloid P, ferritin, mannose binding protein and fibrinogen [75]. IL-6 is the main driver of this response, and systemic levels of IL-6 and those of several acute-phase proteins (e.g. CRP) are strongly correlated [76,77]. Furthermore, IL-6 levels are often correlated with the extent of tissue damage [78].

Table 3. *An overview of animal models examining the role of classical/ IL-6 trans-signaling in malignant, inflammatory and autoimmune disorders.*

Disease	Mouse model	Key findings.
Rheumatoid arthritis	IL-6 knockout vs IL-6wt treated with methylated bovine albumin, complete Freund's adjuvant and *Bordetella pertussis* toxin.	IL-6 caused only a minimal increase in disease activity, whereas Hyper-IL-6 significantly increased clinical disease activity, joint erosion and histological changes in IL-6 knockout mice. Administration of sgp130 suppressed all histological disease severity parameters [79,80].
Rheumatoid arthritis	Antigen-induced arthritis mouse model.	Treatment with trans-inhibitor reduced the severity of inflammation and joint destruction compared with control animals. Joint morphology was nearly normalized after the treatment [81].
Sepsis	Standardized cecal ligation and puncture (CLP).	IL-6 blockade had no effect on survival, while sgp130Fc increased survival from 45% to 100%. Administration of sgp130Fc post-CPL increased survival. A significant reduction of the acute phase response was only detected after anti-IL-6 an not after sgp130Fc therapy [70].
Asthma	Ovalbumin (OVA) sensitization and subsequent intranasal ovalbumin challenge.	Administration of sgp130Fc resulted in downregulation of IL-4, IL-5, and IL-13 levels in alveolar lavage fluid from OVA-sensitized mice. Treatment with sgp130Fc reduced the airway hyperresponsiveness and induced local expansion of $Foxp3^{+}CD4^{+}CD25^{+}$ Tregs [71].
Atherosclerosis	Mice prone to arteriosclerosis (LDL receptor-deficient (LDLR-/-) mice) treated with high-fat, high-cholesterol diet.	Treatment with sgp130Fc resulted in significant reduction in the aortic root and a slight decrease in the aortic lipid deposition after 8 weeks; after 16 weeks a significant reduction of atherosclerotic plaque size in the aortic root and no progression of the atherosclerotic burden in thoracoabdominal aorta were observed. Treatment with sgp130 also reduced arteriosclerosis in mice with established arteriosclerosis. IL-6 trans-signaling blockade inhibited infiltration of macrophages but not $CD3^{+}$ T cells within the plaques [74].
Inflammatory bowel disease	T cell-reconstituted SCID mice, IL-10-knockout mice.	Administration of sgp130Fc resulted in reduced colitis in T cell-reconstituted SCID mice and IL-10-knockout mice; the treatment then induced T cell apoptosis [82].
Inflammatory bowel disease	SAMP1/Yit mice.	Mice treated with Hyper-IL-6 had increased tissue weight of the ileum and increased histological severity of disease at 28 weeks. Destruction of the epithelial layer and inflammatory infiltrates in the lamina propria were more prominent in mice treated with hyper-IL-6. Blockade of IL-6-trans-signaling by sgp130Fc reduced tissue weight and histological changes [73].
Ovarian cancer	Ovarian cancer cells in NOD/SCID mice.	Hyper-IL-6 enhanced endothelial migration and reduced the vascular hyperpermeability. Paclitaxel-induced reduction of cell viability was antagonized by hyper-IL-6, whereas sgp130Fc increased the antitumor effect of paclitaxel [72].
Colon cancer	Mouse colitis-associated premalignant cancer model.	Treatment with sgp130Fc reduced the incidence and number of tumors; this treatment also seemed to induce histologically milder forms [83].

Although IL-6 is the main driver of the acute phase response, other cytokines (e.g. IL-1, IL-8/CXCL8, TNF-α) are also involved [75]. A persistent response is often detected in patients with inflammatory or malignant disorders, and IL-6 released by normal leukocytes or malignant cells is believed to be the main driver [84,85]. Pharmacological neutralization of IL-6 or blocking of IL-6R has a strong inhibitory effect on response [86,87]. However, a significant acute phase response can also be detected in IL-6 knockout mice and in patients treated with IL-6R antagonist, and experimental studies suggest that these responses are caused by other IL-6-family cytokines that are able to interact with IL-6R [88-90]

7.3.2 **Effects of IL-6 on leukocyte migration during local inflammation**

IL-6 is important for regulation of T-cell trafficking, including local recruitment of primed T cells to inflamed tissues and entry of naive T-cells to lymphoid organs [91-98]. Primary antigen encounter occurs predominantly in secondary lymphoid organs; adequate guidance of T cell migration from specialized high endothelial venules is therefore essential to establish cell-to-cell contact between antigen-presenting cells (APCs) and T cells. High body temperature alone, without other inflammatory signals, is sufficient to increase this leukocyte extravasation though a gp130 dependent mechanism [92]. L-selectin and integrin α4β7 on the T-cells then secure their binding to the mucosal vasculature through specific adhesion molecules (MAdCAM-1) expressed by the high endothelial venules [93,94]. IL-6 trans-signaling is important to secure high L-selectin expression on the T cells [94]. However, this process is independent of IL-6 levels, and animal studies suggest that other members of the IL-6 cytokine family can replace IL-6 in IL-6 deficient mice [96]. The L-selectin expression is mediated through the MERK1/ERK-1/2 pathway and seems less dependent on STAT3 activation [94]. IL-6 also leads to increased vascular expression of other adhesion molecules involved in leukocyte extravasation, including ICAM-1, VCAM-1, and CD62E [96-98].

7.3.3 **Interleukin-6 and T cell differentiation**

IL-6 is important for local recruitment, antigen-driven proliferation, polarization and later regulation of T cell responses. It can be released by APCs during the early stages of T-cell activation [45], but is also secreted from other cells, such as MSCs, that are important for the

later stages of T cell maturation. Naive T cells and memory effector T cells express membrane-bound IL-6 receptors, responding to classical IL-6 signaling, whereas mIL-6R expression is lost following activation [40].

IL-6 enhances the development of the Th2 and Th17 T cell subsets, whereas it suppresses the development of Th1 and Treg cells [99-102]. The molecular basis for this regulation appears to be orchestrated largely through STAT3 signaling. IL-6 leads to STAT3 activation, resulting in SOCS1 expression in naive T cells [99]. SOCS1 strongly inhibits Th1 polarization by impairing INF-γ signaling in the T cell [103]. At the same time, IL-6 promotes polarization towards a Th2 phenotype by activation of STAT3-independent NFAT expression and STAT3-dependent expression of c-maf, both required for the production of IL-4 and subsequent Th2 commitment [104,105].

The expression of RORγT in Th17 cells also depends on STAT3, and patients with inherited inactivating STAT3 mutations show Th17 deficiency [106]. In mice Th17 development depends on simultaneous IL-6 and TGF-β stimulation [107,108]; IL-6 then activates STAT3, whereas TGF-β inhibits the transcription of SOCS3, thereby allowing sustained STAT3 activation [109]. Th17 development also depends on STAT3 activation by IL-21 [110]. Finally, differentiation of naive T-cells to Th17 cells relies largely on classical IL-6 signaling, whereas maintenance of Th17 cells depends on trans-signaling [40,45]. IL-6 cluster signaling has recently been described for Th17 cells [45].

Th22 T cells show similarities to Th17 cells. Production of IL-22 is always present, but this is not unique since Th17 cells can also release IL-22. In contrast to Th17 cells, Th22 cells do not express the transcription factor RORγT and do not release IL-17. Development of Th22 cells is thought to depend on the combined actions of IL-6, TNFα, IL-1β and the aryl-hydrocarbon receptor, which acts as a transcription factor. The main targets of Th22 cells are epithelial barriers, but understanding of the role of this T cell subset in GVHD is currently limited [111-113].

Regulatory T cells counteract the proinflammatory activity of Th17 cells. While TGF-β induces both Foxp3 and RORγ, which are essential for Treg and Th17 cell differentiation, respectively, the IL-6-induced STAT3 activation inhibits FOXP3 expression and stimulates

RORγ expression. In contrast, Tregs depend on IL-2 induced STAT5 activation. Although it seems that IL-6 favors proinflammatory Th17 differentiation with suppression of Tregs, the final effect of IL-6 on overall immune homeostasis is difficult to predict since IL-6 also increases the release of anti-inflammatory cytokines.

7.4 SYSTEMIC IL-6 EFFECTS AND EFFECTS IN GVHD TARGET ORGANS

7.4.1 Effects on liver cell regeneration and their metabolic regulation

The liver is capable of complete recovery even after substantial loss of cell mass. Impaired IL-6 function significantly reduces the potential of the liver to regenerate, and this effect seems to depend on IL-6 trans-signaling [88]. Furthermore, pharmacological blockade of IL-6R is associated with a transient increase in transaminases, whereas this is uncommon during treatment with IL-6 neutralizing antibodies [114-117].

7.4.2 IL-6 and STAT3 signaling in the gastrointestinal mucosa

IL-6/STAT3 signaling is important for regeneration of intestinal epithelium and plays a role in inflammatory bowel disease and intestinal carcinogenesis [82,118-122]. STAT3 activation is required to maintain sufficient intestinal barrier integrity, ensure adequate secretion of antimicrobial polypeptides, support proliferation of intestinal epithelial cells and facilitate migration of intraepithelial lymphocytes [118,123]. However, IL-6 levels also correlate with severity of inflammatory bowel disease, and animal studies have shown that IL-6 neutralization, especially blockade of IL-6 trans-signaling, decreases inflammation and suppresses colitis through induction of T-cell apoptosis [82,124]. Furthermore, although IL-6 may have direct proapoptotic effects on intestinal cells in certain experimental models, IL-6 stimulation seems to be essential for regeneration/proliferation of intestinal epithelium after injury of the colon [42]. Early after colonic injury, there is a local IL-6 increase caused by resident intestinal lymphocytes; inhibition of this early IL-6 burst leads to epithelial cell cycle arrest and subsequent impaired healing [119]. The importance of IL-6 for intestinal epithelial cell proliferation could thus partially explain the increased incidence of spontaneous bowel perforation in patients treated with IL-6 blockade. It is not known whether such mechanisms

would increase the risk of severe gastrointestinal complications if IL-6 targeting is used in the treatment of GVHD. Taken together, these observations suggest that the role of IL-6 in gastrointestinal acute GVHD (aGVHD) is complex and may involve both systemic and local immunoregulation as well as direct effects on the intestinal epithelium.

7.4.3 **IL-6 as a metabolic regulator and a myokine**

IL-6 may influence metabolic regulation through its effects on liver regeneration (see above), but also through other mechanisms. First, IL-6 is a regulator of insulin resistance in muscle and liver cells. Second, IL-6 has indirect effects on adipocytes by orchestrating crosstalk between specific anti-inflammatory macrophage subsets and adipocytes. These interactions are frequently altered in metabolic syndrome and obesity-induced inflammation [125,126]. Third, treatment of rheumatoid arthritis patients with the IL-6R antagonist tocilizumab is frequently associated with dyslipidemia and insulin resistance, but this effect seems to be weaker in healthy individuals [127]. Taken together, these examples clearly illustrate the complex IL-6 effects on metabolic regulation [128].

IL-6 is released by muscle cells and is important for the growth and function of normal muscle cells; IL-6 is therefore regarded as a myokine [129]. During exercise, local levels of IL-6 in muscles may increase up to 500-fold, and systemic levels may rise up to 100-fold [130]. Increased IL-6 levels are often observed in patients with cachexia and muscle wasting, but the role of IL-6 in the development of muscular atrophy seen during chronic inflammation is controversial. Mice develop muscular atrophy only after exposure to high-dose IL-6, and several observations suggest that IL-6 is probably not the main driver of this muscular atrophy [129].

7.4.4 **IL-6 and gp130 signaling in haematopoiesis**

Adequate signaling by IL-6 family cytokines through gp130 is essential for normal hematopoiesis [131]. Complete loss of gp130 in mice is lethal due to severe bone marrow hypoplasia and reduced numbers of hematopoietic progenitors in the fetal liver. Interestingly, mice expressing genetically modified gp130 that have abolished STAT1/3 signaling survive postpartum and exhibit increased numbers of myeloid progenitor cells in the spleen and peripheral blood [132]. These observations suggest that gp130 mediates its effects on the

proliferation and differentiation of normal hematopoietic cells, both through non-STAT1/3 and STAT1/3 mediated mechanisms. Mice with overactive IL-6 signaling have massive extramedullary hematopoiesis [133]. The effects on hematopoietic stem cells then rely on IL-6 trans-signaling because these cells lack membrane-bound IL-6R.

IL-6 is used as a growth factor for *ex-vivo* cultured hematopoietic stem cells. Used alone, IL-6 does not sufficiently support hematopoiesis, but it improves the effects of other hematopoietic growth factors (e.g. IL-3) [134]. Other IL-6 family cytokines have similar *in vitro* effects [135]. One previous study also suggests that IL-6 administration to lethally irradiated mice accelerates hematopoietic recovery [136], but the role of IL-6 in hematopoietic reconstitution after stem cell transplantation still needs to be clarified, and there are conflicting results from human studies of IL-11 administration to improve thrombocytopenia [137,138].

7.4.5 **IL-6R polymorphisms and IL-6 trans-signaling in human diseases**

Acute and chronic inflammation leads to increased shedding of IL-6R through increased cell surface expression of ADAM17 proteases by neutrophils and monocytes. Several studies indicate that IL-6 trans-signaling is genetically influenced by IL-6R polymorphisms [139-152]. This is best described for SNP rs2228145 and results in an amino acid change in the juxtamembrane region of IL-6R at the site of proteolytic cleavage; this change increases affinity to ADAM10/17 proteases and subsequently increases IL-6R shedding [153]. In addition, rs2228145 may influence sIL-6R levels through induction of an alternative IL-6R mRNA splice variant [154]. Individuals who are homozygous for rs2228145 have significantly increased levels of sIL-6R [153]. Finally, the increased proteolytic cleavage of IL-6R seems to blunt the effects of classical IL-6 signaling, and is associated with proinflammatory effects along with reduction of acute phase reaction mediated by classical IL-6 signaling [153,155,156].

rs2228145 has an allele frequency of approximately 30% in individuals of European descent. The observed allele frequencies in African and Asian populations are much lower [157]. This difference may at least partly explain the higher levels of proinflammatory mediators in Europeans compared to individuals of African descent [157]. Genome-wide association studies (GWAS) have shown that rs2228145 explains 51% of the total variance of sIL-6R levels

observed in the European population [157]. Other SNPs in the 3' untranslated regulatory region also seem to influence sIL-6R levels and risk of certain diseases (Table 4), but the functional effects of these polymorphisms are not known. Most studies investigating these effects have significant limitations due to sample size and study design. The influence of rs2228145 on disease severity is best documented for coronary heart disease, inflammatory bowel disease and atopic dermatitis [144,149,151,158].

7.5 THE CURRENT STATUS OF CLINICAL IL-6 TARGETING THERAPY

Over the last decade, IL-6 blockade has emerged as a potent therapy for several autoimmune disorders. Tocilizumab is a humanized monoclonal antibody that binds membrane-bound and soluble IL-6 receptors; it was initially approved for the treatment of rheumatoid arthritis [159]. Randomized studies comparing tocilizumab alone or in combination with methotrexate (MTX) have shown that tocilizumab significantly reduces symptoms of arthritis but long-term data on radiological progression are lacking [114-116]. The treatment is well tolerated without increased risk of severe infections, but certain laboratory abnormalities are common [114-116]. First, 5-6% of these patients experience reversible increases in serum levels of liver transaminases. Second, increased serum cholesterol levels requiring cholesterol-lowering intervention is seen in up to 26% of patients. Third, transient decreases in peripheral blood neutrophil counts are relatively common; the treatment seems to interfere with intestinal epithelial regeneration and is associated with increased risk of intestinal perforation, especially for patients with predisposing lesions (i.e. diverticulitis) [160].

Tocilizumab has also been approved for treatment of giant cell arteritis and polyarticular juvenile idiopathic arthritis [161-165]. However, treatment with tocilizumab is associated with higher rates of infectious complications and discontinuation for these patients. Several case reports suggest that tocilizumab has beneficial effects for a wide variety of autoinflammatory and autoimmune disorders [166-169]. Finally, tocilizumab is the first-line treatment for severe cytokine release syndrome due to chimeric antigen receptor T-cell therapy (approved indication) and bi-specific T cell-engaging therapy (off-label use) [170,171].

Table 4. *An overview of important studies investigating associations between SNPS in the IL-6R and disease. GWAS: Genome-wide association study*

Disease	SNP	Individuals	Observation
Crohn's disease Ulcerative colitis	rs2228145	20,550 Crohn's disease 17,647 ulcerative colitis 40,000 healthy controls	rs2228145 was associated with reduced risk for Crohn's disease and ulcerative colitis [141].
Rheumatoid arthritis	rs2228145, rs4537545, rs4845617	60 Rheumatoid Arthritis 60 Healthy controls	rs2228145 and rs4845617 were associated with rheumatoid arthritis in a very small cohort from Pakistan [148].
Rheumatoid arthritis, response to tocilizumab	rs12083537, rs2228145, rs4329505		AAC-haplotype for rs12083537, s2228145 and rs4329505 were associated with poor response to treatment [152].
Systemic lupus erythematosus (SLE)	rs4845617, rs4845374 rs2228145	300 SLE patients 299 healthy controls	The 48864 A>C polymorphism was associated with rash. The haplotype HT2 was associated with frequency of rash and lymphopenia [143].
Atopic dermatitis	GWAS	2895 patients 2448 control subjects 3 additional control groups	Asp358Ala; rs2228145 was associated with atopic dermatitis [149].
Asthma	rs2228145	510 asthma patients 45 healthy controls	rs2228145 minor C was associated with reduced lung function. rs2228145 C allele appeared more frequently in patients classified as severe [150].
Asthma	rs12083537 rs2228145	394 patients 395 controls	rs2228145 C allele was associated with higher sIL-6R, higher serum IgE levels and reduced lung functions. rs12083537 G allele was associated with poor lung functions in patients with asthma [142].
Cardiovascular death in Rheumatoid arthritis	rs2228145	1948 patients with RA	There was no association between rs2228145 genotype and cardiovascular death in patients with RA [144].
Aortic valve stenosis	rs2228145	284 patients	There was evidence of an association between the rs2228145 genotype and the mean and maximal transvalvular gradients [147].
Coronary heart disease	rs2228145	51,441 patients 136,226 controls	rs2228145 was associated with a 3.4% reduction in the risk of coronary heart disease [151].
Myeloma	rs2228145	626 patients	rs2228145 minor allele C together with amplification of chromosome 1q21 correlated to an increase in sIL-6R levels and lower overall survival in a subgroup of myeloma patients [145].
Mastocytosis	rs2228145	66 mastocytosis 99 healthy controls	The rs228145 AA genotype had a 2.5-fold lower risk for mastocytosis compared to AC and CC genotypes [146].
Nonalcoholic steatohepatitis (NASH)	rs2228145	115 patients, 124 controls 115 NASH	CC genotype rs2228145 was associated with higher risk [140].
Depression/ psychosis	rs2228145	576 controls and 3251 patients	Asp358Ala was associated with decreased risk of severe depression and/or psychosis [139].

Sarilumab is another humanized monoclonal antibody that binds membrane-bound and soluble IL-6 receptors, but with a higher affinity than tocilizumab. It is currently approved for rheumatoid arthritis where it is effective in patients having either inadequate response or intolerance to TNF inhibitors. It has safety profile comparable to that of tocilizumab [172-174].

Siltuximab is a chimeric monoclonal antibody that binds and inactivates free IL-6; it has been approved for the treatment of HIV/ HHV8-negative multicentric Castleman's disease. The treatment is generally well tolerated, although minor adverse reactions are common [117,175].

Several drugs targeting IL-6 signaling have now been developed, including antibodies targeting IL-6R or IL-6, as well as small molecules that block intracellular signaling downstream to gp130 [176]. All these strategies inhibit both IL-6 trans- and classical signaling. In contrast, TJ301 (also known as FE 999301 or Olamkicept) is a selective inhibitor of IL-6 trans-signaling, similar to sg-130-FC [177]; it consists of two complete extracellular gp130 domains, and it traps IL-6/sIL-6R but not IL-6 alone or mIL-6R. Safety and efficacy of TJ301 is currently being investigated in clinical trials for ulcerative colitis and Crohn's disease (NCT03235752). The goal of these studies is to investigate whether TJ301 is equally effective as available therapies with fewer problematic side effects .

7.6 CURRENT USE OF ALLOGENEIC STEM CELL TRANSPLANTATION

The first documented cure of refractory leukemia by allogeneic stem cell transplantation (ASCT) was reported in 1971, and in 1977 Thomas showed that long-term, disease-free survival could be achieved by ASCT for patients with relapsed leukemia or aplastic anemia [178,179]. ASCT is now regarded as a highly effective therapy for several hematological malignancies; this is due to the combination of high-dose chemotherapy, which under normal circumstances is intolerable due to bone marrow toxicity, and the antileukemic immune reactivity mediated by donor immunocompetent cells through the graft-versus-leukemia/tumor reactivity. However, ASCT is usually associated with a relatively high rate (15-20%) of disabling or life-threating complications during the first year posttransplant [180,181].

Long-term survival without severe complications is reliant on both the underlying malignant disease and the comorbidity of the patient [182-186]. Thus, each decision to proceed to

transplantation is based on an individual assessment of patient– and disease-related factors. Generally accepted guidelines issued by the European Blood and Marrow Transplantation Society (EBMT) outline the indications for ASCT for each specific disorder [186,187].

In 2014 a total of 16,949 ASCTs were performed in Europe [188]. Approximate 70% of them were performed for hematological malignancies (e.g. AML; ALL, MDS and MPD), 9% for lymphoid malignancies (e.g. CLL, Hodgkin and non-Hodgkin lymphomas) and 5% for bone marrow failure syndromes (e.g. aplastic anemia). ASCT is also employed for other conditions such as inherited immunodeficiency syndromes (e.g. chronic granulomatous disease), inherited disorder of metabolism (e.g. adrenoleukodystrophy), hemoglobinopathies and certain solid tumors (e.g. medulloblastoma). However, due to the low incidence of these disorders the total number of such transplants is low; the highest being hemoglobinopathies that constitute 3% of total ASCTs in Europe [186,188].

Improved supportive care, development of more lenient treatment regimes, increased availability of suitable donors through better donor registries and the use of haploidentical donors are the main reasons for the increased use of ASCT over the last 15 years. An increased number of indications (e.g. lymphomas) and an increased use of ASCT for patients above 60 years of age have also contributed to the overall increase [189-191].

7.7 TRANSPLANTATION PROCEDURE

The ASCT procedure consists of initial conditioning therapy, followed by stem cell infusion, and completed by a period of posttransplant engraftment and expansion of the donor hematopoiesis and the immune system of the donor in the recipient. Interventions in each of these three periods influence the risk of GVHD/relapse and subsequently the possibility of long-term survival [192].

7.7.1 Myeloablative versus reduced-intensity conditioning

The role of conditioning therapy is to support the engraftment through eradication or at least significant reduction of the malignant cell burden and to modify the hosts' immune system,

thereby reducing the risk of graft rejection. The conditioning treatment has a dual anticancer effect with a direct toxic effect on the malignant cells and an additional indirect effect through modulation of the posttransplant graft-versus-leukemia (GVL) reactivity. In contrast, in autologous stem cell transplantation, the anticancer effect of the conditioning is mainly a direct toxic effect [186,193].

MAC regimes includes near lethal or maximum-tolerated chemotherapy/radiation therapy to achieve a maximal anticancer effect [193]. This treatment usually includes busulfan, melphalan, or total body irradiation (TBI) at doses that would cause irreversible damage to the recipient hematopoiesis without the benefits of stem cell transplantation. Reduced-intensity conditioning (RIC) includes drugs with strong immunosuppressive effects but weaker cytotoxic effects (e.g. fludarabine or T cell-specific monoclonal antibodies) [193,194]. However, some of the RIC regimes are also myeloablative, whereas others rely only on their immunosuppressive effects to support engraftment. The most common regimes are summarized in Table 5.

The intensity of the conditioning therapy influences risk of early treatment-related morality and risk of relapse. MAC regimes are associated with high rates of treatment-related complications (infections, GVHD, organ toxicity), which leads to a TRM of at least 15%, thus limiting the effective use of MAC regimes to younger patients (usually below the age of 55 years) without significant comorbidities. MAC regimes have high antitumor activity but are associated with increased early mortality due to GVHD and infections [186,193]. Conversely, RIC regimes have a fairly low rate of early mortality (1-5%), but the lower antitumor activity results in increased early relapses. However, a comparison of the overall effects of different conditioning regimes is hampered by a significant selection bias; for elderly patients with comorbidities, RIC regimes are the only suitable alternative whereas for younger adults with a significant risk of relapse, MAC regimes are often the first choice of treatment.

RIC regimes can be adapted more easily with posttransplant immunomodulatory drugs, low-dose chemotherapy and donor lymphocyte infusion than can MAC regimes, for which the posttransplant period is often hampered by several complications. This has led to an increased use of RIC regimes over the last decade, especially for patients with MDS and AML.

Table 5. *An overview of commonly used myeloablative, reduced-intensity and nonmyeloablative conditioning regimens. Commonly used myeloablative regimens usually include either busulfan > 8 mg/kg or total body irradiation (TBI) >5 Gy (days administered means days before ASCT [193]).*

Regime	Agent and dose	Days administered
Myeloablative regimes associated with high degree of acute toxicity		
BuCy	Busulfan 16mg/kg	-7 to -4
	Cyclophosphamide 120 mg/kg	-3 and -2
CyTBI	Cyclophosphamide 120 mg/kg	-6 and -5
	TBI 12-14Gy	-3 to -1
BEAM	BCNU 300mg/m^2	-6
	Etoposide 800mg/m^2	-5 to -2
	Cytarabine 800mg/m^2	-5 to -2
	Melphalan 140mg/m^2	-1
Reduced intensity but still considered myeloablative		
FluBu	Fludarabine 150 mg/m^2	-8 to -5
	Busulfan 8-10 mg/m^2	-6 to -4
FluTre	Fludarabine 30mg/m^2	-6 to -3
	Treosulfan 14 000mg/m^2	-6 to -4
Non-myeloablative regimes		
Cy low-dose TBI	Cyclophosphamide 600mg/m^2	-6 to -2
	Fludarabine	-6 to -2

7.7.2 **Stem cell transplantation; donor selection and stem cell source**

Donor selection. HLA matching of donor and recipient is used to identify the optimal donor, and donors are only accepted if specific criteria are fulfilled. At most transplant centers, a 5/6 match at HLA-A, HLA-B and HLA-DR is the minimum requirement for a sibling donor, while a 9/10 match at HLA-A, HLA-B, HLA-C, HLA-DR and HLA-DQ is the minimum requirement for a matched unrelated donor (MUD) [186]. However, over the last decade, the use of haploidentical related donors (sharing one haplotype with the recipient, i.e. 5/10 HLA match) has increased [191,195]. The average probability for having a matched sibling donor is between 20% and 30% [196]. In developed countries with ethnically homogeneous populations such as Norway, 70% of patients without a matched sibling donor have a suitable MUD in the bone marrow registries [196].

The selection of donor and graft source has significant impact on transplant outcomes. The most important factor affecting outcome is the degree of HLA mismatch [197-199]. Survival decreases approximately 10% points for each mismatched HLA antigen. With current matching techniques, the outcomes with 10/10 matched related donors (MRD) and 6/6 sibling donors (SIB) are regarded as equal [200-203]. Most patients have only a limited number of suitable donors, but donor-related factors (Table 6) are considered when choosing between matched family donors.

Table 6. *Donor characteristics that influence outcomes after allogeneic stem cell transplantation*

Characteristics	**Impact on outcome**	**Ref.**
HLA mismatch	Mismatch of HLA-A, -B, -C, and -DRB1 is associated with inferior survival, increased risk of GVHD and graft failure. HLA-DP mismatch may also influence risk of GVHD. Single mismatch at the HLA-DQ locus does not seem to influence outcome [197-199].	
KIR genotype	Presence or absence of specific killer-cell immunoglobulin-like receptors (KIR) seems to influence risk of relapse [204].	
Donor age	High donor age reduces overall survival and increases risk of aGVHD [205].	
Donor sex	Conflicting results, female donor to male recipients is associated with higher rates of cGVHD, but the effect on long-term survival is uncertain due to a reduced risk of relapse [206,207].	
Ethnicity	Does not seem to influence outcome.	
Parity	Conflicting results. Some studies have reported a high rate of acute or chronic GVHD, while others have reported that multiparous female donors seem sensitized or tolerant to other HLA types and do not confer a higher risk [208].	
ABO	ABO mismatch is associated with delayed hemolysis and development of pure red cell aplasia. However, ABO mismatch does not seem to significantly impact GVHD or survival [209].	
HLA antibodies	The presence of HLA antibodies in the recipient is associated with a higher rate of graft failure [210].	
CMV status	Conflicting results. Some studies have demonstrated a negative impact of seropositive donors to seronegative recipients [211].	

Stem cell grafts. Stem cells for ASCT can be obtained from different sources, including aspirated cells from bone marrow, umbilical cord blood stem cells or peripheral blood mobilized stem cells (PBSC) mobilized by Granulocyte-colony stimulating factor (G-CSF). PBSC harvested after G-CSF therapy is now the most commonly used graft both for autologous and allogeneic transplantation [212]. These three graft types differ significantly in their composition of stem cells and immunocompetent cells, and those differences significantly influence transplant outcomes (Table 7). Both cord blood and bone marrow grafts contain

immunoregulatory cells that are not present in PBSC grafts (e.g. mesenchymal stem cells) [186]. In addition, cord blood grafts are associated with increased risk of graft failure and late immune reconstitution activity (increased risk of severe infection) due to low number of both stem cells and mature T-cells [148]. In contrast, PBSC grafts contain more stem cells and 10-20 times more activated effector and memory T cells compared with bone marrow grafts. Hence, the use of PBSC grafts is associated with earlier engraftment and increased incidence of chronic GVHD (cGVHD), but long- term survival is comparable with that of bone marrow grafts [213-218].

Table 7. *Stem cell grafts used in allogeneic stem cell transplantation; comparison of infused graft volume, stem cell dose ($CD34^+$ cells), the characteristics of other graft cells and practical considerations. Adapted from the EBMT handbook [186].*

Volume	$CD34^+$ cell dose	$CD4^+$ cell dose	Other cell types	Practical advantages/ disadvantages
Bone marrow				
10-20 ml/kg	2-3 x 10^6/kg	25 x 10^6/kg	Low proportion of effector and memory T cells compared with PBSC grafts; the graft contains mesenchymal stem cells.	Risk associated with general anesthesia, postoperative pain, and risk of infection.
Peripheral blood progenitor/stem cells				
150-400 ml	8 x 10^6/kg	250 x 10^6/kg	High numbers of effector and memory T cells.	Risks associated with G-CSF stimulation and apheresis procedure.
Umbilical cord blood stem cells				
80-160 ml/kg	0,2 x 10^6/kg	0,5-2 x 10^6/kg	The grafts contain mainly naive T cells, but also mesenchymal stem cells.	No risk for the donor. Grafts readily available and can be transported with minimal delay.

7.7.3 **Early complications after ASCT; toxicity, inflammation and infections**

A wide range of complications can occur during the first 4-8 weeks posttransplant [186,219]; most complications (e.g. emesis, vomitus, mucositis, pain and diarrhea) are common, self-limited and caused by the toxicity of high-dose chemotherapy or radiation [219-221]. However, several other severe and clinically distinct complications have also been observed, and, along with aGVHD and severe infections, these complications account for the high rate of early

posttransplant morbidity and mortality (Table 8) [186,222-226]. Due to the important role of IL-6 in idiopathic pneumonia syndrome, this entity is discussed more in detail in a separate chapter.

Table 8. *A short overview of important early posttransplant inflammatory complications.*

Hemorrhagic cystitis [224]
• Early hemorrhagic cystitis is mainly due to the toxic effects of the cyclophosphamide metabolite acrolein, but it may also be caused by other agents (e.g. etoposide, TBI). • Late hemorrhagic cystitis is usually caused by BK virus, adenovirus and CMV infections. • Persistent severe hemorrhagic cystitis is associated with a high rate of morbidity and mortality.
Engraftment syndrome (ES) [223]
• ES occurs at the time of hematopoietic engraftment and is characterized by fever, pulmonary edema, skin rash, diarrhea and transient encephalopathy. • Diagnosis is based solely on clinical criteria and the time of onset. • ES occurs frequently after auto-transplants but is rarely seen after allotransplantation. • It usually resolves quickly without sequela when identified early and treated promptly with glucocorticoids.
Veno-occlusive disorder/sinusoidal obstruction syndrome (VOD/SOS) [225]
• This clinical syndrome is characterized by jaundice, fluid retention and tender hepatomegaly. • It occurs in 3-54% of allotransplant recipients (mainly MAC); 0-3% of auto-transplants. • It is characterized by injury of endothelial cells in the liver acini with altered microcirculation; pre-existing liver disease is among the predisposing factors. • Diagnosis is based on time of onset, clinical and laboratory criteria (EBMT-criteria). • Mortality without adequate treatment is 70-80%; with adequate treatment 25%.
Diffuse alveolar hemorrhage (DAH) [222]
• Definition: Pulmonary bleeding originating from alveoli and due to disruption of the alveolar-capillary basement membrane in the absence of infection, heart failure or severe thrombocytopenia. • DAH is characterized by increasing blood-containing fluid during sequential bronchioalveolar lavage. • It occurs in 2-17% in allo-SCT cases and in 1-21% in autotransplantation cases. • Histopathology: Capillaritis with neutrophil infiltration and necrosis of alveoli and capillaries. • Initially symptoms are shortness of breath and coughing. Hemoptysis occurs in less than 33% of patients. • Mortality rate is 80-100% in patients requiring mechanical ventilation; it is 50% in patients diagnosed early and treated with glucocorticoids.
ASCT-associated thrombotic microangiopathy (TMA) [226]
• The clinical syndrome is characterized by generalized endothelial dysfunction with microangiopathic hemolytic anemia, thrombocytopenia, proteinuria, hypertension, renal dysfunction and hemorrhagic diarrhea. • The cause is probably multifactorial but the conditioning treatment and toxicity related to calcineurin inhibitors as well as GVHD are probably important in the pathogenesis. • It is seen in up to 14% of allotransplant recipients but is rare in autotransplantation. • Currently, there is no effective treatment; severe cases are usually fatal.

As seen in Table 8, even though these complication can involve various organs, several show pulmonary affection. There is a risk of developing irreversible multiorgan failure, and they have high mortality rates even when the patients receive early, adequate treatment.

Infectious complications contribute significantly to morbidity and mortality after ASCT. The risk of infectious complications depends on several factors including graft source, immunosuppressive treatment and whether a T cell-depleting conditioning regime is used [227,228]. During the preengraftment period up to 30 days after the transplantation; the most important predisposition is neutropenia with mucositis, and infections with gram-negative bacteria and yeasts (candida species, *Aspergillus*), along with herpes simplex infections, are most common. Both the duration of neutropenia and the degree of mucositis are influenced by the intensity of the conditioning regime. During the postengraftment period (from day +30 to +100) T– and B-cells defects, aGVHD and prolonged exposure to immunosuppressive drugs are the most important predisposing factors [229,230]. The use of T cell-depleting therapies (e.g. ATG) or grafts with reduced numbers of T cells (e.g. umbilical cord blood grafts) also contributes to the risk of infection [231-234]. Latent virus reactivation (CMV and EBV) and mold infections are generally seen during this later period after transplantation [235-237]. During the late phase, after day +100, T– and B-cell defects, together with cGVHD and its treatment, are the most important predispositions. Patients with cGVHD often show impaired humoral immunity with an increased risk of infections with encapsulated bacteria; infections with *Aspergillus* species*, Pneumocystis jiroveci* and *Herpesviridae* are also frequent [235,236]. Thus, these most prominent infections follow a predictable chronology after allotransplantation. However, seasonal viral infections (e.g. influenza, adenovirus) have been observed throughout the posttransplant period, and they constitute a significant risk for all patients until adequate immune reconstitution has been established, a process which occurs gradually over the first two years after transplantation [238]. Adequate neutrophil count and function typically occur within the first 2 to 4 weeks, while normal numbers of circulating NK-cells and total T cells are typically seen within the first 100 days posttransplant [239,240]. However, quantitative $CD4^+$ T cell defects with decreased numbers of T cells capable of autocrine proliferation in response to activation signals can be seen for several

months posttransplant, and it may take as long as 2 years before fully functional cellular and humoral immune systems have been developed.

7.7.4 **Idiopathic pulmonary syndrome and IL-6**

Idiopathic pulmonary syndrome (IPS) is defined as bilateral pulmonary infiltrates without evidence of infection, cardiac dysfunction, renal failure or iatrogenic fluid overload [186,241]. IPS typically occurs between day +9 and +14 posttransplant. The incidence of IPS depends on several transplant– and patient-associated factors but is usually estimated to be between 3 and 15% [242]. Among the factors associated with IPS are immunological parameters (e.g. GVHD, HLA disparity), patient characteristics (e.g. age) and toxicity due to radiation or chemotherapy (e.g. MTX) [241,242]. The cause is probably multifactorial, and the current concept of its pathophysiology is that several independent pulmonary insults collectively result in IPS. Although the lung has traditionally not been regarded as a target organ in GVHD, clinical observations and animal models suggest that the initial insults result in immune-mediated tissue damage and dysfunction [243-246]. The release of proinflammatory cytokines has been linked to both immune dysfunction and pulmonary injury; the patients usually show increased levels of TNF-α, IL-6 and IL-8 in serum and bronchoalveolar lavage, but IL-6 together with TNF-α have been shown to be most important because they both contribute to the altered immunoregulation in addition to mediating direct cytotoxicity to the lung [247-249].

IL-6 levels are increased in allotransplant recipients experiencing IPS compared with patients without this complication, and the levels are highest for patients being refractory to treatment [249]. In a study of 240 patients increased levels of IL-6 and the soluble interleukin 1 receptor-like 1 (ST-2) could differentiate patients with IPS from unaffected controls without complications, but IL-6 levels could not be used to discriminate between patients with IPS and viral pneumonia [250]. Finally, increased IL-6 levels on day +7 posttransplant were associated with later IPS and increased mortality.

The effect of IL-6 on the development of IPS has been investigated in an animal model [249] which indicated that the development of IPS depends on IL-17 secreting donor $CD4^+$ T-cells. The non-hematopoietic compartment of host pulmonary cells was most important for the local

IL-6 release, and this was different from other proinflammatory cytokines (e.g. IL-21 and TGFβ) that were released both by host and donor cells. Systemic pharmacological blockade of IL-6 resulted in a significant reduction in absolute numbers of TNF-α-releasing Th17 cells. Genetic blockade of IL-6 production in the recipient also resulted in a significant reduction of Th17 cells in the lung and attenuated TNF-α release. Taken together, these observations suggest that IL-6 and TNF-α are among the key components in the pathogenesis of IPS, and combined blockade or neutralization of these two cytokines may be a possible strategy for treatment of IPS. Clinical studies have also reported favorable effects of TNF-α blockade in these patients, but no reports of IL-6 targeting treatment in IPS are available [251-254]. However, in two single-arm studies investigating the effects of standard GVHD prophylaxis plus tocilizumab, the overall incidence of IPS was less than 2 % [255,256].

7.8 CLINICAL PRESENTATION AND GRADING OF GVHD

GVHD can be divided into acute and chronic forms, each with distinct pathophysiological and clinical features [257-259]. The two forms were originally defined by differences in the time point of clinical manifestation; aGVHD was defined as occurring within the first 100 days posttransplant whereas all later manifestations were termed cGVHD. However, patients receiving nonmyeloablative/RIC regimes often show later T cell engraftment and often present with classic aGVHD beyond day 100 [260]. This led to revisions in the criteria and the following definitions of acute and chronic GVHD [257]: (i) Classic aGVHD presents within 100 days posttransplant and has clinical features of aGVHD and no features of cGVHD; (ii) Persistent, recurrent or late aGVH*D* shows the first of subsequent episode(s) of aGVHD later than 100 days posttransplant and has no features of cGVHD; (iii) Classical chronic GVHD has the features of cGVHD without any features of aGVHD and this is irrespective of time point; and (iv) Overlap syndrome with features of both acute and chronic GVHD and this is irrespective of time point.

7.8.1 **A short overview of clinical manifestations of acute GVHD**

Presentation of aGVHD occurs in the skin, gut and liver, whereas cGVHD has also been described in lymphoid organs and mucous membranes of the airways [257,261]. GVHD can theoretically also be directed against the hematopoietic systems of the recipient, but these T cell responses will not be clinically apparent because hematopoiesis after transplantation is mainly of donor origin [262]. Nevertheless, severe immunological manifestations of graft and host interactions do also occur in other organs (especially the lungs but also the thymus [243,263]). These manifestations are generally not termed GVHD, (see sections 7.7.3 and 7.7.4).

Skin. Acute skin GVHD is observed in about 75% of patients and often coincides with engraftment (10-30 days posttransplant) [186]. Skin manifestations range from a local to generalized rash and vary in intensity from light maculopapular rash to generalized bullous and desquamating toxic epidermal necrolysis. The first histological changes are infiltration of mononuclear cells (mainly $CD8^+$ and $CD4^+$ T cells) and degeneration of the basal layer, especially in the hair follicles and at the rete ridges at the site of epithelial stem cells. In established skin GVHD, the epidermis is thinned with lymphocyte infiltrates accompanied by scattered damaged and apoptotic keratinocytes [264].

Gastrointestinal. Acute GVHD of the GI tract is seen in about 30-50% of patients and is categorized into two forms based on the area affected [38]. GVHD of the upper tract usually presents with stomach cramps, nausea and vomiting, while lower GVHD presents with diarrhea with or without melena/hematochezia and abdominal pain. Histological features of gastrointestinal GVHD are infiltration of lymphocytes in the lamina propria, apoptotic cell death and atrophy of the normal structures, which may lead to ulceration. Gastrointestinal GHVD is important for the amplification of systemic GVHD reactivity since the GI tract is a main site for antigen presentation and T cell activation [264].

Liver. Liver involvement is observed in less than 20% of patients, and isolated involvement is rare [99]. The most common manifestation is jaundice without increased transaminases, coagulopathy and hepatic encephalopathy. Early histopathological changes involve infiltration of lymphocytes within the portal triads with apoptosis and destruction of epithelial cells in the small bile ducts. This leads to dysplastic changes of the bile ducts with cholestasis [264].

7.8.2 **Diagnosis, grading and prognosis of acute GVHD**

The diagnosis of GVHD is based on clinical findings and exclusion of other conditions that mimic GVHD. Histological evaluation is often done to rule out infections or drug toxicities [264]. However, GVHD is a result of a complex, dynamic and multifactorial process that often changes over time, and affected organs often have histopathological changes evoked both by pretransplant factors (e.g. conditioning therapy), pre- and posttransplant immunosuppression and antibiotics. For these reasons, histopathological changes are often cited as support for a diagnosis of GVHD, but alone they are not considered sufficient evidence for diagnosis. In addition, manifestations are often patchy, making it easy to miss relevant lesions by biopsy [264,265].

Table 9. *Staging and grading of acute GVHD.*

Organ	**Stage 1**	**Stage 2**	**Stage 3**	**Stage 4**
Skin	Maculopapular rash <25% of BSA	Maculopapular rash 25 to 50% of BSA	Generalized erythroderma	Generalized erythroderma with bullous formation and often with desquamation
Liver	Bilirubin 34 to 51 µmol/L; ASAT 150 to 750 international units	Bilirubin 52 to 103 µmol/L	Bilirubin 104 to 257 µmol/L	Bilirubin >257 µmol/L
Gut	Diarrhea: >30 mL/kg or >500 mL/day	Diarrhea: >60 mL/kg or >1000 mL/day	Diarrhea: >90 mL/kg or >1500 mL/day	Diarrhea >90 mL/kg or >2000 mL/day; or severe abdominal pain with or without ileus
Glucksberg scale				
Grade 1: Stage 1 or 2 skin involvement; no liver or gut involvement; ECOG Performance status 0				
Grade II: Stage 1 to 3 skin involvement; Grade 1 liver or gut involvement; ECOG PS 1				
Grade III: Stage 2 or 3 skin, liver, or gut involvement; ECOG PS 2				
Grade IV: Stage 1 to 4 skin involvement; Stage 2 to 4 liver or gut involvement; ECOG PS3				
PS, performance status				

Grading of aGVHD is important for evaluating when to initiate steroid therapy and for assessing response to therapy. Several systems have been developed based on performance status plus evaluation of the effects on the skin, the GI tract and the liver. The Glucksberg grading from I to IV and the International Bone Marrow Transplant Registry grading from A to D are the most widely used grading systems [266,267]. Both these systems are based on assessments of the degree (termed stage) of the effects on the liver (the bilirubin value), skin

(affected body surface area and clinical features), GI tract (amount of diarrhea/day) and the overall performance status. The stages determined for each organ system are then combined to an overall grade of aGVHD. Grade I GVHD is characterized as mild disease, grade II GVHD as moderate, grade III as severe, and grade IV as life-threatening. A detailed description of the grading system is given in table 9.

The overall grade and response to steroid therapy are the most important prognostic factors for patients with GVHD [186]. Grade II-IV is associated with significant increases in infections and the development of cGVHD with a reduced survival rate. This is especially true for patients with steroid-refractory GVHD, which has a dismal prognosis and long-term survival of 10-15%. Patients with positive responses to steroid treatment have still inferior outcomes compared with patients without GVHD [268-270].

7.8.3 **The pathogenesis of acute graft-versus-host disease**

The graft-versus-host reaction includes initial recognition and subsequent destruction of host tissue by donor leukocytes. The main effector cells in GVHD are T cells, but several other cell types such as macrophages, granulocytes and NK-cells are also directly involved. The process that eventually leads to GVHD is a multistep process that has traditionally been divided into three phases (summarized in Figure 4) [259,271,272].

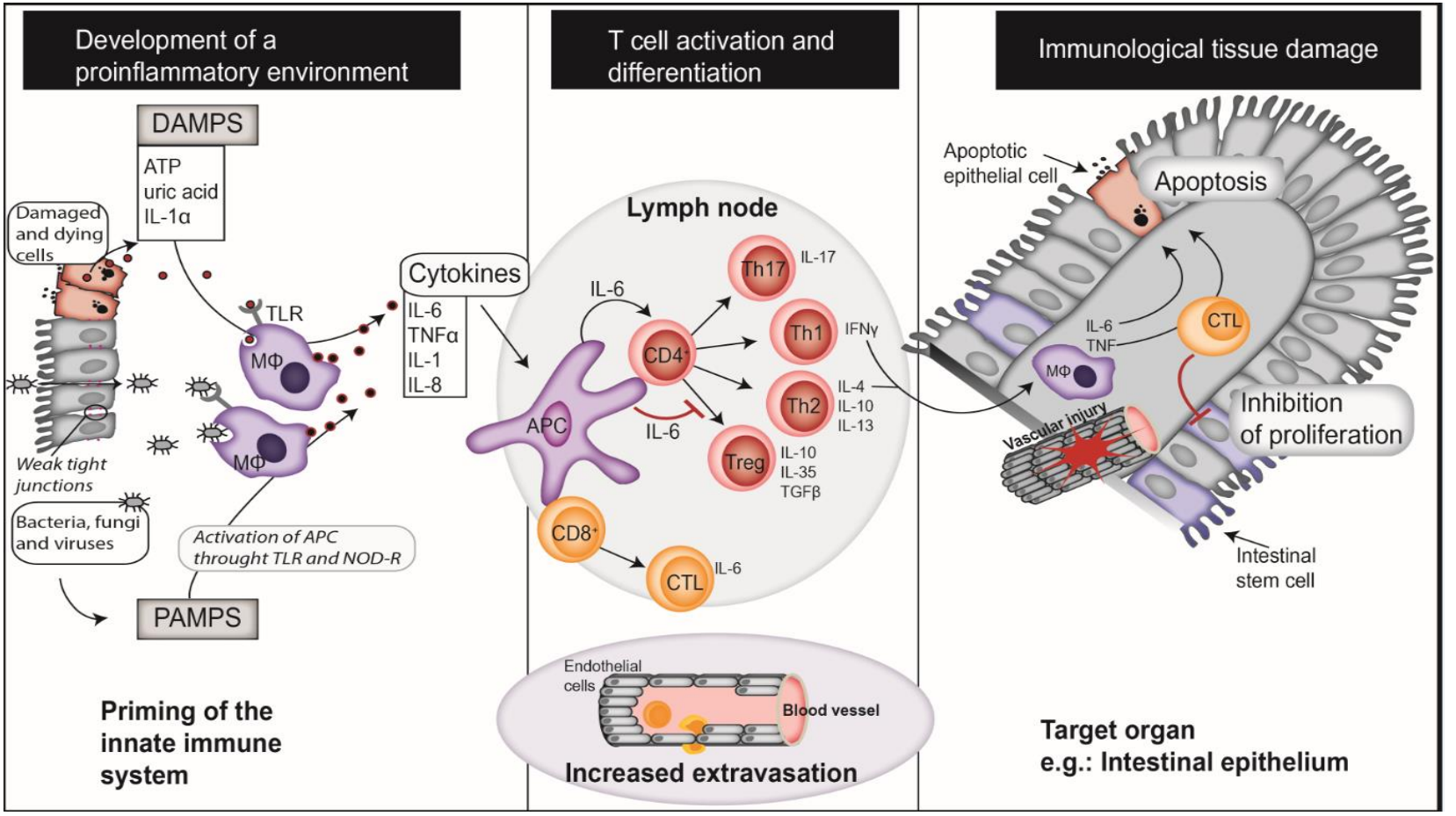
The pretransplant phase 1: Activation of the innate immune system. This is the pretransplant phase; the cytokine environment formed during this phase will later influence the recruitment, proliferation and differentiation of donor T cells in secondary lymphoid organs. Radiation and chemotherapy result in sterile inflammation caused by the release of damage-associated molecular pattern (DAMP) mediators that activate NOD-like receptors. The disruption of endothelial and epithelial barriers results in the translocation of intestinal bacteria and fungi that causes pathogen-associated inflammation with ligation of Toll-like receptors [273]. Ligation of both NODs and TLRs finally results in the release of proinflammatory cytokines (e.g. IL-6, TNF, IL-1) and subsequent activation of APCs [274]. These activated cells then increase their phagocytic capacity, the presentation of foreign and self-peptides on MHC and their expression of T-cell costimulatory molecules (e.g. CD80 and CD86) .

Several factors seem to influence the responsiveness to DAMPs, including specific genetic polymorphism in NOD-receptors, reducing the cytokine response as well as dysregulated production of mucus and antibacterial peptides that regulate the intestinal microbiome [275,276]. Pre-existing risk factors associated with infections or inflammation, such as advanced stage leukemia and history of viral infections, enhance these processes [277].

The early posttransplant phase 2: Donor T cell recruitment, activation and proliferation. After infusion of the stem cell graft, donor T cells are recruited to inflamed tissues and lymphoid organs where they encounter APC expressing MHC loaded with self and non-self/foreign proteins [278]. This is secured through endothelial adhesion molecules that are upregulated as a consequence of the proinflammatory environment created during the first phase [272,278,279]. The interaction between TCR and MHC molecules requires additional stimulation through co-receptors expressed by APC (e.g. CD80, CD86) to complete T cell activation [278]. Blockade of this costimulation significantly reduces GVHD in murine models [280].

Although cytotoxic T cells are the major effector cells in GVHD, T-helper cells also contribute and significantly influence the outcome [259,272,278]. First, Th1 cells release IFN-γ at high levels; they express the transcription factors STAT4 and STAT1 [281]; and their differentiation seems important for the development of aGVHD of the gastrointestinal tract [282]. A majority of these early posttransplant circulating TCRαβ$^+$ CD4$^+$ and CD8$^+$ T cells release IL-6 at relatively high levels, in addition to IFNγ and TNFα [283]. Second, Th2 cells are characterized by secretion of the anti-inflammatory cytokines IL-4, IL-10 and IL-13 [284]; the available studies of Th2 cells in GVHD have shown conflicting results, but some studies describe an association between Th2 differentiation and pulmonary and skin involvement in aGVHD [282,285]. Third, Th17 cells characterized by IL-17 secretion and expression of the transcription factor RORγt are possibly important factors in the severity of aGVHD and severe, early transplant-related lung injury [249,286]. Finally, Tregs are suppressed during GVHD and resolution of GVHD is associated with restored Treg function [287]. As described in chapter 7.3.3, IL-6 is an important regulator of the balance between Th17-cells and Tregs [102].

Figure 4. *A brief overview of the three different steps in GVHD pathogenesis. The first phase is characterized by activation of the innate immune system and antigen-presenting cells and the second by stimulation and proliferation of alloreactive T cells. During the third phase host tissues are damaged by cytotoxic T cells and the release of proapoptotic cytokines (for additional details see section 7.8.3).*



The later posttransplant phase 3: Cellular and cytokine-mediated tissue damage. End-organ damage is mediated by cytotoxic T cells but also by cytokine secretion ([278,284]. Cytotoxic T cells induce apoptosis either through their release of Fas Ligand and through the perforin/granzyme pathway [278,288]. Furthermore, several of the cytokines secreted during the early phases of GVHD increase the expression of Fas-receptors as well as other proapoptotic receptors that augment the proapoptotic effects mediated by the cytotoxic T cells [284]. The inflammatory microenvironment created during the earlier phases of GVHD also augments T cell-mediated cytotoxicity through several additional mechanisms including upregulation of adhesion molecules, release of chemoattractants with increased migration of cytotoxic T cells to the target tissues and increased MHC expression in target tissues.

7.8.4 **IL-6 in animal models of acute and chronic GVHD**

Several mouse models have been created to examine the role of IL-6 in acute and chronic GVHD [136,249,289-293]. Givon et al. examined the effect of IL-6 on bone marrow reconstitution after ASCT [136]. They showed that posttransplant addition of recombinant IL-6 significantly improved survival, and IL-6 also improved WBC reconstitution, but only after transplantation with low stem cell doses. However, IL-6 increased the severity and mortality of GVHD.

Chen et al. studied the role of IL-6 in a GVHD-specific mouse model. They showed that L-6 and IL-6R levels increased early during posttransplant and these levels remained high in mice that later developed GVHD [290]. IL-6 and IL-6R expression increased in the liver and colon but not in the spleen. Selective knockout of IL-6 in recipient or donor cells did not influence the clinical characteristics or outcomes of GVHD. Conversely, anti-IL-6 treatment led to amelioration of GVHD with less weight loss, less histopathological damage in the colon, liver and lungs, reduced levels of Th1 and Th17 cells and increased levels of Treg cells. The increased Treg levels were independent of the thymic function. Similar results were also reported by Noguchi et al [289]; they showed that anti-IL-6 treatment reduced T cell infiltration, transaminase levels, organ failure and also mortality. The treatment also decreased levels of Th1 and Th17 cells and increased Treg levels.

Tawara et al. investigated the effects of IL-6 derived from donor T cells, and they showed that IL-6 levels were higher in animals transplanted with allogeneic cells compared to animals transplanted with syngeneic cells [292]. Furthermore, animals receiving grafts with T cell-selective knockdown of IL-6 developed less severe GVHD and had prolonged survival compared to grafts with normal IL-6 expression; serum cytokine levels and levels of circulating T cells subsets were not altered by this treatment, and selective knockout of IL-6 in the recipients BM did not have a similar effect. However, pretransplant treatment with IL-6 neutralizing antibodies also improved survival as well as clinical and histopathological severity of GVHD without altering Treg levels. Finally, the GVL effect was maintained despite the reduced GVHD. The observations in this model are different to the study by Chen et al. that did not observe any effect of IL-6 neutralization on GVHD manifestations [290].

Belle et al. examined the effects of IL-6 on GVHD-mediated cerebral inflammation in mice [291]. The authors demonstrated that alloreactive T cells accumulated within the CNS when mice were transplanted with cells from MHC-mismatched donors. This was accompanied by increased mRNA levels of proinflammatory cytokines (IFN-γ, TNF-α, IL-6). Pharmacological IL-6 blockade reduced donor-derived $CD4^+$, $CD8^+$, and $TCR\alpha\beta^+$ T cell infiltration. Furthermore, silencing of IL-6 production in the host significantly reduced neuroinflammation, whereas selective IL-6 knockout in donor cells did not have any effect. The accumulation of Treg cells in the CNS was not affected by any of the strategies. However, an IL-6-regulated expansion of microglial cells expressing the immunoregulatory enzyme Indoleamine-pyrrole 2,3-dioxygenase (IDO-1) was observed, but IL-6 blockade did not reverse the reduction of neuroprotective IDO-1 metabolites that is seen in GVHD. Thus, this study suggests local IL-6 release plays a role in the development of GVHD; this is similar to the observations in previous studies in experimental models of IPS [249]. However, these CNS observations are difficult to translate into a clinical context since the brain is generally not regarded a target organ of acute or chronic GVHD.

Animal models have also shown that IL-6 levels increase during progression of sclerodermal GVHD [293], and anti-IL-6 treatment to disease manifestation resulted in decreased severity of the disease. However, anti-IL-6 treatment started after the onset had no effect on disease severity. The IL-6 targeting therapy increased the number of Treg cells

and decreased the expressions of IFN-γ, TNF-α, IL-6, IL-18, TGF-β1, CCL2, CCL3 and CCL5 in the skin.

Taken together, these animal studies suggest that the IL-6 system is important for the development and regulation of allogeneic T cell reactivity after ASCT, but it should be emphasized that the contribution of the IL-6 system differs between models.

7.9 THE ROLE OF BIOMARKERS IN ASCT

7.9.1 Overview and general consideration

There are several potential uses of biomarkers in the ASCT setting [259,294,295]. First, biomarkers could be used for pretransplant identification of patients with increased risk of posttransplant complications; this would then allow for interventions to reduce this risk. Second, biomarkers may allow earlier diagnosis of GVHD. This diagnosis is mainly based on clinical symptoms and signs, together with the exclusion of other causes. The use of biomarkers could allow for earlier therapeutic interventions when GVHD may be more responsive to treatment. Third, biomarkers may be used to predict the response to treatment and/or identify patients at risk of complications secondary to immunosuppressive therapy. Furthermore, identification of biomarkers would likely increase our knowledge about GVHD pathogenesis. However, one has to remember that the incidence of various transplant-related complications is highly dependent on transplantation procedures, disease-related factors and patient characteristics. Candidate markers therefore need to be validated in different patient cohorts that are relatively homogeneous, at least for the most important transplant- and patient-associated factors (e.g. stem cell donor, conditioning therapy, stem cell graft, GVHD prophylaxis), but one should also investigate population-based cohorts of unselected patients to evaluate whether a biomarker can be used for allotransplant recipients in general.

Several technical aspects should be considered [294]. Samples evaluated in clinical studies have usually been stored for a relatively long time period, and the effect of storage is not known. The issues of assay sensitivity as well as assay specificity, including interference and cross-reactivity, should also be considered. The identification of a wide range of potential biomarkers for GVHD is possible due to several new, large-scale analytical technologies, e.g. genomic, proteomic, transcriptomic and metabolomics methods. Several

new biomarkers have now been validated and are entering clinical studies; a summary of the most important markers is given in Table 10. The following discussion will focus on the IL-6 system and acute phase response as a biomarker for GVHD.

7.9.2 **IL-6 as a biomarker for ASCT outcome**

Pretransplant IL-6 levels. Only a limited number of studies have investigated how pretransplant cytokine levels affect outcomes after ASCT. Although increased levels of other proinflammatory cytokines (e.g. TNF-α) at the time of transplantation are associated with an increased risk of posttransplant complications, we have not been able to identify any larger studies that have evaluated the effect of pretransplant IL-6 systemic (i.e. plasma or serum samples) levels on posttransplant outcomes.

Posttransplant IL-6 levels. The course of posttransplant IL-6 serum/plasma levels has been evaluated in several studies [249,296-302]. IL-6 levels usually increase significantly during the first 2 weeks posttransplant; peak IL-6 levels often coincide with the leukocyte nadir before they return to baseline (i.e. near undetectable) in patients without clinical complications [299,302-304]. One study suggested that IL-6 levels during this early posttransplant period were associated with clinical symptoms like fatigue, poor appetite, pain, drowsiness, dry mouth, and sleep disturbances [305]. One study observed elevated IL-6 level on day +6 or +7 posttransplant in patients that later developed sinusoidal obstruction syndrome [306]. Furthermore, the posttransplant IL-6 levels were observed to correlate with the degree of mucositis, but the intensity of the conditioning regime did not seem to influence IL-6 levels [305,307]. TBI-based conditioning seems to be a potent inducer of IL-6 during the early posttransplant period [308].Treatment with ATG or alemtuzumab strongly influences the release of several proinflammatory cytokines [309], while ATG induces transient increases in IL-6 levels posttransplant the effects of alemtuzumab have not been investigated.

Several studies have demonstrated that IL-6 significantly increases during GVHD and when clinical signs of posttransplant infections are present [300,310]. This may be due to tissue damage associated with the conditioning regime and the concurrent risk for aGVHD. McDonald et al. demonstrated that IL-6 levels at the onset of GVHD predict the later severity of GVHD [311].

Table 10. *A summary of potential biomarkers for development of GVHD or survival after allogeneic stem cell transplantation.*

Biomarker	Organ or function	Biological function and significance in GVHD
ST-2 (Interleukin 1 receptor-like 1)	Epithelium	Soluble ST-2 is a decoy receptor for IL-33 that has both pro- and anti-inflammatory properties [312]. It seems to augment the proinflammatory activity of IL-33. Elevated ST-2 level at day +7 posttransplant is associated with an increased risk of GVHD and poor response to steroids [313,314].
Reg3α (Regenerating islet-derived protein 3α)	Epithelium	Reg3α is an antimicrobial peptide produced in the GI tract; it enters systemic circulation after damage to intestinal epithelium, and the level correlates with the degree of endothelial denudation. High levels early after transplantation and early after onset of GVHD are associated with intestinal GVHD and poor response to therapy [310,313].
Ang-2 (Angiopotein-2)	Endothelial damage	Angiopoietin-2 is a marker of endothelial damage. High pretransplant levels are associated with steroid-refractory GVHD and increased mortality [315].
sIL-2R (Soluble IL-2 receptor)	T cell development	SIL-2R is a marker of T cell activation and sIL-2R levels increase 1–2 weeks prior to clinical onset of aGVHD [316].
TNFR1 (Tumor necrosis factor receptor 1)	Proinflammatory activity	TNF-α participates in the initial amplifications of GVHD [259]. TNFR1 increases in response to tissue damage and is a surrogate marker of TNF-α production. An increase in TNFR1 posttransplant correlates with in increased GVHD severity and reduced overall survival [317,318].
IL-6	Proinflammatory activity	IL-6 levels reflect early inflammatory status and are increased at the onset of aGVHD [296-298].
Elafin	Skin	One study reported that eElafin plasma levels at onset of GVHD correlated with severity of skin GVHD. Another study reported that increased elafin levels were observed in cutaneous aGVHD and chronic lichenoid GVHD but not chronic sclerotic GVHD. Elafin levels did not discriminate between aGVHD and drug hypersensitivity reactions [319,320].
TIM-3	Proinflammatory activity	Posttransplant levels predict grade III-IV aGVHD. Tim-3 plasma levels were higher in patients with mid-gut compared with upper-gut GVHD patients and those without GVHD. TIM-3 levels seem to be highest at onset of aGVHD [321].
alpha-1- antitrypsin	Damage to intestinal epithelium	Fecal levels of alpha-1-antitrypsin reflect intestinal damage and intestinal protein loss; these levels correlate with GVHD stage and predicts steroid-refractory aGVHD [322].

Even though the results from other studies regarding posttransplant IL-6 levels and the risk of GVHD are conflicting and difficult to interpret due to study design, low patient numbers, heterogeneity of patient population and/or lack of validation cohorts, several recent review articles conclude that increased IL-6 levels in the early posttransplant period predict later severe GVHD [259,294].

7.9.3 **Acute phase response and risk of GVHD**

Pretransplant CRP levels. Several studies investigating the role of pretransplant CRP serum/plasma levels are summarized in Table 11 [323-330]. Most of these studies indicate that increased CRP levels are associated with increased TRM and reduced long-term overall survival. The results from studies of CRP and the risk of GVHD are conflicting. Three studies identified pretransplant CRP level as an independent risk factor for aGVHD [324,327,328], and one study showed that higher levels conferred a higher risk of cGVHD [330], but most studies did not detected any significant association between pretransplant CRP levels and later GVHD. However, several factors influence CRP levels, especially active malignancy and infections, and almost all these studies either included a high number of patients with active malignancies or the information about disease status was missing or incomplete. Furthermore, the different patient cohorts were highly heterogeneous with regard to conditioning therapy or stem cell donor/graft, making it difficult to draw robust conclusions. The study by Pavlu et al. is an exception [325]; it only included allotransplanted CML patients, and data on patient comorbidity were also presented. These investigators observed a highly significant association between increased pretransplant CRP levels and increased TRM, but not with the overall risk of GVHD. Finally, even though IL-6 and CRP serum levels are usually highly correlated, no existing studies have evaluated both these factors.

Table 11. *Summary of studies investigating the effects of pretransplant CRP levels on outcomes after allogeneic stem cell transplantation.*

Author	Year	Patient number	Non-malignant disease	Related/ unrelated	Stem cell source		Remission/ high risk	HCT-CI	Effect on				
		(Number)	(Number)	(Number)	BM	PBSC			aGVHD	OS	TRM	RRM	cGVHD
Artz [324]	2008	112[a]	5	68/44	7	105	NR/ 52	Yes	Yes	Yes[b]	Yes	NR	NR
Pavlu [325]	2010	271	No, only CML	130/141	256	15	NR/ 113	Yes	NR	Yes	Yes	No	NR
Remberger[326]	2010	504	~16%	196/229	156	312	NR	NR	No	Yes	Yes[dc]	Yes[ed]	NR
Sakamoto[330]	2012	211	8/211	86/95	95	86	NR, 90/121	NR	No	No	Yes	No	Yes
Aki[323]	2012	106	No	97/9	0	106	22	Yes	NR	Yes	No	NR	NR
Sato[327]	2013	90	NR	39/51	58	24	NR/12%	NR	Yes	Yes	Yes	NR	NR
Jordan[329]	2013	349	11%	170/179	227	121	NR/ 113	NR	No	Yes	Yes	No[ce]	NR
Yamamoto[328]	2016	78	0	24/54	47	3[f]	NR/46	Yes	Yes	Yes	Yes	Yes	No

a: Data only for 81 patients. b: Effect of CRP only, shown in univariat analysis. c: Data only for the RIC cohort. d: Effect only in univariat analysis. e: Increased CRP at day of stem cell infusion showed significantly higher relapse-related mortality. f: 28 patients transplanted with unrelated umbilical cord blood as stem cell source.

Abbreviations: BM: Bone Marrow, PBSC: Granulocyte colony-stimulating factor (G-CSF) mobilized peripheral blood stem cells, NR: Not reported, RRM: Relapse-related mortality

Posttransplant CRP levels. Serum/plasma CRP levels usually show only minor variations during conditioning treatment, but one study described a 5-50 fold increase for a minority of patients treated with ATG [331]. For most patients this increase was transient; the levels normalized rapidly after cessation of ATG, and the increases were not associated with adverse prognoses. Thereafter, CRP levels showed similar increases as did IL-6 in the first 2 weeks posttransplant; this inflammatory response was seen also for patients without clinical or microbiological evidence of infections [332]. This inflammation seems to be caused by conditioning therapy, and the type of conditioning seems to significantly influence the course of posttransplant CRP levels. However, despite this early posttransplant increase, systemic CRP levels are still a sensitive early marker for systemic infection (especially bacterial and fungal infections) [333]. Persistent elevation of CRP above 160 mg/L for more than 5 days seems to be an independent risk factor for death due to infections [334].

Data on posttransplant CRP levels and prediction of later GVHD are conflicting. One study described that patients with maximum CRP level above 150 mg/L during neutropenia were more likely to suffer from GVHD grades II-IV [335]. In contrast, Schwaighofer et al. observed increased CRP levels after bone marrow transplantation (BMT) during severe infections or fevers of unknown origins, but not in aGVHD grade III/IV [336]. Other studies have not been able to demonstrate any significant correlation between CRP levels and GVHD either [296,337]. Furthermore, Min et al. described mean CRP levels during the first week posttransplant that were lower in patients that subsequently relapsed [338]. Thus, even though pretransplant CRP levels seem to be associated with posttransplant outcome, associations between posttransplant CRP levels and GVHD are less obvious.

7.10 GENETIC POLYMORPHISM AND OUTCOME AFTER ASCT

Genetic polymorphism is defined as the occurrence of a distinct DNA base sequence in a given population with an allele frequency of at least 1% [339]. The only difference between genetic polymorphisms and mutations is that mutations have allele frequencies below 1 %. This 1% distinction is an arbitrary limit [339]. Polymorphisms are classified as either (i) SNPs, where the base sequence varies only by a single nucleotide; (ii) tandem repeats, where a sequence of up to 1000 nucleotides is repeated; and (iii) copy number polymorphisms that show a difference in the copies of one or more sections of the DNA

consisting of 10^3-10^6 base pairs [340]. In the ASCT setting, genetic polymorphisms are divided into HLA gene and non-HLA gene polymorphisms.

7.10.1 HLA gene polymorphisms and risk of GVHD

The HLA system is a gene complex located on the short arm of chromosome 6 (6p21) and this complex carries the coding for the major histocompatibility complex (MHC) proteins. Different MHC proteins (HLA-A, B, C, DP, DQ and DR) present foreign and self-peptides to T cells, and the genetic regions encoding the peptide-binding groves are highly polymorphic [341]. These variations in the amino acid sequences of the groves determine which peptides are presented by the various MHC molecules on the surfaces of APCs. Polymorphisms in these regions also alter the affinity of MHC molecules to T-cell receptor molecules [341]. The matching of the different HLA polymorphisms between the allogeneic stem cell donor and recipient is the basis for selection of an appropriate donor according to generally accepted criteria (see section 7.7.2). HLA mismatch is the strongest predictive genetic polymorphism for GVHD [342,343].

The HLA complex also includes a large number of other genes that are important for the regulation of both the innate and the adaptive immune system. Inheritance of the various genes in this region is not random; these genes are in what is referred to as a *linkage disequilibrium* [344,345]. This means that there is a non-random association of various alleles at different loci; that is, different loci show linkage disequilibrium when the frequencies of association of their different alleles are higher/lower than what would be expected if they were associated randomly. Since almost all ASCTs are based on matching of at least 6-10 HLA loci the possible importance of other immunoregulatory genes within the HLA gene complex is difficult to evaluate in small cohorts, but recent studies indicate that other polymorphisms within the HLA gene complex also influence transplant outcome [346,347].

7.10.2 Non-HLA polymorphism

Several polymorphisms in genes outside the HLA-region also influence posttransplant outcome [340]. These polymorphisms have been detected either by examining selected candidate genes or through GWAS. Candidate gene analyses are usually restricted to a limited set of polymorphisms within a specific gene; such analyses are thus hypothesis-

driven and require in-depth knowledge of functions and interactions of the encoded protein. In contrast, GWAS investigates a large number of polymorphisms within the genome without considering the biological functions of the encoded proteins. The first non-HLA polymorphisms shown to influence posttransplant outcome were within genes that regulate the expression, function or downstream signaling effects of cytokines and their receptors [340]. Further studies have now shown that non-HLA polymorphism influence both the risk of GVHD and the susceptibility to severe infections after ASCT. [340,348]. An overview of relevant polymorphisms of the IL-6/IL-6R system is given in Table 12.

7.10.3 **AGVHD and IL-6 polymorphism**

Several SNPs in both the IL-6R and the IL-6 gene are associated with altered levels of IL-6 and sIL-6R. First, The SNP rs1800795 (also termed SNP 174 G<C and is in complete linkage disequilibrium with rs1800797, rs1800796) in the promotor region of the IL-6 gene influences the synthesis of IL-6 [34,35]. Previous studies have shown that this SNP is associated with the risk and/or severity of autoimmune disorders [345,349,350]. Ten studies have examined the role of this specific SNP in aGVHD (Table 12) [351-363]. The largest study investigating the role of IL-6 polymorphism in GVHD was performed by Chien et al. [362]. After correction for several other variables they demonstrated that the donor genotype rs1800795 was associated with a 20-50% increase in the risk of grade II-IV aGVHD. Similar observations have been made in four other studies, and a meta-analysis of seven studies concluded that patients who received grafts from donors that were either hetero- or homozygous for the IL-6 G allele of rs1800795 had an increased risk of severe aGVHD. Despite these observations, rs1800795 does not seem to influence the overall survival, as only two studies reported inferior survival [360,361]. It is not known whether this can be explained by associations between rs1800795 and higher response rates to steroid, lower risk of relapse or lower risk of severe infections. In contrast, data on the effects of rs1800795 on the rate of cGVHD are conflicting. Of the available studies, six reported no effect, whereas only three reported an increased risk of cGVHD.

Table 12. *A summary of studies investigating the influence of different SNPs in IL-6 and IL-6R genes on outcome after allogenic stem cell transplantation.*

Study	Year	Patients (Number)	Donor type (Number)	Stem cell source		aGVHD		cGVHD	Survival
				PB[1]	BM[1]	Genotype	Risk		
IL-6 rs1800795 or rs1800797									
Martínez-Laperche [364]	2018	359	Sibling 359	250	109	R	Increased	Increased	No
Alam [363]	2015	268	MRD 184	184	NR	D	Increased	NR	No effect
Chien [362]	2012	1298	Related 612 Unrelated 686	377	921	D	Increased	NR	NR
Ambruzova [361]	2008	56	Sibling 56	NR	NR		Increased	No	Decreased,
Ambruzova [360]	2009	166	Related121 Unrelated45	144	22	R		No	Borderline decreased
Karabon [359]	2005	93	Sibling	NR	NR	D/ R	Increased	NR	No
Laguila Visentainer [358]	2005	118	Sibling	36	82		No effect	Increased,	No effect
Mullighan [357]	2004	160	Sibling154	100	60	D	Increased	No	No effect
Lin [356]	2003	993	Sibling 993				No effect	NR	NR
Rocha [355]	2002	107	Sibling 107		107		No effect	No effect	No effect
Socié [354]	2001	100	Sibling 100				No effect	Increased	NR
Cavet 2001 [353]	2001	80	Sibling 80	80		R	Trend for higher grade	R or D increased	NR
IL-6-R polymorphism, rs4845617									
Kim [351,352]	2012 and 2014	394	MRD 288 MMD19 MUD84	276	118	Increased risk chronic eye GVHD. Recipient genotype associated with increased NRM.			

Abbreviations: PB: Peripheral Blood stem cell collection, BM: Bone marrow, D: Donor, R: Recipient, MRD: Matched related donor, MUD: Matched unrelated donor. MRD Mismatched related donor. NR: Not reported, NRM: Nonrelapse mortality. [1] Number of individuals that received BM or PB.

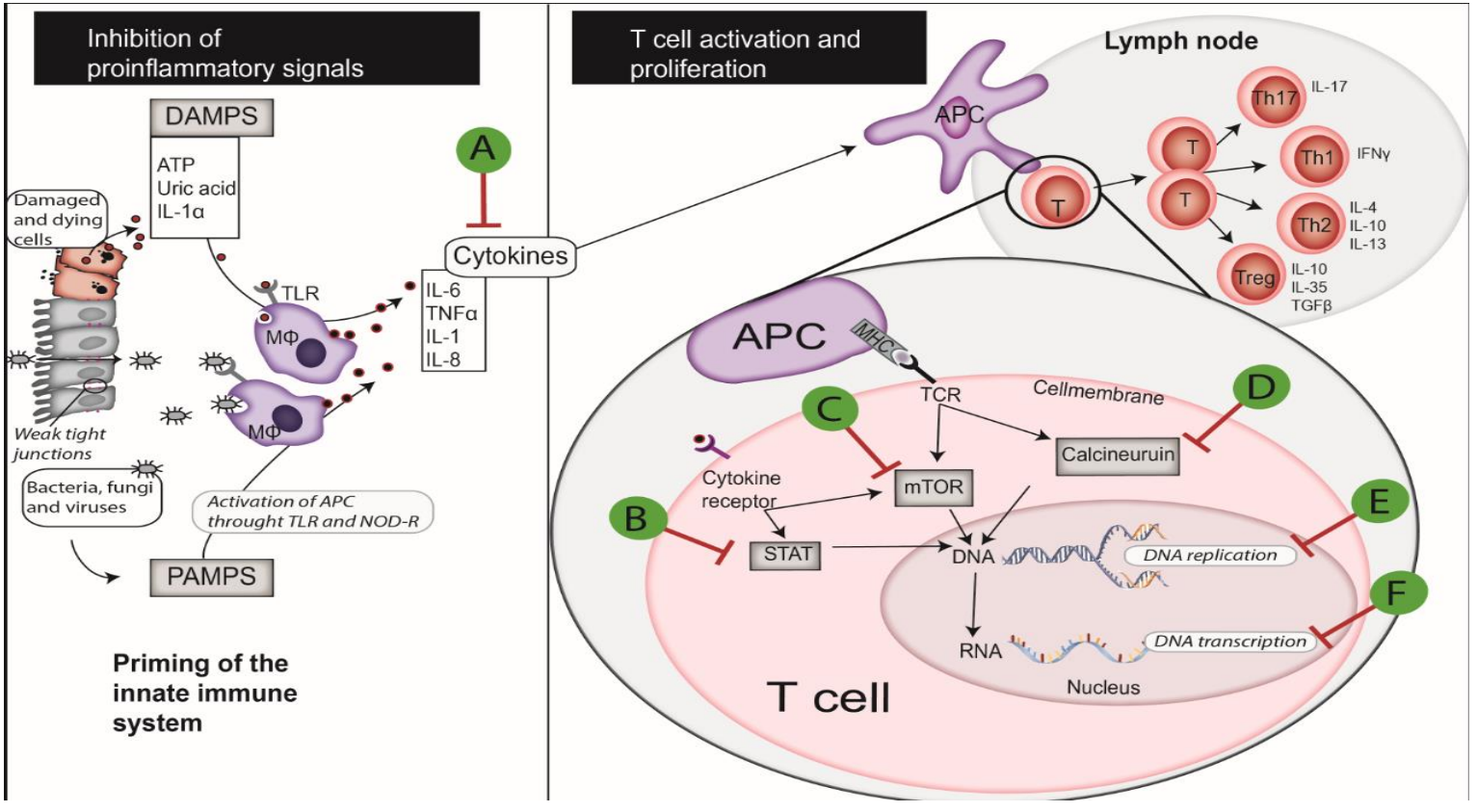
Only a few studies have investigated the effects of SNPs in IL-6R on outcomes after ASCT. Kim et al. [351] investigated the effects of 259 different SNPs on outcomes after allotransplantation and did not observe any effect of SNPs in the IL-6 gene, but patients with an SNP in the IL-6R gene (rs4845617) had decreased relapse-free survival. In a new study of the same patients, univariate analysis identified several SNPs in the IL-6R (rs2229238, rs4072391, rs4379670, rs7514452) that were associated with an increased risk of aGVHD, but they could not predict aGVHD in a multivariate analysis. SNP rs4845617 is in linkage disequilibrium with rs2228145 ($r^2 = 0.0155$) and may predict cGVHD of the eyes [352].

7.11 PROPHYLAXIS AND TREATMENT OF ACUTE GVHD

7.11.1 General principles for prophylaxis and treatment of GVHD.

GVHD has traditionally been regarded as a complication mediated by donor T cells, and most strategies for prevention and treatment of GVHD have aimed to deplete T cells or to reduce activation, proliferation, migration and differentiation of T cells (Figure 5) [259,271,278]. Thus, several therapeutic approaches are available, but there is still no general consensus on what are the optimal preventive and treatment strategies [265,268,365,366]. The following chapters will summarize the most commonly used therapeutic alternatives.

Figure 5. An overview over possible targets for GVHD prevention and treatment. ***(A)*** Inhibition of lymphocyte activation and proliferation by inhibiting proinflammatory cytokines, e.g. tocilizumab. ***(B, C, D)*** Specific blocking of intracellular signaling pathways crucial for T cell activation e.g. JAK1/JAK2 inhibition through ruxolitinib (B) or the AKT/mTOR pathway through tacrolimus (C) or cyclosporine (D). ***(E, F)*** Blocking of DNA synthesis (e.g. MTX), replication (E) and translation (F) (e.g. corticosteroids) in proliferating T cells.



7.11.2 **Pharmacological inhibition of T cell activation**

Cyclosporine A and tacrolimus. Cyclosporine A and tacrolimus are structurally different and have distinctive pharmacological and pharmacodynamical properties, but both drugs seem to exhibit their immunosuppressive effects by blocking calcineurin in T cells. Calcineurin is normally activated following T-cell receptor ligation, and it secures transcription of cytokines (e.g. IL-2, TNF-alpha, IL-3, IL-4 and CD40L) that are required for adequate T cell proliferation [367,368]. Thus, these drugs should be regarded as selectivity inhibitors of T cell proliferation.

Both drugs have small therapeutic windows, and drug monitoring is required to ensure adequate effects without serious adverse events. Most common acute adverse effects are impaired renal function, neurotoxicity, hypertension and metabolic changes [369]. Long-term use can be associated with irreversible progressive renal disease. Both drugs are used extensively in combination with MTX or mycophenolate mofetil (MMF) for GVHD prophylaxis [370]. Very few studies have directly compared tacrolimus and cyclosporine [371,372]. One study suggested that tacrolimus is more potent in preventing aGVHD, but it has not been possible to show that this translates into reduced TRM or increased overall survival [371]. Thus, the two drugs should be regarded as equally potent.

Methotrexate. Combination of MTX with either cyclosporine A, tacrolimus or MMF has been regarded as the standard GVHD prophylaxis for the last 30 years [370]. MTX is an antimetabolite that inhibits dihydrofolate reductase, and it seems to interfere with immune responses through inhibition of T cell proliferation, increasing T cell responsiveness to proapoptotic signals and modulation of T cell trafficking [373,374]. Prolonged administration of MTX posttransplant is not possible due to unacceptably high rates of mucositis, myelosuppression, hepatotoxicity and renal impairment, but low-dose MTX therapy (single daily doses of 5-15 mg/m^2) with or without leucovorin rescue is usually well tolerated. Prophylactic MTX is usually administered on days +1, +3, 6 and +11 posttransplant [370,375,376].

Mycophenolate mofetil. MMF is a prodrug which is metabolized in the liver to the active metabolite mycophenolic acid that has both antibiotic, antiviral and cytotoxic effects and specifically inhibits the inosine-5'monophosphate dehydrogenase [377]. This enzyme is crucial for synthesis of the mononucleotide GMP that is essential for DNA synthesis and

proliferation [377]. Mycophenolic acid selectively inhibits B and T cell proliferation because these cells cannot synthesize GMP during proliferation [378]. Mycophenolic acid also has T cell-independent immunosuppressive effects through its interference with glycosylation of adhesion molecules [379].

MMF is typically used in combination with a calcineurin inhibitor as aGVHD prophylaxis, usually in RIC transplantations or transplantation with umbilical cord stem cells [370]. There is no evidence that MMF is more effective than MTX in preventing aGVHD, and many regard them as equally effective for GVHD prophylaxis [370]. All studies investigating the effect of MMF in aGVHD are retrospective, and complete response rates vary between 0 and 31% [380-385]. MMF is usually well tolerated, and the major side effects (myelosuppression and diarrhea) are usually dose-dependent and rapidly reversible after discontinuation.

MMF is one of a few drugs that have been tested in randomized control trials for cGVHD. Although several previous small retrospective studies indicated that MMF was effective and well tolerated, this randomized trial was stopped early due to a lack of efficacy and increased risk of death from MMF [386]. However, several small case series have reported high response rates [381,383,384]. Thus, even though MMF is well tolerated and effective as a GVHD prophylaxis, its role in steroid-refractory acute GI-GVHD and cGVHD is questionable.

7.11.3 **Strategies for targeting the cytokine network**

Cytokine targeting in GVHD seems reasonable as many cytokines increase the proliferation of alloreactive T cells. Targeting of a wide range of cytokines has been explored in murine models, but only TNF-α, IL-2 and IL-6 targeting has been investigated in larger clinical trials [387-393].

IL-2 is important for T cell proliferation and differentiation. At least four IL-2 targeting agents have been tested, but no beneficial effect has been demonstrated, and they may even be associated with adverse outcomes [387,394-396].

Etanercept is a recombinant human TNF-α receptor fusion protein that binds free TNF-α. It is less effective as a TNF-α inhibitor than infliximab, a TNF-α specific monoclonal antibody [397]. Both agents have been tested in steroid-refractory GVHD (SR-aGVHD)

[390-392,398-401]. Infliximab has then been associated with significantly increased risk of infections and no improvement in survival and response rate [399,402]. In one study, treatment with etanercept was associated with a higher rate of response without increased frequency of infections [398].

7.11.4 **T cell depletion as an anti-GVHD strategy**

Depletion of T cells from the graft reduces the incidence of acute and chronic GVHD by prohibiting or delaying immune recovery posttransplant, but the strategy is associated with an increased risk of infectious complications (mainly viral and fungal infections), posttransplant lymphoproliferative diseases and relapse [403]. T cell depletion can be done either *in vivo* with poly- or monoclonal antibodies directed against T cells or *ex vivo* with depletion of the stem cell graft [404]. Only in-vivo T cell depletion was employed for the transplants included in our analysis. We have therefore limited the overview to the three most commonly employed drugs for in-vivo T cell depletion.

Antithymocyte globulin (ATG). ATGs are polyclonal antibodies derived from animals immunized with different lymphoid cells, i.e. these antibodies target molecules expressed by T cells (e.g. CD3, CD8, CD7, CD107) as well as other immunocompetent cells, proinflammatory cytokines and molecules involved in the trafficking of immunocompetent cells [405]. Only three products have been tested in the GVHD setting: ATGh (ATGAM) derived from horses, ATG-T (Thymoglobulin) derived from rabbits immunized with human thymocytes, ATG-F (Grafalon) derived from rabbits immunized with the human Jurkat cell line [405]. The different ATG products differ significantly in antigen specificity and strength and do not have the same dose equivalency.

ATGs can be used (i) as part of conditioning therapy to secure adequate immunosuppression and allow stem cell engraftment, (ii) in addition to standard GVHD prophylaxis, or (iii) in the treatment of acute or chronic GVHD. Addition of the different rATG to standard GVHD prophylaxis reduces the risk of cGVHD. Experience with thymoglobuline showed that the dose of ATG must be carefully selected; single doses below 2.5 mg/kg are less effective in preventing GVHD, whereas single doses above 7 mg/kg significantly reduce the risk of cGVHD, but increase relapse rates. The optimal dose of ATG-T is probably between 4.5 and 6 mg/kg [405-409]. In contrast, several studies have

shown that addition of horse ATG to standard GVHD prophylaxis does not confer any benefit [405].

Alemtuzumab. This monoclonal antibody targets CD52, which is expressed by all lymphoid cells, monocytes and dendritic cells, and causes prolonged lymphodepletion [410,411]. Alemtuzumab in combination with other immunosuppressive drugs seems to reduce the risk of acute and chronic GVHD to below 20%, even with multiple HLA mismatches. However, alemtuzumab increases the risk of graft rejection and disease relapse and it is associated with high rate of posttransplant viral infections due to slow immune reconstitution [410,411]. Alemtuzumab may have some advantages compared with ATG in transplantation for bone marrow failure syndromes where the GVL effect is less important [411].

Posttransplant cyclophosphamide. Cyclophosphamide is a prodrug which is metabolized into its active metabolites that induce cell death by DNA cross linking. The donor alloreactive-reactive T cells in the stem cell grafts become activated early after graft infusion; this activation is caused by the presentation of recipient antigens in a proinflammatory microenvironment, and early posttransplant treatment with high-dose cyclophosphamide kills proliferating alloreactive T cells but not non-proliferating T cells [412]. Stem cells express aldehyde dehydrogenase that metabolizes the various cyclophosphamide metabolites, giving them protection against this cyclophosphamide effect. Such cyclophosphamide therapy in combination with other immunosuppressive drugs seems to be effective as prophylaxis against acute and chronic GVHD [413-416]. High-dose cyclophosphamide is not recommended for GVHD treatment.

7.11.5 **Immunomodulation**

Extracorporeal photopheresis is now increasingly used in treatment of cGVHD and is regarded as an effective treatment [417]. Ongoing studies are investigating whether this treatment should be used for prophylaxis and/or treatment of aGVHD. The use of mesenchymal stem cells in the treatment of aGVHD should be regarded as an experimental procedure [418].

7.11.6 **GVHD prophylaxis**

The current EBMT-ELN recommendations for GVHD prophylaxis after myeloablative conditioning state that the standard procedure is cyclosporin plus a short course of methotrexate; tacrolimus plus methotrexate is regarded as equivalent. Cyclosporine is tapered from 3 months onward if no GVHD is present; the overall duration then being 6 months. Pretransplant ATG can be included in the prophylaxis at least when using matched unrelated donors [370].

For RIC, the recommended prophylaxis is cyclosporine plus mycophenolate mofetil [370]; ATG can be added as described for myeloablative conditioning. Cyclosporine is tapered from 3 months onward if no GVHD is present; the overall duration then being 6 months. Early posttransplant cyclophosphamide is commonly used for ASCT with haploidentical donors [186].

7.11.7 **Steroids as first-line treatment of aGVHD**

As outlined above cyclosporine and tacrolimus are used for GVHD prophylaxis. The first action to be taken during an episode of aGVHD is to ensure that the levels of cyclosporine or tacrolimus are within the therapeutic range [186,365]. Although these agents are thought to act through the same mechanisms, some patients exhibit no clinical response to cyclosporine but respond to tacrolimus and *vice versa*.

Stage 2 GI-GVHD or overall grade III-IV GVHD is regarded as severe and immediate treatment with high-dose steroids should be initiated, preferably 1 mg/kg methylprednisolone for stage 2 GI or 2 mg/kg methylprednisolone (HDMP) for grad III-IV. HDMP treatment leads to a complete resolution of symptoms in approximately 50% of patients, and for the responding patients the steroid dose is gradually reduced over a period over 1-2 weeks [186,365]. Patients that either have progression after 3 days, no improvement after 7 days or incomplete response after 14 days are defined as steroid-refractory aGVHD (SR-aGVHD).

7.11.8 **Treatment of steroid-refractory acute GVHD**

Despite numerous new therapeutic strategies the long-term survival in SR-GVHD remains dismal [269]. The major problems are lack of response and increased risk of severe

infections due to the severe GVHD-associated immune dysfunction that is caused by complex mechanisms and results in impaired physiological barriers of the GI tract and the skin. The additional immunosuppression needed for patients with SR-GVHD is almost always associated with a significant increase risk of opportunistic infections [186]. The further discussion will focus on standard prophylactic approach and treatment of GVHD including the available clinical experience of IL-6 blockade in GVHD. However, a brief overview of important therapeutic strategies treating SR-aGVHD is given in Figure 5.

There is no general agreement on what should be the second-line treatment of aGVHD [186,365,366]. Due to cost, efficacy and safety many transplant centers regard etanercept as the first-line therapy for SR-aGVHD [186,365,366]. MTX has been tried in the treatment of steroid-refractory aGVHD. It was usually well tolerated, but most patients included in these studies had either low grade GVDH (i.e. grade 1 or 2) or MTX was combined with steroids [419,420]. For these reasons its effect should therefore be regarded as poorly documented [365]. As previously described can mycophenolate induce a remission in up to on third of patients with SR-aGVHD, but is associated with a significant increase in infectious complications [381-386]. The use of ATG in the treatment of aGVHD has also been investigated in several studies, but due to a high rate of fungal and viral infections some guidelines regard the role ATG in SR-GVHD as questionable [365]. The EBMT-ELN guidelines therefore conclude that there is no standard second-line treatment for aGVHD; but the components of this treatment will often be continuation of calcineurin inhibitor and steroid and addition of MMF or TNF-targeting therapy [186].

7.11.9 **Treatment of chronic GVHD**

The EBMT-ELN guidelines state that the first-line treatment is steroid, possibly together with cyclosporine. If additional treatment is needed no standard therapy is available, but ECP seems to be increasingly used [186].

7.11.10 **IL-6 targeting therapy in prophylaxis and treatment of GVHD**

The efficacy and safety of IL-6 targeting in GVHD has only been evaluated using tocilizumab. IL-6 blockade as GVHD prophylaxis has been investigated in two single-arm phase 2 studies (Table 13). Kennedy et al. added a single dose of tocilizumab 8 mg/kg (maximum dose 800 mg) to standard GVHD prophylaxis with cyclosporine A and MTX

[256]. Of 48 patients that underwent T cell-replete allotransplantation, 2/3 received RIC while 1/3 were transplanted using TBI-cyclophosphamide. Addition of tocilizumab seemed safe without evidence for increased graft rejection, delayed neutrophil regeneration, reduced chimerism or early relapse compared to historical controls. None of the typical side effects of tocilizumab (see section 7.5) were observed, but three patients experienced severe liver toxicity during the first month after transplantation. The authors concluded that the observed rate of 4% acute grade III-IV GVHD should be regarded as low. Furthermore, Drobyski et al. also added tocilizumab to standard GVHD prophylaxis with tacrolimus and MTX in 35 patients receiving busulfan-based conditioning therapy [255]. The treatment was well tolerated, and graft rejection was not observed. Nine patients experienced a transient increase of transaminases that peaked early (7-10 days) after infusion. During the first 100 days, 14% experienced grade III-IV aGVHD. However, GVHD manifestations were confined to skin or upper gastrointestinal tract. The tocilizumab therapy significantly reduced the risk of aGVHD at day +180 compared with historical controls, but there was no difference in the incidence of cGVHD, relapse or overall survival. To the best of our knowledge, there are no ongoing trials investigating the addition of IL-6 blockade to standard GVHD profylaxis. The effect of tocilizumab on the rate of cytokine release syndrome following haploidentical SCT with post–cyclophosphamide GVHD prophylaxis is currently under investigation (NCT02057770).

The efficacy of tocilizumab in the treatment of aGVHD has been reported in a limited number of case reports and four published case series [421-428]. Taken together, these reports suggest that tocilizumab may be effective in the treatment of severe and/or steroid-refractory aGVHD. Transient increases in liver transaminases were observed, but severe liver toxicity was uncommon; However, as expected, infectious complications were common in these patients. Furthermore, a study (NCT01475162) of tocilizumab in the treatment of steroid-refractory aGVHD was prematurely stopped since the monitoring board felt that the risks of complications outweighed the potential benefits. Finally, no data are available on IL-6 targeting in the treatment of cGVHD.

Table 13. *Patient characteristics and results from two clinical studies evaluating the safety and efficacy of tocilizumab added to standard GVHD prophylaxis.*

Patient characteristics	Kennedy (n=48) [256]	Drobyski (n=35) [255]
Age (median and range)	48 (22–64)	66 (22-76)
Gender (male/female)	30/18	22/13
Diagnosis: AML/ALL/others	26/10/0	19/4/12
Conditioning regimes		
Flu/Mel	32	0
TBI/Cy	16	0
Flu/Bu	0	30
Bu/Cy	0	5
Graft source		
Marrow	0	6
PBSC	48	29
AGVHD		
Acute grade II-IV	12%	14%
Acute grade III-IV	4%	3%
Involving skin	10%	10%
Involving GI tract	8%	8%
Involving liver	0%	0%
Chronic GVHD	51%	38% at 12 months
Overall survival	84% at 24 months	68% at 12 months
TRM	4%	14% at 12 months

8. AIMS OF THE STUDY

Outcomes after ASCT depend on both donor-associated factors and the pretransplant characteristics and posttransplant factors of the recipient [204-208,210,214]. The aim of the thesis was to investigate the possible importance of IL-6-family cytokines for outcome after ASCT based on these three perspectives: contributors to donor heterogeneity, pretransplant risk factors and posttransplant immunoregulators. The objectives of the individual articles reflect this aim:

- The objective of Article I was to investigate the biological context of the IL-6 family cytokines and how systemic cytokine levels together with inflammatory parameters (i.e. CRP levels, endothelial function) correlate with posttransplant outcomes.
- The objective of Article II was to investigate how genetic variations within the IL-6/IL-6R genes are associated with pre- and posttransplant levels of sIL-6R, gp130, CRP and with important posttransplant outcomes.
- The objective of Article III was to investigate whether stem cell mobilization, with G-CSF administration to healthy PBSC donors, contributes to donor heterogeneity by modulating the systemic levels of IL-6-family cytokines.
- Previous aGVHD is a risk factor for later cGVHD [376,429,430], and the objective of Article IV was to compare the early intracellular signaling events (i.e. the phosphorylation of intracellular mediators) after IL-6 stimulation for T cells derived from allotransplant recipients with and without previous aGVHD.

9. SUMMARY OF RESULTS

Article I: Pretransplant levels of CRP and interleukin-6 family cytokines; effects on outcome after allogeneic stem cell transplantation

Background: Previous studies have demonstrated that pretransplant immunoregulatory and inflammatory factors have an effect on outcomes after allogeneic stem cell transplantation. We therefore investigated whether pretransplant levels of IL-6 family cytokines as well as other inflammatory markers (CRP levels, endothelial dysfunction) correlated with outcomes after allotransplantation.

Methods: We included 100 consecutive allotransplant recipients transplanted with allografts from related donors. The levels of IL-6, IL-11, IL-27(p28), sIL-6R (sCD126), LIF and IL-31 for CNTF and OSM were determined by Luminex technology in pretransplant serum samples from patients and in samples from healthy controls.

Results: Pretransplant IL-6 and sgp130 levels were significantly correlated and differed significantly from the levels found in healthy controls; both levels were also associated with time to neutrophil engraftment. However, only CRP levels were associated with increased TRM at days +100 and +700, but CRP levels did not influence overall survival after 2 years or for the entire period. The only IL-6 family cytokine that seemed to influence clinical outcome was IL-31; high IL-31 levels were associated with increased TRM. Finally, extensive fluid retention (probably due to endothelial dysfunction with capillary leaks) during the first 4 weeks posttransplant was an independent risk factor for aGVHD, TRM and overall survival.

Conclusion: High pretransplant IL-6 levels seem to be a part of a high-risk pretransplant phenotype together high CRP levels, but pretransplant IL-31 level was the only IL-6 family cytokine that correlated with transplant outcome. Furthermore, early posttransplant fluid retention was also associated with adverse prognoses, but it is not known whether or how pretransplant factors contribute to this complication.

Article II: A pilot study of single nucleotide polymorphisms in the interleukin-6 receptor and their effects on pre- and posttransplant serum mediator level and outcome after allogeneic stem cell transplantation

Background: Several IL-6R SNPs seem to be important for immunoregulation. SNP rs228145 influences IL-6R receptor shedding and is associated with risk of autoimmune diseases. Two other SNPs (rs4329505 and rs12083537) are also associated with outcome in inflammatory disorders. We investigated whether SNPs within the IL-6R gene influenced levels of the interleukin-6 Family cytokines, pretransplant levels of CRP and posttransplant outcome in cohort of 101 allotransplant recipients.

Methods: We investigated how SNPs in the IL-6 receptor influenced serum levels of the IL-6-family cytokines, pretransplant levels of CRP and posttransplant outcome in a cohort of 101 unselected allotransplant recipients. Our study included SNP rs228145, rs4329505 and rs12083537, together with five other SNPs (rs4379670, rs6698040, rs4845374, rs4453032 and rs4845618) that were used as tagging SNPs (i.e. each SNP independently correlated with 100 different SNPs (r2 above 0.7) within the IL-6R gene). The SNP rs1800975 in the promotor region of the IL-6 gene was included because it is associated with increased risk of aGVHD. SNPs were investigated in both PBSC donors and in their recipients.

Results: Patients homozygosity for the major alleles of the IL-6R SNPs rs2228145 and rs4845618 was associated with high pretransplant CRP serum levels and decreased sIL-6R levels; these differences persisted 6 months posttransplant. Recipient homozygosity for the minor allele of rs4379670 was associated with decreased pretransplant CRP levels. Furthermore, the recipient IL-6R genotype SNP rs432950 was associated with late immunological complications and increased NRM. Finally, rs4845618 donor genotype was associated with aGVHD, whereas donor genotype for the IL-6 SNP rs1800795 was associated with decreased survival +100 days posttransplant.

Conclusions: Our study suggests that SNPs in the IL-6R/IL-6 genes of allotransplant recipients and their donors modulate IL-6 signaling and outcome (especially immune-mediated complications) after ASCT.

Article III: Immunological heterogeneity of healthy peripheral blood stem cell donors-effects of granulocyte colony-stimulating factor on inflammatory responses

Background: G-CSF administration not only mobilizes normal hematopoietic stem cells to the peripheral blood, this treatment also seems to alter the circulating levels or modulate the function of various immunocompetent cells.

Methods: We investigated how G-CSF administration influenced CRP levels and systemic levels of IL-6-family cytokines in healthy PBSC donors. We also investigated whether priming of monocytes or mesenchymal stem cells with TLR ligands influenced IL-6 release in the presence of G-CSF.

Results: PBSC mobilization with G-CSF 10 μg/kg/day for 4 days significantly increased CRP levels especially for elderly donors and donors with high CRP levels prior to G-CSF administration (101 donors investigated). Systemic levels of IL-6-family cytokines were also analyzed after 4 days of G-CSF administration, immediately after stem cell harvesting and 24 hours after harvesting for 20 consecutive donors. Graft supernatants were also analyzed. IL-6 serum levels increased significantly during G-CSF therapy, but IL-6 levels showed no correlation with CRP levels and normalized within 24 hours after the end of G-CSF treatment. The other IL-6 family members showed wide variation (especially oncostatin M) but were not significantly altered by G-CSF/harvesting. IL-6 and oncostatin M levels showed significant correlations during G-CSF therapy: a subset of donors was characterized by high IL-6/oncostatin M serum levels during G-CSF therapy. Finally, G-CSF increased the amount of IL-6 release by in vitro cultured monocytes, fibroblasts and mesenchymal stem cells stimulated by various TLR-agonists.

Conclusions: G-CSF administration to healthy PBSC donors increases serum levels of IL-6/oncostatin M as well as inflammation-associated CRP. These responses contribute to the heterogeneity of healthy PBSC donors.

Article IV: IL-6 responsiveness of $CD4^+$ and $CD8^+$ T cells after allogeneic stem cell transplantation differs between patients and is associated with previous acute graft-versus-host disease and pretransplant antithymocyte globulin therapy

Background: Experimental studies suggest that IL-6-induced STAT3 phosphorylation in T cells derived after ASCT is associated with GVDH. This may also be true for allogeneic PBSC recipients, but available studies in humans have included few patients. Furthermore, it is not known whether this STAT3 response is caused by classical IL-6 signaling or trans-IL-6 signaling and whether the IL-6 responsiveness differs between T cell subsets, involves additional intracellular mediators or is modulated by concomitant T cell activation.

Methods: We investigated the phosphorylation of STAT3(Ser727), Akt(Thr308), mTOR(Ser2442) and STAT3(Tyr705) in circulating T cells derived from 31 allotransplant recipients at day +90 posttransplant. Our studies included effects of IL-6-induced cis and trans-signaling on constitutive phosphorylation (exposure to IL6 alone) and effects of IL-6 signaling in the presence of TCR activation (anti-CD3+anti-CD28). T cells were stimulated with IL-6 alone, hyper-IL-6, IL-6+IL-6R and IL-6+IL-6R+gp130Fc. The protein kinase C activator PMA (12-O-Tetradecanoylphorbol-13-acetate) was used as a T cell activation signal.

Results: PMA stimulation increased the phosphorylation of STAT3(Ser727), Akt(Thr308) and mTOR(Ser2442) both for CD3+CD4+ and CD3+CD8+ T cells, but these responses were generally stronger for patients with previous aGVHD. A significant PMA-induced increase in STAT3(Tyr705) phosphorylation was seen only for CD3+CD8+ T cells. We also investigated effects of IL-6 in unstimulated (i.e. constitutive phosphorylation) and TCR-activated posttransplant T cells. Both cis and trans-IL-6 signaling increased STAT3(Tyr705) phosphorylation. These responses were seen for constitutive phosphorylation and during TCR stimulation, and the responsiveness was independent of previous GVHD. However, the STAT3(Tyr705) responses reached lower levels of significance for CD3+CD8+ than for CD3+CD4+ T cells, especially in relation to constitutive phosphorylation, and CD3+CD4+ showed a broader IL-6 responsiveness with concomitantly increased phosphorylation of STAT3(Ser727) phosphorylation in response to both cis and trans-signaling. Finally, CD3+CD4+ T cells derived from aGVHD patients showed increased STAT3(Ser727) phosphorylation in response to cis signaling whereas CD3+CD4+ cells derived from patients

without aGVHD showed increased mTOR(Ser2449) phosphorylation in response to trans-signaling.

Conclusion: Circulating T cells derived at day +90 posttransplant respond to cis and trans-IL-6 signaling with increased phosphorylation of STAT3, Akt and mTOR. However, the phosphoresponses differ between the CD3+CD4+ and CD3+CD8+ T cell subsets, and they also differ between patients with and without previous aGVHD.

10. MATERIAL AND METHODOLOGICAL CONSIDERATIONS

10.1 SAMPLE AND DATA COLLECTION

10.1.1 Ethical considerations

All biobanks were approved by the Regional Ethics Committee and registered by Norwegian authorities. The use of all samples and the patient information registered in the biobanks was also approved by the Regional Ethics Committee.

10.1.2 Patient selection and construction of databases with patient characteristics and transplant outcomes

All individuals included in article I and article II were patients that underwent ASCT at Haukeland University Hospital. Article IV also included patients that underwent ASCT at Oslo University Hospital Rikshospitalet. Control samples were collected from healthy donors at Haukeland University Hospital. Patient information was available at the transplant centers. Allotransplant recipients are generally scheduled for routine consultation every third month during the first posttransplant year and then yearly for the following 4 years. For follow up, we registered the results of the allotransplantation, including survival, occurrence of GVHD and relapse status. Since allotransplant recipient may experience multiple endpoints, verification of each clinical event is important to avoid discordance between reported and adjudicated cause-specific events [431]. To assure consistent reporting for the patients, clinical outcome data were extracted by two independent reviewers. The definitions of the three outcomes analyzed from the data are given below:

- *Treatment-related mortality.* No generally accepted definition of TRM exists for allotransplant recipients. However, most studies define TRM as death due to complications other than relapse, and our studies also used this definition; all deaths related to GVHD, from infections, early or late multiorgan failure and secondary malignancies, were included. In the competing risk analysis relapse was defined as the competing risk factor.
- *Acute GVHD.* Diagnoses and grading were determined in accordance with published guidelines [186,261]. However, previous studies have reported significant deviance

between reported occurrence of aGVHD by clinicians and the occurrence of aGVHD evaluated by expert panels [431]. Clinically significant GVHD was therefore also recognized on the basis of intention to treat with high-dose steroids, defined as at least 1mg/kg of methylprednisolone equivalents. Furthermore, relapse patients may have had therapeutic interventions aimed at inducing GVL (and subsequent GVHD), such as the tapering of steroid doses or the administration of donor lymphocyte infusions. In a retrospective analysis, it is not always possible to identify the exact time these interventions were made. Hence, relapse patients were censored simply at the time when relapse was diagnosed.

- *Chronic GVHD.* Clinical guidelines exist for the diagnosis and grading of chronic GVHD. The current classification and grading system of cGVHD is complex and requires extensive clinical evaluation of several organ systems. For these reasons cGVHD grading is regarded as inconsistent with large inter-individual variation [432]. Rates of immunosuppression tapering vary significantly among patients. Patients who do not experience aGVHD usually begin tapering 60-90 days posttransplant, while patients treated with high doses steroids for aGVHD cannot begin tapering steroids doses until well after day 100.

  By including only patients with either (i) no previous aGVHD or (ii) previous GVHD that did not require steroid therapy before day +100 and then developing a clinical picture consistent with cGVHD after this time point, we defined a relatively homogeneous patient group for our analysis of cGVHD (i.e. classic cGVHD without overlap with previous aGVHD). This ensured a much simpler and reliable readout, which we describe as the “development of a clinical picture consistent with chronic GVHD requiring either an additional immunosuppressive agent/treatment or prolongation of the cyclosporine A prophylaxis”.

10.1.3 **Collection and handeling of patient samples.**

For preparation of serum samples, venous blood was collected in sterile plastic tubes (BD Vacutainer® SST™ Serum Separation Tubes, Becton-Dickenson; Franklin Lakes, NJ) and allowed to coagulate for up to 120 minutes at room temperature before centrifugation. Samples then were stored at -80°C for later analysis.

For collection of cells, venous blood was collected into sterile plastic tubes containing ACD-A solution (Greiner Bio-One, Kremsmünster, Austria). Cells where then harvested after density gradient separation (Lymphoprep; Nycomed, Oslo, Norway) and cryopreserved in liquid nitrogen, using highly standardized methods for storage, for later analysis. The freezing medium contained final concentrations of 10% dimethylsulfoxide (DMSO) and 20% inactivated fetal calf serum. The cryopreserved samples were thawed rapidly, washed once to remove DMSO and resuspended in RPMI 1640 (ThermoFisher, Waltham, MA), then allowed to rest for 1 hour at 37°C with 5% CO_2. DNase (Sigma-Aldrich, St Louis, MO) was added to the medium during thawing and stimulation to prevent clumping.

10.2 LABORATORY METHODS

10.2.1 **Analysis of soluble mediators in serum samples**

Due to the limited amount of serum available, we used the Luminex/Multiplex platform for analysis of the various soluble mediators. This technology allows for detection of up to 50 different proteins in a small sample volume. At the time of our analyses, the following members in the IL-6 family were available on the Luminex/Multiplex platform from Bio-Rad (Hercules, CA) and CNTF and OSM from Millipore (Burlington, MA): IL-6, IL-11, IL-27(p28), sIL-6R (sCD126), LIF and IL-31. All analyses were performed strictly according to the manufacturer's instructions and in duplicate. Samples were analyzed using the Luminex®200™ Bio-Rad platform with program version 6.1. All other biochemical tests in the current studies were performed as routine analysis at the central laboratory for clinical biochemistry at the corresponding hospital. CRP was analyzed using an immunoturbidimetric method from Roche (Basel, Switzerland), and a lower limit of detection of 1 mg/L was used for CRP throughout the observation process.

10.2.2 **Preparation of donor/ recipient DNA and SNP genotyping.**

For all patients and corresponding donors, reference DNA samples are collected pretransplant and stored at Haukeland University Hospital for later use in routine chimerism analyses. After approval from the Regional Ethics Committee, excess DNA from these samples was used for SNP analyses. The required amount of DNA was 5-10 µL, and

spectrophotometric analysis (NanoDrop™, ThermoFischer Scientific, Waltham, MA) was used to ensure DNA concentration between 2 and 20 ng/µL.

SNP genotyping requires two different techniques; one for the detection of the SNP and another for reporting the presence of the specific DNA sequence [433,434]. Genotyping for candidate gene SNP analysis is typically done by traditional PCR-based assay like TaqMan™ SNP genotyping (ThermoFischer Scientific Waltham, MA). In our present study we chose the KASP™ genotype assay (LGC, Teddington, UK) that employs a similar technology to the TaqMan™ SNP genotyping assay but at a significantly lower cost.

10.2.3 **Selection and analysis of the different SNP in IL-6R**

The IL-6R gene is located on chromosome 1 and is therefore inherited independently from other genes known to influence transplant outcomes (e.g. the MHC genes on chromosome 6) or genes directly influencing IL-6 signaling (IL-6 located on chromosome 7, gp130 located on chromosome 5 and STAT3 located on chromosome 17). Hence, an association between clinical outcome and IL-6R gene would probably not be affected by genetic linkage with these genes. The main goal of the analysis was to evaluate the effect of SNP rs2228145 on transplant outcome; as described earlier, this polymorphism results in altered sIL-6R serum levels and modulation of IL-6 effects.

Other SNP within IL-6R have been associated with autoimmune diseases or altered levels of inflammatory markers. The IL-6R gene harbors more than 2000 SNPs, when promotor and non-coding regions are included. The majority of the SNPs occurs at minor allele frequencies below 5%, with only 325 SNP having a minor allele frequencies above 1% [435] (genome assembly GRCh37, p.1305, accessed the 1st of September, 2016). To evaluate the effects of independent SNPs in the IL-6R gene, we employed a previously used strategy [436]. Briefly, we selected five tagging SNPs (rs4379670, rs6698040, rs4845374, rs4453032, rs4845618) with each tagging approximately 100 different SNPs (r^2 above 0,7). After a review of the literature, we also decided to include rs4845617 rs4329505 and rs12083537 in our studies. Other identified SNPs were excluded because they showed strong linkage disequilibrium (r^2 >0,7) with rs2228145 or the selected tagging SNPs. All selected SNPs had an allele frequency of at least 10%.

10.2.4 **Stimulation of cells and detection of protein phosphorylation**

The main goal of this study was to analyze the effects of classical and hyper-IL-6 signaling on the phosphorylation of intracellular proteins in human T cells. Since HLA-restricted posttransplant activation of donor T cells requires stimulation of T-cell receptors, we also wanted to examine the effects of IL-6 signaling both on resting T cells and in combination with T-cell receptor ligation.

After resting, the cells were washed twice and allowed to rest on ice for additional 15 minutes before they were divided into tubes A and B. Cells in tube A were incubated with anti-human CD3 (clone UCHT1; BD, Fanklin Lakes, NJ) and anti-CD28 (clone 28.1; BD) for 15 minutes; the cells were then washed and incubated with polyclonal goat anti-mouse antibodies (BD) for 15 additional minutes. During this time, cells in tube B underwent the same washing and waiting steps as cells in tube A but were not incubated with antibodies.

Cells from tubes A and B were each divided into six different tubes and stimulated as described in Table 14 for 10 minutes. Briefly, hyper-IL-6 induces only IL-6 trans-signaling and IL-6 in the absence of the sIL-6R induces only classical signaling, while IL-6 combined with sIL-6R induces classical and trans-signaling simultaneously and the presence of sgp130FC blocks IL-6 trans-signaling but leaves classical IL-6 signaling intact. PMA was included as a positive control for PI3K/AKT/mTOR activation. Stimulation of the cells was initiated by transferring the tubes into a 37°C water bath; stimulation was stopped after 10 minutes by adding formaldehyde directly into the tubes.

Table 14. *Analysis of the phosphorylation of intracellular T cell mediators; a summary of the various incubation condition used for activation of the T cells and for initiation of IL-6 signaling.*

	Tube A						**Tube B**					
Incubation conditions	1	2	3	4	5	6	7	8	9	10	11	12
CD3/CD28 T cell ligation	■	■	■	■	■	■						
Hyper-IL-6 5ng/mL	■						■					
IL-6: 20ng/mL		■	■	■				■	■	■		
sIL-6R 50ng/mL			■	■					■	■		
sgp130-Fc 500ng/mL				■						■		
PMA 100ng/mL					■						■	
Unstimulated control						■						■

10.2.5 **Flow cytometry**

Flow cytometry analysis is a semi-quantitative method that allows detection of proteins in single cells stained with specific fluorochrome-conjugated antibodies. The number of lasers and detectors in the flow cytometer limits the maximum number of simultaneously detectable parameters, and modern flow cytometers allow the detection of 8-16 parameters simultaneously. For the current study, we used a BD FacsVerse flowcytometer that can detect up to ten different parameters simultaneously.

As described previously, activation of the IL-6 pathway leads to signaling through the JAK1/STAT3, ERK/MAPK and PI3K/AKT/mTOR pathways [16]. However, the most prominent intracellular activation events are phosphorylation of STAT3, especially at Y705 but also at S727. The TCR receptor activates different signaling cascades, including the PI3K/AKT/mTOR pathway, but does not typically activate the JAK/STAT pathway directly [437]. To assess the intracellular phosphorylation events after classical IL-6 signaling, we selected the four events: STAT3(Ser727), Akt(Thr308), mTOR(Ser2442) and STAT3(Tyr705).

Preparation of cells with permeabilization procedures allows for detection of intracellular proteins, such as cytokine expression or the phosphorylation status of signaling proteins. While detection of intracellular cytokines requires treatment with a mild detergent (saponin) to facilitate penetration of antibodies intracellularly, the detection of phosphorylated proteins requires permeabilization with ice-cold methanol [438,439]. Although this last permeabilization method allows the detection of additional targets, this procedure strips the cells of cell-surface proteins, making simultaneous detection of these surface markers and phosphorylation events impossible [440]. Therefore, in the current study we had to employ three different flow cytometry protocols. One panel was used for evaluation of the surface expression of IL-6R, a second panel for evaluation of intracellular cytokine expression and a third panel for detection of phosphorylation events. The different antibody panels used are described in detail in article IV.

10.3 STATISTICAL ANALYSES

Statistical analyses were performed using the SPSS version 22.0 (IBM Corp.; Armonk, NY) for descriptive statistics; GraphPad Prism 5 (Graph Pad Software, Inc.; San Diego, CA)

for graphical presentation of data; and Stata Statistical Software, Version 14 (StataCorp; College Station, TX) for survival and competing risk analyses. Flow cytometry data were analyzed using FlowJo software (Tree Star, Inc.; Ashland, OR).

10.3.1 **Evaluation of outcomes after transplant (article I and II)**

A major goal of the study was to evaluate the effects of different factors on overall survival, relapse and treatment-related complications (including GVHD) following ASCT. The following sections briefly describe the statistical methods employed.

Product limit estimator (Kaplan-Meier Survival Estimates). The Kaplan-Meier method calculates the survival rate (percentage of individuals still alive) for a given time point. The advantage of the Kaplan-Meier curve is that the method corrects for patients where the status after a specific time point is not fully known (i.e. patients not included in follow-up). However, the limitation of Kaplan-Meier estimate is that it can only be used to study the effect of one factor at a time and cannot correct for the effects of other factors [441].

Cox proportional hazard model. The Cox model is a statistical regression model that is frequently used to investigate the association between survival and different variables. This method calculates the hazard rate ratio (HR). The hazard ratio is the ratio of the hazard rate (event per time interval) in the group of interest divided by the hazard rate in a predefined control group [442]. The underlying assumption for the Cox model is that the hazard rate is constant throughout the observed time interval. This assumption must be validated by different statistical methods for each data set to conclude that the proportional hazard model is valid [442]. However, the advantage of the proportional hazard model is that it allows the analysis of more than one statistical outcome variable at a time. Parameters included in the multivariate model are defined in advance. We decided that age, CRP and variables with a p-value <0.1 in univariate analyses would be included in the final multivariate analysis. In the final model a p-value <0.05 was regarded as statistically significant.

Competing risk mode Fine and Gray. Often a patient will experience a posttransplant event of interest that alters the probability of experiencing another second event of interest (e.g. patients who die due to relapse have a de facto probability of zero of dying of any other complication). Such situations are defined as competing risks in statistical analyses, and the Cox proportional hazard model cannot correctly account for this in the

hazard ratio. The subdistribution of hazards approach proposed by Fine and Gray is the most commonly applied method to correct for competing risks [443]. This method produces a subdistribution HR (SHR) that can be interpreted in the same way as the HR ratios. It can also be used to calculate cumulative incidences and allows for comparison of cumulative incidence between groups

Entering continuous variables into regression models has several limitations. First, a statistical regression model works under the assumption that the effect of a variable is linear, which is almost never the case. Second, imputing a variable with a large range (e.g. CRP levels) often results in very low hazard ratios, making it difficult to interpret the actual clinical effects of the parameter. Continuous variables are therefore often dichotomized to make a simplified risk classification. To overcome this problem, all variables were included in the analysis as continuous variables. Variables with a significant effect were then split into three dummy variables and entered into the model to better evaluate where each group could be dichotomized.

Correction for multiple comparisons. At a significance level of 5%, one out of 20 significant observations will not be correct [444]. In case of multiple testing, the significance levels can be adjusted to correct for this effect. The most commonly used method, known as the Bonferroni correction, is done by multiplying the significance level by the total number of comparisons (hypotheses)[445]. This method, or similar methods, are typically employed when analyzing large-scale data comparisons, such as in GWAS, where the analysis does not incorporate any knowledge of underlying biological processes. Required significant levels for GWAS are typically set as $<5 \times 10^{-8}$ [446]. However, there is ongoing debate about the need for making adjustments for multiple comparisons, especially when the test is not “unfocused” but is based on a biological rationale [445,447,448]. Furthermore, the majority of other studies evaluating the effect of SNP in the IL-6 receptor alone have not consistently performed any correction for multiple testing, and the effects of IL-6 SNP polymorphism have been demonstrated consistently in several studies [353-363]. For these reasons, we choose to report p-values and confidence intervals that were not adjusted for multiple comparisons.

11. DISCUSSION

The current knowledge of the effects of IL-6 on aGVHD is derived through three different approaches: murine models of GVHD with IL-6 knockout; patient studies of associations between IL-6 serum levels, SNP frequencies and transplant outcome; and clinical experience with IL-6 targeting in treatment of GVHD or as addition to standard GVHD. Each of these previous studies regarded IL-6 solely as a proinflammatory cytokine, whereas more recent studies have shown that both classical and IL-6 trans-signaling are important for maintaining the balance between chronic inflammation and tissue regeneration [16,449], especially in the GVHD target organs, liver or gut. The main goal for this thesis was to focus not only on IL-6, but also on other IL-6-family cytokines in allotransplantation. Our studies had an additional focus on CRP levels because the levels of this proinflammatory biomarker are usually strongly correlated with IL-6 levels and may reflect the balance between classical proinflammatory signaling and regeneration-supporting IL-6 trans-signaling.

The two first articles in the present thesis were also based on studies of associations between serum levels or SNPs with outcome after allotransplantation, and we investigated serum levels of all IL-6 cytokine family members (article 1) and several SNPs that have not been investigated in allotransplant recipients before. Furthermore, article IV investigates IL-6 effects on posttransplant immunocompetent cells and supports the further investigation of JAK-STAT3 inhibition in GVHD treatment. Finally, article III suggest that IL-6-family cytokines contribute to the heterogeneity of healthy allogeneic stem cell donors.

Article I

In contrast to many other cytokines, IL-6 as well as most other IL-6 cytokine family members have not only local effects, but also systemic or distant effects reflected through variations in their serum/plasma levels [450]. We therefore investigated associations between their serum levels and clinical outcomes after ASCT. Although we could not find any significant associations between the levels of the main modulators of IL-6 trans-signaling and transplant outcomes, this does not exclude the possibility that the IL-6 buffer has a role in GVHD pathophysiology. First, local and systemic concentrations of

sIL-6R are regulated by different mechanisms; systemic levels are predominantly determined by IL-6R shedding from hepatocytes and leucocytes [61,62], whereas sIL-6R level in inflamed tissues depends on local recruitment of immunocompetent cells that shed mIL-6R [43,62]. This shedding is enhanced by upregulating ADAMTS17 by neutrophils. Second, in the absence of IL-6, other IL-6 family cytokines compensate and secure adequate IL-6-like signaling through gp130 [90,451]. However, such redundancy between different IL-6 cytokines has been described in cell cultures, and it is therefore difficult to know the net effect of IL-6-family cytokines in a clinical setting.

In this study we analyzed pretransplant serum levels; an important question is whether the pretransplant time period is the optimal time to investigate the effects of the IL-6 buffer. Pretransplant IL-6 levels are generally low, but significant increases are observed in almost all patients during the first 2 weeks posttransplant, with greater increases in patients developing inflammatory complications [298,301-303,311]. These serum levels later normalize in patients without complications but remain high in patients with aGVHD. However, most patients suffer from severe conditioning-induced neutropenia early posttransplant, and since neutrophils are a main source of sIL-6R it is not clear that high IL-6 levels are associated with high IL-6 transactivation during neutropenia. Thus, IL-6 transactivation effects may be most important during the pretransplant phase rather than during the early posttransplant period.

Our study showed that pretransplant CRP level was an independent risk factor for TRM. Previous studies, and a subsequent study published in 2016, yielded similar results on TRM, but conflicting results with regard to associations between CRP and GVHD [323-330]. A meta-analysis published in 2019 included our study together with 13 other studies and concluded that pretransplant CRP level was an independent risk factor for inferior overall survival and NRM [452]. A weaker association with aGVHD was also observed. The pooled hazard ratio analysis for overall survival and TRM was consistent throughout the different studies. These data clearly suggest that our findings are robust and reproducible.

A new and interesting aspect of this article was the association between IL-31 and TRM. IL-31 is an inflammatory cytokine that is important for the development of cellular immunity and especially T cell functions in the skin. Elevated serum levels are observed

in patients with allergic skin disorders [19,453]. High IL-31 levels may indicate inadequate barrier functions.

Article II

In the second study, we demonstrated that rs2228145 and rs4845618 genotypes correlated with pre and posttransplant sIL-6R and CRP levels, but we did not observe a clear effect on GVHD or other outcomes for these SNPs. One possible explanation is that the main sources of sIL-6R are hematopoietic cells and hepatocytes [61,62] (i.e. in allotransplant recipients, the levels are determined both by donor and recipient cells). Alternatively, to assess the effect of three various genotypes combinations on recipient posttransplant sIL-6R and CRP levels, the study is possibly underpowered with regard to analysis of clinical outcome. Finally, our study included only patients with related donors, and the concordance between donor and recipient SNP genotypes is therefore higher than for recipients of grafts from MUDs.

The SNP rs2228145 contributes 70% of the variation in CRP level in Europeans, whereas the frequency of this allele is much lower in Asian and African populations, for which other factors seems to be more important for the variation ([157]. Most of our patients were of European heritage, and a similar study in patients of different ethnicity would possibly give additional information about the impact of the genetic variation of the IL-6R in allotransplantation.

IL-6 signaling is also modified by genetic variations in gp130, JAK2 and STAT3 [454-456]). The JAK2 46/1 haplotypes are associated with aGVHD. Although SNPs within the gp130 and STAT3 genes influence outcomes of inflammatory disorders [455,457,458], no effects on GVHD have been reported. The G148C polymorphism in the gp130 gene may influence IL-6 trans-signaling, but it was not included in our study [456]. This last polymorphism occurs at low frequency, with only 22.7% being hetero- or homozygote for the rare C allele in a Norwegian population, but it was associated with significantly altered gp130 levels. Thus, IL-6, IL-6R, gp130 and the JAK2 SNPs should be included in future studies.

A major problem in genetic association studies is defining an adequate threshold for p-values [446]. In the current study, we performed up to ten comparisons, and the probability

of one being false positive is approximately 40% [444]. Several strategies can be used to adjust for multiple comparisons, but corrections of p-values increase the risk of neglecting significant results. Alternative approaches include a Bayesian study design or a hierarchical test procedure [459,460]. In the current study we employed a predefined testing procedure in which we analyzed effects of specific SNPs on predefined outcomes. In this context, an important observation is that our study identified an association between the IL-6 SNP rs1800975 and outcome; this is similar to several other studies [353-364].

Article III

The use of G-CSF-mobilized stem cell is associated with an increased risk of cGVHD; the main reason for this seems to be the higher levels of mature T cells and NK-cells in these grafts[186]. However, G-CSF administration leads to altered systemic (i.e. serum/plasma) cytokine and metabolite profiles of both the donors and the graft supernatants [461,462], but it is not known whether these effects influence outcome of allotransplant recipients. A recent study identified IL-6 as a cytokine that was exceptionally influenced by leukapheresis, and G-CSF may also have a proinflammatory effect, reflected as increased CRP serum levels [461]). These two observations prompted us to investigate the levels of IL-6-family cytokines during stem cell mobilization and harvesting. Our third article verified that CRP and IL-6 levels are significantly altered by G-CSF administration [463], but similar effects were not observed for any of the other IL-6-family cytokines.

G-CSF effects were most prominent in our elderly donors and donors with increased CRP levels before therapy. There is ongoing debate whether well-matched younger (below 40 years of age) MUDs should be preferred over elderly sibling donors [464], and our study suggests that donor heterogeneity with regard to biological signs of inflammation should be considered, especially when selecting an elderly donor. However, we would emphasize that systemic CRP and cytokine levels are only a part of this heterogeneity, together with variations in levels of circulating immunocompetent cells and differences in SNPs of immunoregulatory genes.

Article IV

We investigated how various forms of IL-6 signaling influence downstream phosphorylation events in T cells. IL-6 seemed to potentiate activation of mTOR after T-cell receptor ligation. T cells undergo adaptations in energy, nucleotide and protein metabolism following T-cell receptor activation [465], and mTor is an important intracellular regulator that ensures these metabolic requirements are met [466]. Direct or indirect mTor inhibition through cyclosporine, tacrolimus or sirolimus is a part of standard GVHD prophylaxis. Furthermore, during the posttransplant period, IL-6 promotes development of proinflammatory Th17 cells and inhibits immunosuppressive regulatory T cells [102]. However, our findings indicate that IL-6-mediated mTor inhibition is also capable of reducing general alloreactivity through mTor. This might explain why the addition of IL-6 blocking tocilizumab to standard GVHD prophylaxis leads to an additional reduction in alloreactivity [256,422].

We observed that the degree of STAT3 activation following various IL-6 stimuli was greater in patients with previous GVHD. Betts et al. observed a correlation between the degree of IL-6-induced STAT3 phosphorylation in $CD4^+$ T cells early after transplant and later development of GVHD [467]. However, different T cell subsets show significant variation in their expression of membrane-bound IL-6R; it is highly expressed only in the naive and memory T cell subsets. Hence, stimulation with IL-6 alone (as was done by Betts et al. [467]) only induces classical IL-6 signaling predominantly in naive and memory $CD4^+$T cells. Thus, our observations may simply reflect the profile of the various T cell subsets being present rather than a specific IL-6-linked abnormality of intracellular T cell signaling or STAT3 activation as a late effect of previous aGVHD. An analysis of T cell subsets and expression of the membrane-bound IL-6R was included in our study. However, we were not able to detect any significant correlations between specific T cell subsets and phosphorylation status. There are several possible reasons for this. First, a limited amount of sample material was available for most patients due to lymphopenia, and a more detailed study of various T cell subsets was not possible. Second, subset studies may also be difficult to interpret because phosphorylation analysis requires methanol fixation that can lead to degradation of membrane proteins and the intracellular cytokines that are used for identification of various subsets [439,440]. It is therefore difficult to analyze

phosphorylation events in various T cell subsets beyond the $CD4^+$ and $CD8^+$ subsets. Finally, the number of targets was limited by our use of a standard 8-channel flow cytometer. We therefore chose to focus on pathway activation/phosphorylation events rather than more detailed studies of T cell subsets. Recently developed mass cytometry technology allows for simultaneous detection of up to 40 molecular targets [468]. Future studies could employ this technology to better explore phosphorylation events in various T cell subsets.

Although IL-6-mediated STAT3 activation is associated with an increased risk of GVHD, evidence from other immunological diseases indicates that therapeutic modulation at the level of the IL-6R will not be sufficient to prevent STAT3 phosphorylation. Myeloma cells depend on IL-6/IL-6R/gp130 signaling for survival, but despite this, blocking of IL-6 or IL-6R has relatively weak effects on gp130 mediated signaling, whereas direct gp130 blocking results in a much stronger inhibition, sufficient for induction of apoptosis [469]. Thus, targeting gp130 instead of IL-6/IL-6R may be a more efficient strategy to inhibit proinflammatory Th1 and Th17 cells and increase the effects of Th2 and Treg cells [102]. However, gp130 is important for normal tissue hemostasis in many organs [67], and a general gp130 blockade could lead to excessive side effects outside the targeted organ.

We would emphasize that we included consecutive patients, and this leads to a high degree of patient heterogeneity with regard to diagnoses, conditioning treatment, GVHD prophylaxis and donor types. We cannot exclude the possibility that the importance of IL-6 differs between patient subsets. In this context, one should also remember that sufficient mononuclear cells for a complete flow-cytometric evaluation was achieved only for a subset of patients; a circumstance consistent with the hypothesis that our observations may be relevant only for a subset of allotransplant recipients that have reached certain levels of immunological reconstitution or circulating immunocompetent cells.

12. CONCLUSIONS

Although IL-6 negatively influences outcome after ASCT, it has become increasingly clear that the blockade of IL-6 alone does not sufficiently suppress proinflammatory signals to prevent GVHD. A recent study of IL-6 blockade in severe aGVHD treatment was stopped due to insufficient effects by the investigated IL-6R antibody. The recently described IL-6 cluster signaling indicates that IL-6 signaling can be mediated by direct cell-to-cell contact through an immunological synapse; this may make extracellular IL-6 blockade insufficient. These observations, together with the results from our present studies, suggest that unselective blockade of IL-6 activity in aGVHD will have a limited effect.

However, more sophisticated targeting of IL-6 signaling, such as the direct targeting of proinflammatory IL-6 trans-signaling or inhibition of mediators downstream to sgp130, which have been studied in clinical trials for other inflammatory disorders may be more effective and efficient. However, better animal models that allow for detailed studies of such selective IL-6 blocking strategies should be developed, and the new strategies should be further investigated in such *in vivo* models before they are moved into clinical trials in GVHD. Such models already exist for various autoimmune disorders. Thus, the basis for the design of future clinical trials of IL-6 targeting in GVHD should be further studies in more relevant animal models along with clinical experience from other immune diseases using the recently developed and more sophisticated IL-6 strategies.

13. FUTURE PERSPECTIVE

Numerous previous studies have established a clear link between IL-6 and outcome after ASCT. The goal of the current thesis was to further investigate the effects of IL-6 along with factors that influence the various forms of IL-6 signaling and the events downstream of gp130. Our studies of pretransplant IL-6-family cytokine levels (article I) showed that systemic levels of sgp130 and sIL-6R, as markers of classical and IL-6 trans-signaling, did not correlate with specific posttransplant outcomes. However, genetic variations within the IL-6 system influenced markers of IL-6 trans-signaling and modulated immune reconstitution (article II). We also observed that various forms of IL-6 signaling potentiated the responsiveness of posttransplant T cells, especially IL-6-mediated STAT3 activation that was stronger in patients with previous GVHD (article IV). Taken together, our studies suggest the IL-6 family is one of several factors that contributes to early outcome after allotransplantation. Our studies suggest that early endothelial dysfunction with extensive fluid retention and pretransplant systemic levels of IL-31 are two additional factors that are important for outcome. Finally, our studies of healthy stem cell donors suggest that the IL-6 family may also influence posttransplant outcome through a contribution to donor heterogeneity (article III). However, whether these effects are more relevant for later cGVHD needs further investigations.

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Pretransplant Levels of CRP and Interleukin-6 Family Cytokines; Effects on Outcome after Allogeneic Stem Cell Transplantation

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Abstract: Several pretransplant factors, including CRP (C-reactive protein) levels, reflect the risk of complications after allogeneic stem cell transplantation. IL-6 induces CRP increase, and we therefore investigated the effects of pretransplant IL-6, soluble IL-6 receptors, IL-6 family cytokines and CRP serum levels on outcome for 100 consecutive allotransplant recipients. All patients had related donors, none had active infections and 99 patients were in complete remission before conditioning. The incidence of acute graft versus host disease (aGVHD) requiring treatment was 40%, survival at Day +100 82%, and overall survival 48%. Despite a significant correlation between pretransplant CRP and IL-6 levels, only CRP levels significantly influenced transplant-related mortality (TRM). However, CRP did not influence overall survival (OS). Pretransplant IL-31 influenced late TRM. Finally, there was a significant association between pretransplant IL-6 and early postconditioning weight gain (i.e., fluid retention), and this fluid retention was a risk factor for aGVHD, TRM and OS. To conclude, pretransplant CRP, IL-31 and early posttransplant fluid retention were independent risk factors for TRM and survival after allotransplantation.

Keywords: allogeneic stem cell transplantation; interleukin 6; interleukin 31; C reactive protein; graft versus host disease; comorbidity; fluid retention

1. Introduction

Graft versus host disease (GVHD) and severe infections are the most important causes of non-relapse mortality after allogeneic stem cell transplantation (ASCT) [1,2]. The risk of GVHD is influenced by pre-existing patient-, donor- and disease-specific factors as well as the pretransplant conditioning treatment and GVHD prophylaxis. The pretransplant cytokine network is also important, and experimental models suggest that the conditioning therapy induces the release of pro-inflammatory cytokines that increase the MHC (Major histocompatibility complex) molecule expression on host antigen-presenting cells and thereby activates donor T cells [3]. Several studies also suggest that specific single nucleotide polymorphisms (SNP) in Interleukin-6 (IL-6) genes influence the risk and severity of acute GVHD [4].

Previous analyses have shown that pre-transplant CRP levels correlate with overall survival (OS) and transplant-related mortality (TRM) [5–11]. The molecular mechanisms behind these associations

are largely unknown and only one study included analysis of cytokines together with CRP [5]. IL-6 is produced by macrophages and mesenchymal cells during inflammation and is the main driver of CRP production. The IL-6 cytokine family includes IL-6 together with IL-11, IL-27, IL-31, Leukemia inhibitory factor (LIF), Oncostatin M (OSM), Ciliary neutrophilic factor (CNTF), Cardiotrophin-1, Cardiotrophin-like-cytokine and Neuropoietin [12]. All these cytokines bind to receptors utilizing gp130 for signal transduction and are involved in immunoregulation [13–15]. Mice depleted of IL-6 still retain their ability to produce CRP [16]. Experimental studies suggest that the other IL-6 family members then compensate for the IL-6 response by interacting with IL-6R and causing an acute phase reaction. Cross-reactivity between other IL-6 family cytokine receptors is also possible [15,17].

Animal studies suggest that IL-6 is important in GVHD pathogenesis and inhibits reconstitution of regulatory T-cells, thereby promoting Th17 development [18–21]. However, IL-6 is also linked to anti-inflammatory processes and tissue regeneration [22]. The IL-6 receptor lacks intracellular domains and relies on gp130 for intracellular signal transduction. gp130 is ubiquitously expressed, whereas the membrane-bound IL-6 receptor (IL-6R, also known as CD126) is only found on certain cells. Soluble IL-6R (sIL-6R) does not inactivate IL-6, but binds to and activates gp130 on cells not expressing IL-6R themselves. Activation by sIL-6R is thought to mediate mainly pro-inflammatory effects while activation through membrane-bound IL-6R mainly mediates anti-inflammatory effects.

Under physiological conditions, soluble gp130 (sgp130) levels exceed the sIL-6R levels and thereby act as a physiological buffer against pro-inflammatory IL-6 effects [23]. Specific SNPs in IL-6R lead to higher levels of sIL-6R and are also associated with higher baseline CRP [24,25] and increased incidences of inflammatory and cardiovascular diseases [26]. IL-6R levels are also associated with increased relapse rate in certain cancers [27].

As described above, the IL-6 family cytokines have important immunoregulatory functions, but they also function as regulators of vascular permeability [28–31]. In this context, we have investigated the possible associations between pretransplant levels of CRP/IL-6 family members and posttransplant outcomes, including early weight gain (i.e., fluid retention) as well as GVHD and survival.

2. Results

2.1. The Clinical Characteristics of Patients Included in the Study

During the observation period a total 102 ASCTs were performed, including one ALL (acute lymphoblastic leukemia) and one AML (acute myeloid leukemia) patient who were re-transplanted due to relapse. The characteristics of the 100 patients are summarized in Table 1; 95 of these patients were Caucasians. Pretransplant serum samples were available for 100 transplantations (i.e., 98 patients included in the study). The median time from samples collection until transplantation was 23 days (interquartile range (IQR) 14 days), and average storage time before analysis 1518 days (range 75–3464 days, IQR 1688 days).

At admission for transplantation, 95 patients had Performance Status (PS) 0–1, only one patient had PS 3 due to immobilization secondary to prior cerebrovascular disorder caused by polycythemia vera, and no patients had PS 4. No patient had active infection and all but one AML patient were in remission when conditioning therapy started. With the exception of one patient, GVHD prophylaxis with cyclosporine A plus four doses of methotrexate (Days 1, 3, 6 and 11) was planned, but two of them did not receive methotrexate due to early complications. Antithymocyte globulin (ATG) was given to two patients as additional GVHD prophylaxis due to one HLA-antigen mismatch. All patients received granulocyte colony-stimulating factor (G-CSF) mobilized peripheral blood stem cell grafts except for patients with aplastic anemia ($n = 4$) or a donor younger than 15 years of age ($n = 1$) who all received bone marrow grafts. The majority of patients received conditioning treatment with BuCy (74 patients; busulfan 0.80 mg/kg QID from Day −7 to −5 and cyclophosphamide 60 mg/kg QD on Day −3 and −2) or FluBu (17 patients; fludarabine 30 mg/m^2 QD from Day −9 until −5 and busulfan 3.2 mg/kg QD on Day −3 and −2) (Table S1); busulfan was always given intravenously. Sinusoidal obstruction syndrome was diagnosed according to the Baltimore criteria for six patients.

Table 1. Clinical and laboratory characteristics of the 100 allotransplant recipients included in the study.

Age, Median and Range (Years)	**47.5 (15–70)**
Caucasian/non-Caucasian (number)	95/5
Diagnosis (number)	
AML	43
MDS-AML	16
Myelodysplastic syndrome (MDS), high-risk	4
Acute lymphoblastic leukemia	20
Chronic myeloid leukemia	2
Myelofibrosis	4
Chronic myelomonocytic leukemia	2
Myeloproliferative neoplasia, unspecified	2
Aplastic anemia	4
Chronic lymphocytic leukemia	2
Hodgkin's lymphoma	1
Remission at transplantation (number)	99
aGVHD requiring high dose steroid treatment (number) [1]	46
Conditioning regimes (number)	
Busulfan + cyclophosphamide (myeloablative condition)	74
Fludarabine + busulfan (reduced intensity conditioning)	17
Antithymocyte globulin + cyclophosphamide	4
Others	5
GVHD prophylaxis (number)	
Cyclosporine A + methotrexate	97
Cyclosporine A + mycophenolate mofetil	1
Cyclosporine A + methotrexate + antithymocyte globulin	2
Donor (number)	
Related	100
Sibling	93
Parent	6
Other related	1
Female/male donor	39/61
Female donor to male recipient	21
CMV pos. recipient	65
CMV pos. donor to neg. recipient	18
Stem cell source (number)	
Bone marrow grafts	5
G-CSF mobilized peripheral blood stem cell grafts	95
CRP mg/L (median and range; lower limit of detection being 1.0 mg/L)	5 (<1–120)
Maximum weight gain kg (median, range)	5.0 (0–16.1)

[1] The criteria for high-dose steroid treatment were acute GVHD grade II with gastrointestinal involvement or Grade III/IV acute GVHD.

2.2. *Pre-Transplant IL-6 and sgp130 Serum Levels Were Increased Prior to Conditioning Therapy Whereas the Levels of sIL-6R and Other IL-6 Family Members Did Not Differ from Healthy Controls*

LIF serum levels were only analyzed for 34 unselected patients and five controls and could not be detected for any of them; to save sample material, analysis of LIF was omitted for the remaining patients. IL-11 and IL-28 serum levels were determined for all patients and controls, but since the majority of patients showed undetectable levels or levels close to the detection limit, both these mediators were excluded from the statistical analyses. The serum levels of the other mediators were included in our statistical analyses together with a new parameter referred to as IL-6 difference and defined as the serum level of sgp130 minus the corresponding level of sIL-6R.

Median serum level, variation range and IQR of each mediator for the patients and healthy controls are presented in Table 2 and Figure S1. IL-6 showed significantly higher levels for the patients

compared to the healthy controls (p-value < 0.01); sgp130 levels were also higher in the patients but this difference reached only borderline significance (p-value 0.049), whereas sIL-6R levels did not differ significantly. The other IL-6 family members did not show any statistically significant differences when comparing patients and healthy controls.

Table 2. Pretransplant serum levels of IL-6 family cytokines for the allotransplanted patients (n = 100); a comparison with the levels for healthy individuals (n = 14). Significant values (p < 0.05) are highlighted in bold.

Mediator	All Allotransplant Patients			Healthy Controls			p-Value	LLOD
	Median	Range	IQR	Median	Range	IQR		
OSM	6.7	(6.7–89.3)	2.6	7.3	(6.7–111.9)	25.4	0.13	6.7
CNTF	701	(127–15,464)	1874	502	(127–11,819)		0.67	127
IL-6	12.6	(0.92–581)	19.6	3.0	(0.9–7.2)	4.2	**<0.01**	0.9
sIL-6R	11,580	(609–42,666)	10,722	8427	(4936–22,594)	10,541	0.09	18.7
sgp130	54,808	(8286–226,166)	60,005	39,776	(32,525–134,172)	67,302	**0.049**	81.0
sgp130-sIL-6R difference	4306	(−20,977–206,959)	48,710	32,283	(27,387–1,114,152)	58,499	0.10	NR
IL-31	7.12	(2.59–130.80)	7.52	8.70	(2.59–25.51)	8.62	0.1856	2.59

Abbreviations: Sgp16-sIL-6R diff, Difference between sgp130 and sIL-6R levels; IQR, Interquartile range; LLOD, Lower level of detection, NR, Not relevant.

The correlations between the levels of various IL-6 family members are presented in Table S1. A strong correlation was only seen between IL-6R and sgp130; in addition IL-6 showed significant correlations with both IL-6R and sgp130, whereas sgp130 also showed significant positive correlations with IL-6R and CNTF and an inverse correlation with IL-31.

2.3. Preconditioning Levels of IL-6 Family Cytokines Did Not Differ between Patients with and without Later aGVHD

Patients receiving RIC transplantations were significantly older, showed lower IL-31 levels but higher levels of sIL-6R and sgp130 than the MAC patients (Table S2); in both groups there were no correlation between age and mediator levels. Furthermore, there was no difference in mediator (IL-6 family members, soluble receptor chains) levels between patients experiencing later aGVHD and patients not developing aGVHD (see Table S3), but a non-significant trend of higher IL-31 levels was observed for patients with aGVHD (p-value 0.097).

2.4. sIL-6R and sgp130 Levels Correlates with Time until Neutrophil Reconstitution but Not with Time Until Platelet Reconstitution

Several IL-6 family members regulate normal hematopoiesis [32–35], and we therefore investigated whether their preconditioning systemic levels showed any correlations with preconditioning peripheral blood cell counts or posttransplant engraftment (see Table 3 and the complete data presented in Table S4). Firstly, IL-6 levels correlated inversely to pretransplant hemoglobin concentration (Table S4; Spearman's $\rho = -0.40$, p-value < 0.05); this is similar to previous observations [36]. Secondly, OSM serum levels showed a statistically significant correlation with preconditioning total peripheral blood leukocyte counts (Spearman's $\rho = 0.27$, p-value < 0.05), but without significant correlations to lymphocyte, neutrophil or monocyte counts. For the other IL-6 cytokine family members, no significant correlations were detected.

Preconditioning serum sIL-6R and gp130 levels showed significant positive correlations to time until neutrophil engraftment (Table 3). Furthermore, for patients with CRP level above median we observed a significantly lower pretransplant hemoglobin concentration, IL-6 concentration and leucocyte count; there was also a significantly higher proportion of CMV positive patients in the high CRP group (60.3% vs. 91.0%, p-value < 0.01).

Table 3. Correlation between preconditioning serum levels of soluble mediators and the peripheral blood cell counts tested before and following allotransplantation. The results are presented as the Spearman's ρ and significant correlations ($p < 0.05$) are highlighted in bold. (**Upper part**) Significant correlations between IL-6 family cytokine levels tested before conditioning therapy and peripheral Boblood cell counts tested before immediately before initiation of conditioning treatment; (**Lower part**) Correlations between preconditioning serum mediator levels and peripheral blood cell counts (neutrophils and platelets) tested after allotransplantation. Time to neutrophil engraftment was defined as peripheral blood neutrophils above 0.2×10^9/L on three consecutive days and time to platelet engraftment as peripheral blood thrombocytes above 20×10^9/L on three consecutive days without platelet transfusions.

Preconditioning Peripheral Blood Cell Counts		
Peripheral Blood Parameter	**IL-6 Family Cytokine**	**Correlation**
Hemoglobin level	IL-6	**−0.40**
Total leukocyte count	OSM	**0.27**
Hematopoietic Reconstitution after Allotransplantation		
Mediator	**Neutrophils above 0.2×10^9/L**	**Platelets above 20×10^9/L**
IL-6	0.12	0.19
sIL-6R	**0.283**	0.12
sgp130	**0.238**	0.05
Diff	**0.215**	0.02
CNTF	0.19	0.01
OSM	0.01	−0.06

2.5. Pretransplant IL-6 Levels Correlated with Pretransplant CRP Levels

The lower limit of detection for CRP was 1 mg/L. Median pretransplant CRP serum level was 5 mg/L (IQR 12 mg/L, range LLOD-120 mg/L). CRP correlated significantly to pretransplant IL-6 levels (Spearman's $\rho = 0.68$, p-value < 0.05) and also to pretransplant hemoglobin level (Spearman's $\rho = -0.36$, p-value < 0.05). No significant correlation between age and CRP levels was observed. There was no difference between median CRP levels for patients receiving RIC and MAC treatment (p-value 0.896).

2.6. A Large Patient Subset Shows Early Weight Gain after Conditioning/Transplantation

IL-6 seems to contribute to the increased vascular permeability during inflammation; similar effects have also been suggested for IL-11, IL-21 and possibly LIF [28–31]. For this reason, we investigated both the possible associations between pretransplant levels of IL-6 family members and posttransplant weight increase/fluid retention, and the impact of weight gain on outcome after transplantation. We first analyzed maximal weight gain by comparing contrasting groups. The median value of the maximal weight gain during the first four weeks after the start of conditioning therapy was 5.0 kg (range 0–16.1 kg, IQR 4.0 kg) weight increase compared with baseline. Only one of the patients with weight gain exceeding 5 kg was diagnosed with sinusoidal obstruction syndrome. Both pretransplant CRP (Generalized linear model as described in Material and Methods, $p < 0.02$) and IL-6 ($p < 0.0$) levels showed a significant effect on maximal weigh gain in univariate analyses, but in multivariate analysis no single factor had a significant effect on the weight gain.

The 50 patients with a maximal weight gain exceeding 5 kg could be divided into two groups depending on the time until maximum weight gain was registered; one group (21 patients) had a maximum weight gain prior to stem cell transplantation/infusion, and another group (29 patients) with increasing weight during the first two weeks posttransplant (Figure S2). We then analyzed the data for the whole patient population ($n = 100$); the median weight gain was then significantly higher for patients showing maximal weight after the transplantation compared with patients reaching their maximal weight gain between initiation of conditioning and stem cell transplantation (3.9 kg vs. 5.9 kg, p-value < 0.01), but the proportions of patients dying before Day +100 did not differ

between these two groups (p-value 0.22). In this context, it was not unexpected that the time from start of conditioning treatment until maximum weight and maximal weight gain showed a significant correlation (Spearman's test, $\rho = 0.62$, p-value < 0.01, Figure S2). There were no statistically significant differences in serum levels for any cytokine/mediator, CRP level, age or any other clinical/laboratory parameter when comparing patients with maximum weight gain above or below 5 kg.

We then compared contrasting groups with high and low weight gain; based on the later use of maximal weight gain as a continuous variable and the use of dummy variables to define cut-off in the survival analyses (see section 2.8 below) we then used a cutoff of 6.8 kg to define two contrasting groups, i.e., patients with low and high weight gain, respectively. Firstly, the median pretransplant creatinine kevel for all 100 patients was 72 μM (variation range 42–149 μM). Patients with weight increase exceeding 6.8 kg had a significantly higher creatinine levels prior to conditioning therapy compared with the other patients ($p = 0.02$), and this differences remained significant also when comparing creatinine levels 14 and 28 days after transplantation. Secondly, the preconditioning albumin levels did not differ between these two groups, whereas the albumin levels were significantly lower for patients with maximal weight gain exceeding 6.8 kg both when comparing these two groups 14 and 28 days after transplantation (Figure 1). Thirdly, the cyclosporine A levels did not differ between the two groups.

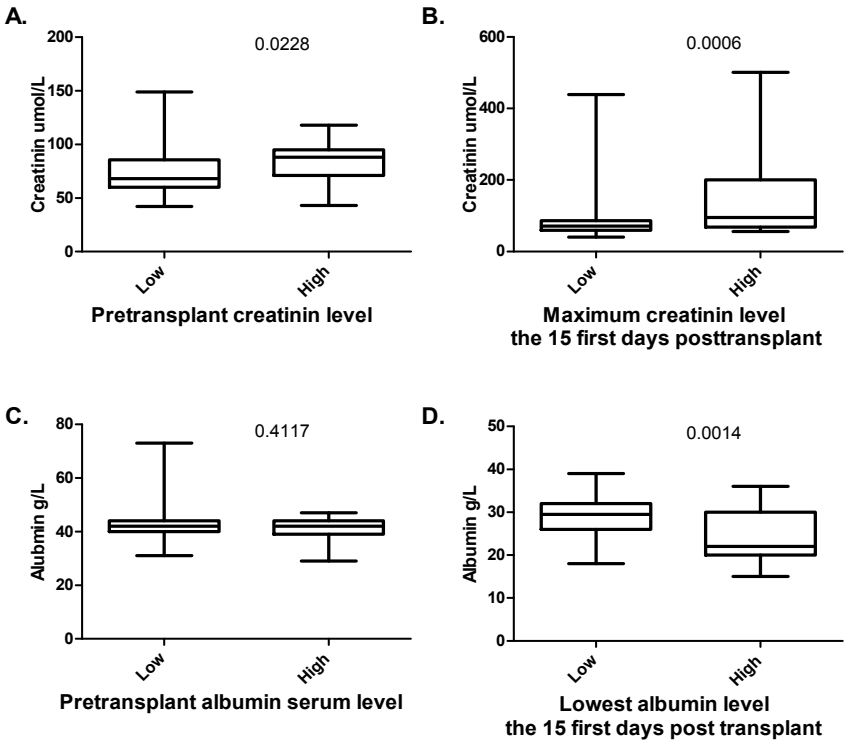

Figure 1. Maximal weight gain (i.e., fluid retention) early after allogeneic stem cell transplantation—a comparison between patients with weight gain below (**low**) or above (**high**) 5 kg. The figure shows the comparison of: (**A**) pretransplant creatinine serum level; (**B**) the highest observed creatinine level before Day +15 posttransplant; (**C**) pretransplant albumin levels; and (**D**) the lowest albumin level before Day +15 posttransplant. The Mann–Whitney U-test was used for the analyses; the corresponding p-value is given in the upper right for each part of the figure.

2.7. The Risk of Steroid-Requiring aGVHD Was Only Associated with Maximum Weight Gain and Sibling vs. Non-Sibling Donor but Not with Preconditioning Levels of Cytokines/Receptors or CRP

The cumulative incidence of aGVHD requiring high-dose steroid treatment was 40%; this included patients with grade II disease with gastrointestinal involvement, and patients with grade III/IV acute GVHD. Only maximum weight gain and sibling vs. non-sibling donor were significantly associated with increased incidences of aGVHD, whereas we could not detect a significant effect for the preconditioning serum levels of any single mediator (IL-6 family cytokines, sIL-6R, and sgp130), CRP level, CMV status, female to male donor or age. The overall results of these univariate analyses are presented in Table S5, while the results from the multivariate analysis are presented in Table S4.

2.8. Transplant-Related Mortality before Day +100 Post-Transplant Was Only Associated with Maximum Weight Gain as Well as Preconditioning CRP and IL-31 Levels in Adjusted/Multivariate Analysis

The Kaplan–Meier plot of overall survival 100 days post-transplant is shown in Figure 2A; the overall survival at Day +100 being 82% The crude analysis of TRM at Day +100 post-transplant showed significant associations with maximum weight gain, pretransplant IL-6 and IL-31 levels, type of transplantation and pretransplant CRP level above median (Table S6; p-values < 0.05). The effect of CRP on overall survival is also presented in Figure 2.

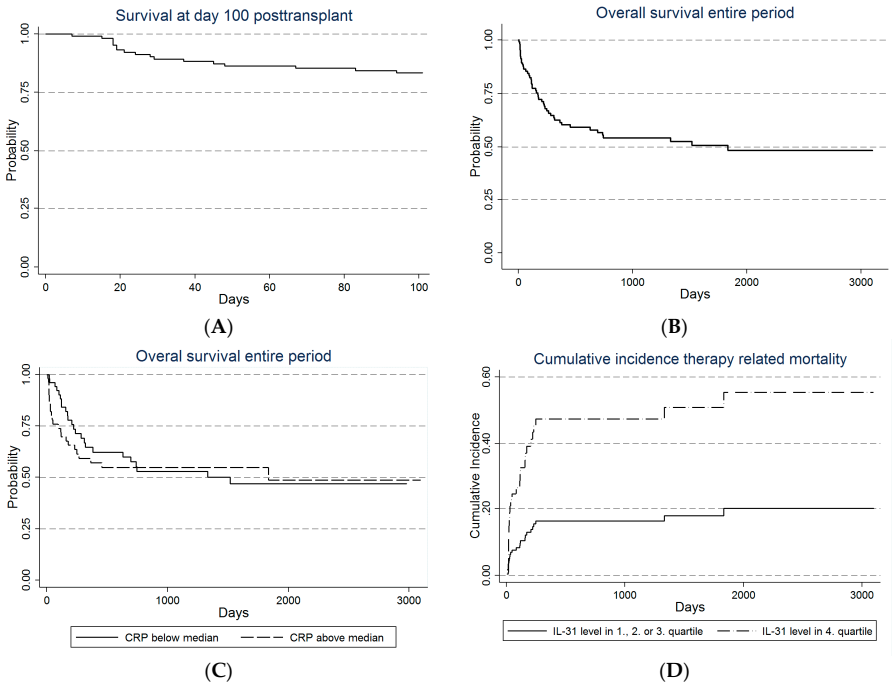

Figure 2. Survival after allogeneic stem cell transplantation for all patients included in our study. The Kaplan–Meier plots show: (**A**) overall survival for the first 100 days posttransplant and (**B**) overall survival for the entire period; (**C**) the effect on the overall survival of pretransplant CRP levels above or below the median CRP serum level; and (**D**) the cumulative incidence of TRM for patients with either low (quartiles 1–3) or high pretransplant IL-31 levels.

By splitting continuous variables into dummy variables it could be shown that possible cut-off points for IL-6 and IL-31 pretransplant levels corresponded to serum levels above the third quartile,

for CRP above 14 mg/L and for maximum weight gain 6.8 kg. An adjusted model then showed a significant effect only of maximum weight gain (above vs. below 6.1 kg), pretransplant IL-31 serum level in the fourth quartile and pretransplant CRP levels above the median (Table S4). Dichotomizing variables were not regarded as optimal due to few events in each group, but an adjusted model with continuous variables showed significant effect of IL-31, CRP, maximum weigh gain and female to male transplantation.

2.9. Recipient Age, Maximum Weight Gain and Preconditioning IL-31 Levels Are Associated with Transplant-Related Mortality and Overall Survival after 2 Years in Multivariate Analysis

OS for the entire cohort at two years was 56%. In univariate analysis maximum weight gain, pretransplant CRP, pretransplant IL-31 serum level and sibling vs. non-sibling donor had significant effects on transplant-related mortality (Table S7). Pretransplant CNTF serum level was also included in the following multivariate analysis because it showed a *p*-value of borderline significance (*p*-value 0.08). In this final model age, maximum weight gain, pretransplant CRP level and pretransplant IL-31 serum level had significant effects on TRM (Table S8). However, only age, maximum weight gain and IL-31 level affected overall survival in uni- and multivariate analysis with no significant effect of CRP level.

2.10. Only Maximum Weight Gain and Preconditioning Serum IL-31 Levels Are Associated with Transplant-Related Mortality and Overall Survival for the Entire Observation Period in Multivariate Analysis

The median observation period of all patients was 477 days (range 7–3098 days). OS for the entire cohort is shown in Figure 2B. In univariate analysis, TRM was significantly influenced by maximum weigh gain, pretransplant CRP, pretransplant IL-31 level and sibling vs. non-sibling donor (Table S9), but in the final model only the effects of weight gain and IL-31 reached significance with no effect of CRP and sibling vs. non-sibling donor. Analysis for OS yielded similar results (Table S10). The effect of CRP on overall survival and IL-31 on TRM are shown in Figure 2C,D.

3. Discussion

Several studies have investigated the pro-inflammatory cytokine network after allogeneic stem cell transplantation. However, relatively few studies have investigated the impact of inflammation and cytokine levels prior to the conditioning therapy, but they suggest that preconditioning signs of inflammation (i.e., CRP levels) are important for the posttransplant clinical course (Table S11). The molecular mechanisms behind this prognostic impact of CRP are largely unknown. The systemic pretransplant cytokine profile, β_2-Mikroglobulin serum levels and levels of endothelial cell markers also seem to reflect the risk of severe posttransplant complications [37,38]. IL-6 is the main driver of CRP production [39,40], and to further characterize the molecular mechanisms behind the pretransplant pro-inflammatory phenotype we investigated whether systemic preconditioning levels of IL-6 family members reflect the risk of posttransplant complications.

Our patient cohort is relatively small, but we would emphasize that our patient cohort represents an unselected and population-based group of patients, and the patient characteristics are in addition described in detail. Our cohort should therefore be regarded as representative for adults transplanted with HLA-matched family donor allografts. Although our patients represent an unselected consecutive cohort, the patient heterogeneity is relatively small compared with many other studies. Only family donors (and for almost all patients sibling donors) were used, nearly all patients received peripheral blood mobilized stem cells and most patients received the same conditioning treatment and GVHD prophylaxis. However, we would emphasize that our results have to be interpreted with care due to the relatively low number of patients and the patient heterogeneity, and future studies have to clarify whether these mechanisms are important also for other allotransplant recipients.

IL-6 can be constitutively released by and also be a growth factor for malignant hematopoietic cells [41,42], and high levels may even reflect an adverse prognosis in various malignancies [43–45]. IL-6 is also an important immunoregulator and sgp130 as well as sIL-6R influence both IL-6 and

CRP levels. For these reasons we investigated whether systemic levels of IL-6, other IL-6 family members or sgp130/sIL-6R reflect a risk of posttransplant complications or disease relapse in allotransplant recipients.

Most previous studies have found the preconditioning CRP level to be an independent prognostic factor associated with increased TRM and subsequently OS; two studies also identified increased CRP levels as a risk factor for later aGVDH but only one study identified high CRP levels as a risk factor for cGVHD (Table S11). Disease status can influence pre-transplant CRP levels, and even though classification of disease status was not clearly defined or differed between these studies, it seems clear that all these previous studies included a relatively high number of patients with active disease. In addition, patient and donor heterogeneity together with several outcome possibilities makes it hard to draw robust conclusions from these studies. Our current study differs from previous studies in that the patient population is more homogeneous with respect of donor type, pretransplant disease status, performance status and conditioning regimens. In our study pretransplant CRP showed a strong association with TRM at Day +100, but this effect was lost over time with no significant effect on overall survival for the whole observation period. Thus, our results suggest that preconditioning CRP is an independent marker for risk of early death in allotransplant recipients with low disease burden.

In our present study, we included the early posttransplant weight gain in our statistical analyses together with preconditioning/pretransplant levels of IL-6 family cytokines. Endothelial cells express gp130 but not membrane-bound IL-6R. During inflammation increased IL-6 and sIL-6 levels cause activation of vascular endothelial gp130 leading to redistribution of VE-cadherin with disruption of adherence junctions between endothelial cells and subsequent capillary leakage [28]. Other members of the IL-6 family also play a role in the regulation of vascular permeability [28–31].

Very few studies have investigated early posttransplant fluid retention as a risk factor after allotransplantation. A weight increase of at least 3% during 24 h is often used as a part of the diagnostic criteria for capillary leak syndrome [46]. This definition was used in a recent study of capillary leak syndrome in elderly allotransplanted pediatric patients whereas a weight criteria alone was used for the smallest children; these authors then described an association between capillary leak syndrome and decreased survival [47]. In our present study we used a maximal increase of 5 kg in the body weight despite diuretic therapy as a cutoff for comparison of contrasting groups with regard to the degree of fluid retention. Furthermore, our previous studies suggest that this cut-off identifies two patient subsets that differ with regard to metabolic regulation of fluid balance and capillary permeability, i.e., altered levels of metabolites involved in regulation of vascular functions, endothelial function/damage, capillary permeability and renal functions [47]. Weight gain should thus be regarded as a posttransplant parameter influenced by the pretransplant status [48]. For these reasons, early posttransplant weight gain was included in our statistical analyses together with other preconditioning factors. Our studies showed that this early posttransplant weight gain was associated with adverse prognosis, but further studies are needed to clarify the biological mechanisms behind these associations.

There is no generally accepted definition for capillary leak syndrome [49], but a definition including at least 3% weight gain during 24 h may be used [46]. As an alternative we therefore analyzed the impact of the maximal weight gain, and in contrast to the definition of capillary leak syndrome our parameter could be handled as a continuous variable in the survival analyses. A high posttransplant weight gain was associated with high preconditioning creatinine level, decreased albumin levels at the time of maximal weight and increased aGVHD/transplant-related mortality later posttransplant. However, both maximal weight gain and the alternative definition of capillary leak syndrome seem to reflect complications that usually develop during the early posttransplant period before Day +15, suggesting that these two parameters at least partly reflect the impact of the same biological mechanisms.

Only Artz et al. [5] incorporated cytokine levels (IL-6) in their analysis of preconditioning CRP levels, and they could not detect any association between IL-6 above the median level and infections or hepatic toxicity (grade 3/4 at Day +100), duration of hospital stay, aGVHD, TRM or OS.

By dichotomizing the IL-6 in the initial analysis one can easily loose the effect of IL-6, and for this reason we deliberately did not choose to dichotomize continuous variables prior to the first univariate analysis. By applying this approach it was possible to identify new cut-off points. Our observations of significant associations with IL-6 levels in univariate analyses suggest that this parameter is a part of a more complex increased-risk pretransplant phenotype, although it cannot be used in the pretransplant risk evaluation.

CRP is not only a biomarker of inflammation, it seems to be an important component of the innate host defense and its monomeric form activates and induces pro-inflammatory cytokine release by endothelial cells [50,51]. The preconditioning CRP levels probably reflect a pro-inflammatory phenotype, but it is likely that the complete risk-associated phenotype is more complex involving different molecular mechanisms including immunoregulatory metabolites and cytokines (including IL-6 family members) as well as damaged or altered endothelial cells [47,48]. Taken together with our previous studies our present observations suggest that the preconditioning CRP levels function as a risk factor that integrates the pro-inflammatory effects of several pretransplant characteristics, including serum IL-6 levels that showed significant associations in univariate analyses, correlated with CRP levels and even may serve as a therapeutic target in aGVHD [52].

In this study, high IL-31 levels were associated with reduced overall long term survival without any association with aGVHD. To the best of our knowledge the role of IL-31 in allogeneic stem cell transplantation has not been investigated previously. Baseline patient characteristics and relapse rate did not differ between the low and high IL-31 groups. IL-31 is released during inflammation by different cell types, including keratinocytes, fibroblast and cells of the innate and adoptive immune system. The main role of IL-31 is in the interaction between epithelial surfaces (i.e., skin, lung, and gut) and the immune system [15]. Serum IL-31 levels correlate with disease activity for pruritic skin disorders, and IL-31 seems important in the pathogenesis of allergic asthma as well as ulcerative colitis and Crohn's disease [53–56]. Increased IL-31 levels are also seen in non-Philadelphia chromosome myeloproliferative disorders [57]. A possible hypothesis is that increased preconditioning IL-31 levels reflect disturbed epithelial barriers (e.g., skin, airways, and gastrointestinal tract) that cause a long-lasting predisposition to inflammation and/or infection.

Our present study further emphasizes the importance of the precondition/pretransplant status of allotransplant recipients with regard to risk of posttransplant complications. The molecular mechanisms behind the adverse pretransplant pro-inflammatory phenotype are probably complex and largely unknown. Identification of CRP as a possible biomarker suggest that pro-inflammatory mechanisms are important, and the suggested link between pretransplant IL-6/inflammation/fluid retention/outcome suggests that altered endothelial function/vascular permeability are also involved.

4. Material and Methods

4.1. Patients

The study was approved by the local Ethics Committee (REK VEST 2013/ 634, Regional Ethics Committee III, University of Bergen, Bergen, Norway) and samples collected after written informed consent from patients at Haukeland University Hospital. In this period only patients with an available family donor was allotransplanted and therefore no transplantations with matched unrelated donors are included. These patients represent all allotransplanted adults from a defined geographic area (Norwegian Health Regions III, IV and V) with an available family donor. The decision to do an allotransplantation was taken by the Norwegian Advisory Board for Stem Cell Transplantation and based on national guidelines. Thus, our study should be population-based and include a random group of well-characterized patients. Samples were collected on the day of pre-transplantation evaluation or on the day of admission for stem cell transplantation.

Acute and chronic GVHD was diagnosed according to generally accepted criteria. All patients with aGVHD were evaluated using Glucksberg score, but patients who required more than

1 mg/kg/day methylprednisolone intravenous or an equivalent dose as GVHD treatment had grade II-IV aGVHD, i.e., patients with grade II disease and gastrointestinal involvement, and patients with grade III/IV acute GVHD. Neutrophil reconstitution was defined as three consecutive days with neutrophil counts of at least 0.2×10^9/L, and platelet reconstitution as stable platelet counts exceeding 20×10^9/L for at least 3 consecutive days without transfusions.

For a subset of patients in this cohort it has previously been shown that increased preconditioning/pretransplant levels of specific metabolites predicts capillary leak syndrome [47,58]. The maximum weigh gain was therefore included in the analysis. Weight at start of conditioning therapy was set as the reference weight, and the weight was thereafter registered prospectively twice daily until hematological reconstitution and thereafter every morning; the maximum weight gain during the first 30 days posttransplant was recorded. As used in previous studies, capillary leak syndrome was defined as a 5 kg weight gain from baseline despite diuretic therapy. The Baltimore criteria were used for diagnosis of sinusoidal obstruction syndrome; ultrasound examination was used when this diagnosis was suspected based on the clinical evaluation. The majority patients were treated with ursodeoxycholic acid from the start of conditioning therapy [59].

Performance status (PS) at time of admission for ASCT was recorded for every patient during the entire period. Standard comorbidity index scores (HCT-CI and EBMT-score) were not systematically implemented or register until after 2012 and were therefore available only for a minority of patients; for these reasons, it was only PS registered.

4.2. Healthy Controls

Control samples from healthy individuals were collected from 14 randomly chosen healthy blood donors at the local blood bank. No additional information about gender or laboratory values was registered.

4.3. Analysis of Soluble Mediator Levels in Serum Samples

Venous blood was collected onto sterile plastic tubes (BD Vacutainer® SST™ Serum Separation Tubes, Becton-Dickenson; Franklin Lakes, NJ, USA) and allowed to coagulate for 120 min at room temperature before centrifugation ($300\times$ *g* for 10 min) and serum collection. Serum was immediately frozen and stored at -80 °C until analyzed. Repeated freezing and thawing were avoided. The samples were analyzed with Bio-Plex kits for IL-6, IL-11, IL-27(p28), sIL-6R (sCD126), LIF and IL-31 (Bio-Rad, Hercules, CA, USA), and Multiplex Assays (Millipore, Billerica, MA, USA) for CNTF and OSM. All samples were analyzed using Luminex®200™ Bio-Rad platform with program version 6.1 and all analyses were performed in duplicates strictly according to the manufacturer's instructions. CRP was analyzed using an immunoturbidimetric method provided by Roche (Basel, Switzerland), and during the entire period the lower limit of detection for CRP was 1 mg/L.

4.4. Statistical Analyses

Statistical analyses were performed using the Statistical Package for the Social Sciences version 22.0 (IBM Corp.; Armonk, NY, USA), GraphPad Prism 5 (Graph Pad Software, Inc.; San Diego, CA, USA) and Stata Version 14 (StataCorp. 2009; Stata Statistical Software, College Station, TX, USA). Spearman's correlation for bivariate samples was used for correlation analyses, the Mann–Whitney *U*-test was used to compare continuous variables and the Chi-Square tests or Fisher's exact test were used to compare categorical variables. Differences were regarded as statistically significant when p-values < 0.05.

Overall survival was calculated using the Kaplan–Meier product limit method. The Cox proportional Hazzard model was used for calculating crude and adjusted hazard ratios (HR) for overall survival (OS). In a similar manner crude and adjusted subdistribution hazard ratios (SHR) were calculated using cumulative incidence regression methods as described in Fine and Gray [60] for therapy related mortality during the first 100 days post-transplant (defined as early TRM), 700 days

post-transplant (defined as late TRM) and for the entire period. For competing risk analysis cause of death was either classified as relapse related or treatment related. In advance it had been defined that age, CRP and variables with p-value <0.1 in univariate analyses would be included in the final model for each defined time period. In the final model a p-value <0.05 was regarded as statistically significant. Generalized linear model was used to analyze the effect of different covariates on maximum weight gain.

For samples with a measured value below the lower level of detection (LLOD), the value was set to the LLOD in the statistical analyses. For the models of OS and TRM each variable was first entered as continuous variables. Variables with a significant effect were split into three dummy variables each corresponding to second, third and fourth quartile to examine if dichotomization was possible.

5. Conclusions

This study confirms that elevated CRP level above baseline increases the risk of early but not late death due to transplant related mortality, but it is not associated with an increased risk of GVHD. Pretransplant IL-6 levels are highly correlated with CRP levels but does not predict outcome after ASCT. IL-31 was the only member of the Interleukin-6 family that had an effect on outcome; in contrast to CRP IL-31 had a significant effect on long-term TRM. The occurrence of capillary leak syndrome was associated with both GVHD and a significant increase in transplant related mortality. The pretransplant pro-inflammatory phenotype is associated with an increased risk of sever posttransplant complications and is characterized by increased levels of CRP, IL-6 and sgp130 and suggests a possible link between pretransplant IL-6 and posttransplant capillary leak.

Supplementary Materials: Supplementary materials can be found at www.mdpi.com/1422-0067/17/11/1823/s1.

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Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

aGVHD	Acute graft versus host disease
ALL	Acute lymphoblastic leukemia
AML	Acute myeloid leukemia
ASCT	Allogenic stem cell transplantation
BM	bone marrow
CD	Cluster of differentiation
cGVHD	Chronic graft versus host disease
CMV	Cytomegalovirus
CNTF	Ciliary neutrophilic factor
CRP	C-reactive protein
EBMT	European Society for Blood and Marrow Transplantation
G-CSF	granulocyte colony-stimulating factor
gp130	Glycoprotein 130
HCT-CI	Hematopoietic cell transplant comorbidity index
HLA	Human leucocyte antigen
IL	Interleukin
IL-6R	Interleukin-6 receptor
IQR	Interquartile range
LIF	Leukemia inhibitory factor
MAC	Myeloablativ conditioning
MDS	Myelodysplastic syndrome
MHC	Major histocompatibility complex
NR	Not reported

OS	Overall survival
OSM	Oncostatin M
PB	peripheral blood
PS	Performance status
RIC	Reduced intensity conditioning
sgp130	Soluble glycoprotein 130
sIL-6R	Soluble interleukin-6 receptor
SNP	Single nucleotide polymorphism
TRM	Transplant-related mortality

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Supplementary Materials: Pretransplant Levels of CRP, Interleukin-6 Family Cytokines and Outcome after Allogeneic Stem Cell Transplantation

Tor Henrik Tvedt, Stein Atle Lie, Håkon Reikvam, Kristin Paulsen Rye, Roald Lindås, Tobias Gedde-Dahl, Aymen Bushra Ahmed and Øystein Bruserud

Table S1. Correlation between the preconditioning serum levels of IL-6 cytokine family members, IL-6R and sgp130. Spearman's rank order correlation test was used for the analyses. The results are presented as the Spearman's ϱ and significant correlations ($p < 0.05$) are highlighted in bold.

Parameter	IL-6R	sgp130	IL-31	OSM	CNTF
IL-6	**0.34**	**0.31**	0.17	0.028	−0.17
IL-6R		**0.71**	**−0.23**	0.03	0.18
sgp130			**−0.26**	0.11	**0.24**
IL-31				−0.06	−0.17
OSM					**0.16**

Table S2. Biological and clinical parameters of allotransplant recipients included in the study; a comparison of patients receiving reduced intensity conditioning (RIC, $n = 17$) and myeloablative conditioning (MAC, $n = 83$) treatment.

Parameter	RIC-Group		MAC-Group		*p*-Value
	Median	Range	Median	Range	
sIL6-R	18,022	4775–33,936	10,353	609–42,666	<0.01
sgp130	107,106	31,493–157,256	54,145	8286–226,166	<0.01
IL-31	LLOD	LLOD–25.5	7.0	LLOD–131	0.03
Gender	Female	5	Female	33	0.324
	Male	13	Male	49	
Acute leukemia (number)	AML	9	AML	50	0.1
	ALL	0	ALL	20	
Age (years)	61	22–70	43	15-62	<0.01
Time to neutrophil engraftment (days)	17	6 to 24	15	10 to 50	0.09

Table S3. Preconditioning serum levels of IL-6 family cytokines, sIL-6R sgp130 for the 100 allotransplanted patients; a comparison of patients with and without later aGVHD (all concentrations are given in pg/mL).

Parameter	No aGVHD		aGVHD		*p*-Value
	Median	Range	Median	Range	
IL-6	13.19	1.32–434.93	10.35	LLOD [1]–580.78	0.602
sIL-6R	10,520	4775–33,936	12,783	609.4–42,666	0.271
sgp130	54,158	31,493–170,849	55,209	8286–226,166	0.447
IL-6 difference [2]	43,502.5	25,002.5–145,868.00	46,843.35	−20,976.5–206,959	0.418
IL-31	7.28	LLOD–25.51	LLOD	LLOD–130.80	0.095
OSM	LLOD (6.68)	LLOD–86.14	LLOD	LLOD–89.29	0.530
CNTF	736	LLOD–15,464	577	LLOD–10,148	0.870

[1] LLOD, lower limit of detection; [2] The IL-6 difference was defined as the serum level of sgp130 minus the corresponding level of sIL-6R.

Table S4. Correlations between pretransplant levels of cytokines and receptors for IL-6 family cytokines, peripheral blood cell counts, serum levels of biochemical parameters and maximal weigh gain. The *p*-values were calculated using Spearman's rank order correlation. The results are presented as the Spearman's ϱ and significant correlations ($p < 0.05$) are highlighted in bold and underlined.

Parameter	IL-6	sIL-6R	sgp130	IL-6-diff	IL-31	OSM	CNTF
Hb	**−0.40**	−0.11	−0.17	0.17	−0.04	−0.12	0.01
Leukocytes	0.05	0.10	−0.01	−0.02	0.08	**0.27**	−0.02
Neutrophils	−0.01	−0.06	−0.10	−0.09	0.10	0.14	−0.03
Lymphocytes	−0.02	0.06	−0.02	−0.03	0.12	0.12	0.13
Monocytes	0.05	0.03	−0.13	−0.14	−0.01	0.01	−0.04
Thrombocytes	−0.25	−0.141	−0.18	−0.16	−0.17	−0.05	−0.15
CRP	**0.68**	0.14	0.07	0.04	0.13	0.15	−0.12
LDH	−0.08	0.17	0.07	0.04	−0.02	0.06	−0.11
Maximal weight gain	0.137	−0.03	0.05	0.05	−0.02	0.07	−0.04

Abbreviations: CRP, C reactive protein (mg/L); Hb, Hemoglobin concentration (g/100 mL); IL-6 diff, IL-6 difference; Lactate dehydrogenase count (U/L).

Table S5. Crude and adjusted subdistribution hazard ratios for aGVHD.

Covariate	Crude				Adjusted			
	p-Value	SHR	95% CI		*p*-Value	SHR	95% CI	
IL-6, continuous variable	0.79	1.00	0.99	1.01				
ILl-6R continues variable	0.31	1.00	1.00	1.00				
sgp130, continuous variable	0.51	1.00	0.99	1.00				
Diff, continuous variable	0.60	1.00	0.99	1.00				
IL-31, continues variable	0.43	1.01	0.98	1.03				
OSM	0.82	1.00	0.98	1.02				
CNTF continuous variable	0.37	1.00	1.00	1.00				
Age/10 year	0.33	1.10	0.89	1.37	0.15	1.16	0.95	1.41
Gender	0.15	1.52	0.86	2.70				
RIC vs. MAC	0.45	0.74	0.34	1.62				
Sibling vs. non-sibling	0.01	3.12	1.39	6.99	<0.01	3.76	1.87	7.54
Female to male vs. other	0.89	0.94	0.45	1.99				
CMV pos. donor to neg. recipient vs. other	0.66	0.82	0.35	1.95				
CRP, continuous variable	0.13	1.67	0.86	3.25				
CRP, value below vs. above median	0.68	1.00	0.98	1.01				
Maximum weigh gain, <6.8 kg vs. >6.8 kg	<0.01	1.14	1.04	1.25	<0.01	1.14	1.05	1.24

Table S6. Crude and adjusted subdistribution hazard ratios for treatment related mortality at 100 days post-transplant.

Covariate	Crude				Adjusted			
	p-Value	SHR	95% CI		p-Value	SHR	95% CI	
IL-6, continuous variable	0.04	1.00	1.00	1.01				
IL-6, all other values vs. value in 4. quartile	<0.01	4.01	1.48	10.93	0.12	2.43	0.78	7.51
IL-6R continues variable	0.49	1.00	0.99	1.01	0.08	2.98	0.85	10.33
s-gp130, continuous variable	0.92	1.00	0.99	1.01	0.02	4.79	1.29	17.67
Diff, continuous variable	0.78	1.00	0.99	1.01				
IL-31, continues variable	<0.01	1.02	1.01	1.02				
IL-31, all other values vs. value in 4. Quartile	0.02	3.43	1.24	9.47	0.01	3.78	0.85	10.33
OSM	0.10	0.93	0.87	1.02				
CNTF continuous variable	0.28	0.99	0.98	1.01				
Age/10 year	0.70	1.06	0.79	1.42	0.83	1.03	0.73	1.47
Gender	0.84	1.11	0.44	3.08				
RIC vs. MAC	0.25	0.30	0.04	2.28				
Sibling vs. non-sibling	0.29	2.10	0.53	8.47				
Female to male vs. other	0.18	2.06	0.71	5.99				
CMV pos. donor to neg. recipient vs. other	0.63	1.45	0.46	4.60				
CRP, continuous variable	<0.01	1.03	1.01	1.04				
CRP, value below vs. above median	0.02	4.59	1.32	15.94	0.04	3.83	1.01	13.75
Maximum weigh gain, <6.8 kg vs. >6.8 kg	<0.01	5.19	1.83	14.72	<0.01	6.18	2.23	17.15

Table S7. Crude and adjusted subdistribution hazard ratios for treatment related mortality at 700 days post-transplant.

Covariate	Crude				Adjusted			
	p-Value	SHR	95% CI		*p*-Value	SHR	95% CI	
IL-6, continuous variable	0.11	0.99	0.99	1.00				
IL-6, all other values vs. value in 4. quartile	0.21	1.00	0.99	1.01				
IL-6R continues variable	0.87	1.00	0.99	1.00				
sgp130, continuous variable	0.41	1.00	0.90	1.00				
Diff, continuous variable	0.35	1.00	0.99	1.00				
IL-31, continues variable	<0.01	1.02	1.01	1.02				
IL-31, all other values vs. value in 4. Quartile	0.01	2.87	1.29	6.40	<0.01	3.78	1.67	8.54
OSM	0.36	0.68	0.30	1.55				
CNTF continuous variable	0.11	2.80	0.99	1.01				
Age/10 year	0.12	1.02	0.99	1.04	0.05	1.27	0.99	1.63
Gender	0.68	0.81	0.29	2.26				
RIC vs. MAC	0.96	1.00	0.98	1.02				
Sibling vs. non-sibling	0.04	2.80	1.02	7.65	0.18	2.14	0.69	6.65
Female to male vs other	0.39	1.43	0.64	3.22				
CMV pos. donor to neg. recipient vs. other	0.97	1.02	0.37	2.83				
CRP, continuous variable	0.03	1.02	1.00	1.03				
CRP, value below vs above median	0.03	2.36	1.07	5.16	0.04	2.14	1.09	5.07
Maximum weigh gain, <6.8 kg vs. >6.8 kg	<0.01	3.35	1.50	7.50	<0.01	3.90	1.69	8.99

Table S8. Crude and adjusted subdistribution hazard ratios for overall survival at day 700.

Covariate	Crude				Adjusted			
	p-Value	SHR	95% CI		*p*-Value	SHR	95% CI	
IL-6, continuous variable	0.52	0.99	0.99	1.00				
IL-6, all other values vs. value in 4. quartile	0.59	1.00	0.99	1.01				
IL-6R continues variable	0.61	1.00	0.99	1.00				
sgp130, continuous variable	0.34	1.00	0.99	1.00				
Diff, continuous variable	0.33	1.00	0.99	1.00				
IL-31, continues variable	0.04	1.02	1.01	1.03				
IL-31, all other values vs. value in 4. quartile	0.04	2.03	1.02	4.06	<0.01	2.76	1.35	5.64
OSM	0.24	0.67	0.35	1.29				
CNTF continuous variable	0.26	1.80	0.64	5.06				
Age/10 year	0.02	1.03	1.00	1.05	<0.01	1.41	1.09	1.84
Gender	0.58	1.24	0.58	2.71				
RIC vs. MAC	0.98	0.99	0.98	1.02				
Sibling vs. non-sibling	0.02	1.32	1.03	1.69	0.34	1.81	0.52	6.20
Female to male vs. other	0.36	1.51	0.63	3.63				
CMV pos. donor to neg. recipient vs. other	0.47	0.73	0.30	1.74				
CRP, continuous variable	0.18	1.01	0.99	1.02				
CRP, value below vs. above median	0.43	1.27	0.70	2.34	0.48	1.26	0.66	2.39
Maximum weigh gain, <6.8 kg vs. >6.8 kg	0.01	2.28	1.89	4.89	0.01	2.30	1.17	4.53

Table S9. Crude and adjusted subdistribution hazard ratios for overall survival whole period.

Covariate	Crude				Adjusted			
	p-Value	SHR	95% CI		*p*-Value	SHR	95% CI	
IL6, continuous variable	0.20	1.00	1.00	1.01				
IL-6, all other values vs. value in 4. quartile								
il-6R continues variable	0.90	1.00	1.00	1.00				
sgp130, continuous variable	0.47	1.00	1.00	1.00				
Diff, continuous variable	0.42							
IL-31, continues variable	<0.01	1.02	1.01	1.02				
il31, all other values vs. value in 4. quartile	0.02	2.68	1.21	5.92	0.01	2.97	1.23	7.14
OSM	0.96	1.00	0.97	1.03				
CNTF continuous variable	0.08	0.99	1.00	1.00	0.22	1.00	1.00	1.00
Age/10 year	0.05	1.30	1.00	1.02	0.01	1.42	1.08	1.87
Gender	0.42	0.72	0.33	1.59				
RIC vs. MAC	0.65	0.79	0.29	2.18				
Sibling vs. non-sibling	0.06	2.68	0.97	7.44	0.09	2.66	0.85	8.31
Female to male vs. other	0.30	1.50	0.69	3.25				
CMV pos. donor to neg. recipient vs. other	0.23	1.54	0.72	3.76				
CRP, continuous variable	0.03	1.02	1.00	1.03				
CRP, value below vs. above median	0.03	2.32	1.09	4.91	0.10	2.08	0.88	4.95
Maximum weigh gain, <6.8 kg vs. >6.8 kg	<0.01	2.89	1.30	6.40	0.01	3.02	1.30	7.03

Table S10. Crude and adjusted subdistribution hazard ratios for TRM whole period.

Covariate	Crude				Adjusted			
	p-Value	SHR	95% CI		*p*-Value	SHR	95% CI	
IL-6, continuous variable	0.72							
IL-6R continues variable	0.689							
sgp130, continuous variable	0.392							
Diff, continuous variable	0.382							
IL-31, continues variable	0.03	1.02	1.01	1.03				
IL-31, all other values vs. value in 4. quartile	0.03	2.00	1.05	3.93	0.02	2.94	1.49	5.81
OSM	0.514							
CNTF continuous variable	0.972							
Age/10 year	0.01	1.39	1.1	1.76	<0.01	3.23	1.17	1.9
Gender	0.351							
RIC vs. MAC	0.228							
Sibling vs. non-sibling	0.368	1.32	1.03	1.69				
Female to male vs. other	0.164							
CMV pos. donor to neg. recipient vs. other	0.948							
CRP, continuous variable	0.305							
CRP, value below vs. above median	0.778				0.895	1.04	0.57	1.89
Maximum weigh gain, <6.8 kg vs. >6.8 kg	0.03	1.97	1.07	3.63	0.02	2.08	1.12	3.89

Table S11. The prognostic impact of pretransplant CRP levels in allotransplant recipients; a summary of previous studies investigating the effect of pretransplant CRP on outcomes after allogeneic stem cell transplantation.

Author	Year	Patient Number	Non-Malignant Disease	Related/Unrelated	Stem Cell Source		Remission/High Risk	HCT-CI	Effect on				
					BM	PBSC			aGVHD	OS	TRM	RRM	cGVHD
Artz [5]	2008	112 [a]	5	68/44	7	105	NR/52	Yes	Yes	Yes [b]	Yes	NR	NR
Pavlu [11]	2010	271	No, only CML	130/141	256	15	NR/113	Yes	NR	Yes	Yes	No	NR
Remberger [6]	2010	504	~16%	196/229	156	312	NR	NR	No	Yes	Yes [d]	Yes [e]	NR
Sakamoto [7]	2012	211	8/211	86/95	95	86	NR, 90/121	NR	No	No	Yes	No	Yes
Aki [9]	2012	106	No	97/9	0	106	22	Yes	NR	Yes	No	NR	NR
Sato [8]	2013	90	NR	39/51	58	24	NR/12%	NR	Yes	Yes	Yes	NR	NR
Jordan [10]	2013	349	11%	170/179	227	121	NR/113	NR	No	Yes	Yes	No [c]	NR

a: Data only for 81 patients; b: effect of CRP only shown in univariate analysis; c: Increased CRP at day of stem cell infusion showed significant higher relapsed related mortality; d: Only for the RIC cohort; e: Effect only in univariate analysis. Abbreviation: BM, Bone Marrow; G-CSF, Granulocyte colony-stimulating factor; NR, not reported; PBSC, peripheral blood stem cells, RRM Relapse-related mortality.

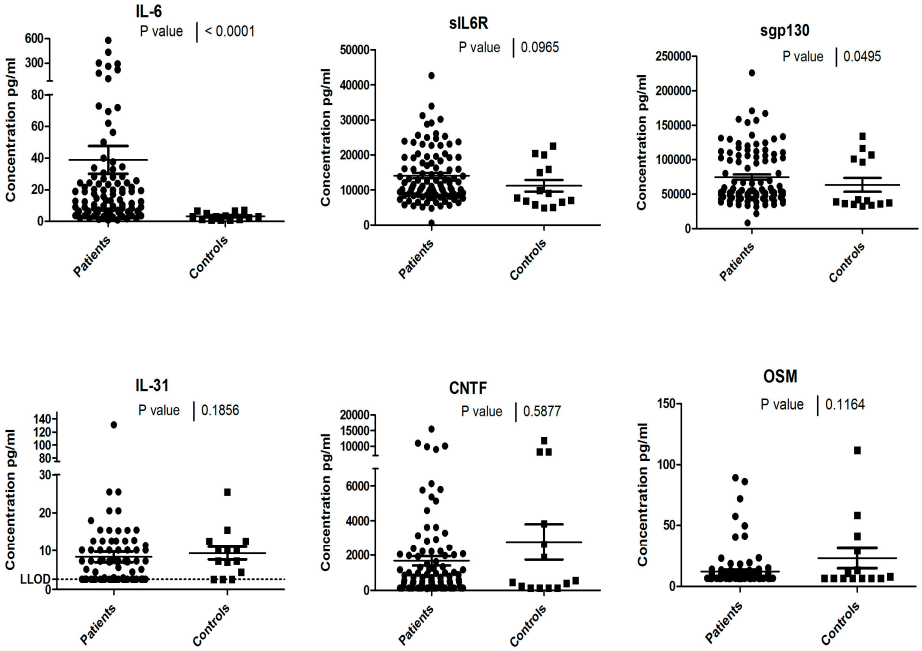
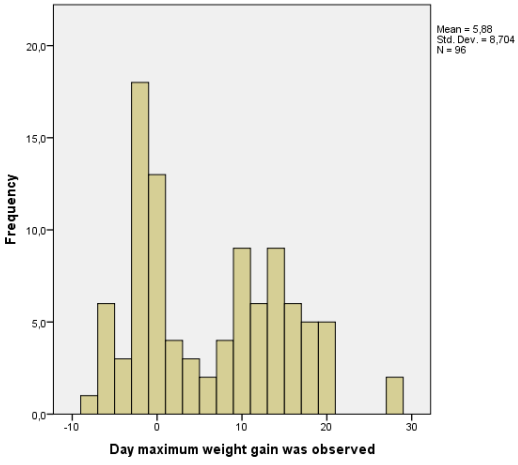


Figure S1. Preconditioning serum levels of IL-6 cytokine family members, IL-6R and sgp130; a comparison of between allotransplanted patients (n = 100) and healthy controls (n = 14) (LLOD = Lower limit of detection).



(A)

Figure S2. *Cont.*

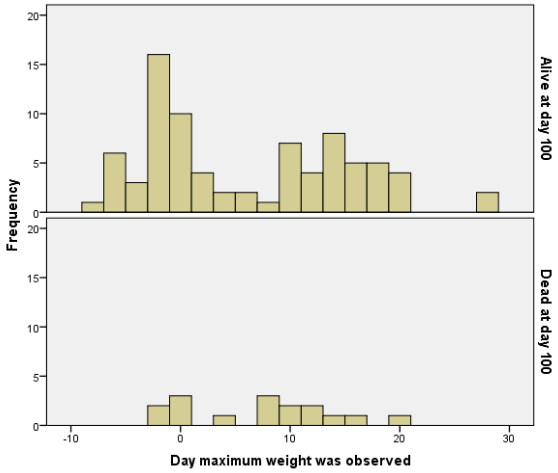


Figure S2. Early posttransplant weight gain in allotransplant recipients. The figures show the day of maximal weight gain after initiation of the conditioning treatment. Day 0 is the day of stem cell infusion. (**A**) This figure shows the day of maximal weight gain for all allotransplant recipients (n = 100); (**B**) The two figures show the day of maximal weight gain for patients being alive on day +100 posttransplant (**upper**) and for the patients being dead at this time (**lower**).

III

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Communication

Immunological Heterogeneity of Healthy Peripheral Blood Stem Cell Donors—Effects of Granulocyte Colony-Stimulating Factor on Inflammatory Responses

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Abstract: Interleukin-6 (IL-6) contributes to the development of immune-mediated complications after allogeneic stem cell transplantation. However, systemic IL-6 levels also increase during granulocyte colony-stimulating factor (G-CSF) mobilization of hematopoietic stem cells in healthy donors, but it is not known whether this mobilization alters systemic levels of other IL-6 family cytokines/receptors and whether such effects differ between donors. We examined how G-CSF administration influenced C-reactive protein (CRP) levels (85 donors) and serum levels of IL-6 family cytokines/receptors (20 donors). G-CSF increased CRP levels especially in elderly donors with high pretherapy levels, but these preharvesting levels did not influence clinical outcomes (nonrelapse mortality, graft versus host disease). The increased IL-6 levels during G-CSF therapy normalized within 24 h after treatment. G-CSF administration did not alter serum levels of other IL-6-familly mediators. Oncostatin M, but not IL-6, showed a significant correlation with CRP levels during G-CSF therapy. Clustering analysis of mediator levels during G-CSF administration identified two donor subsets mainly characterized by high oncostatin M and IL-6 levels, respectively. Finally, G-CSF could increase IL-6 release by in vitro cultured monocytes, fibroblasts, and mesenchymal stem cells. In summary, G-CSF seems to induce an acute phase reaction with increased systemic IL-6 levels in healthy stem cell donors.

Keywords: toll-like receptors; Interleukin-6; C-reactive protein; acute-phase reaction; graft versus host disease; tissue and organ procurement

1. Introduction

Granulocyte colony-stimulating factor (G-CSF)-mobilized peripheral blood stem cell grafts are used for allogeneic stem cell transplantation (ALLO-SCT) [1]. This G-CSF therapy has several immediate effects on the donor immune system but does not seem to have any long-term consequences [2]. It increases levels of various anti-inflammatory cytokines while simultaneously decreasing the production of several proinflammatory cytokines [3,4], inhibits T cell responsiveness and shifts their differentiation towards Th2 responses [5,6], induces IL-10 producing allo-inhibitory regulatory T cells [7], promotes

the development of myeloid-derived tolerogenic dendritic cells [8], and reduces serum levels of the chemotactic C-X-C motif ligand 8 (CXCL8) and C-X-C motif ligand 12 (CXCL12) chemokines [9]. Thus, anti-inflammatory effects are common [8].

The increased risk of graft-versus-host disease (GVHD) for patients receiving G-CSF-mobilized stem cells has been explained by the increased number of donor T cells in these grafts [10]. However, the effects of G-CSF therapy in healthy individuals are complex as illustrated both by the frequent reversible side effects (e.g., musculoskeletal pain) and uncommon but more severe toxicity (e.g., splenic rupture and pulmonary toxicity), including progression of arthritis as an example of a proinflammatory effect [11,12]. A recent study also described metabolic effects of G-CSF therapy in healthy stem cell donors, and these effects may influence immunoregulation [13]. Furthermore, the systemic level of the proinflammatory acute phase stimulant interleukin-6 (IL-6) is also increased for a subset of such donors [9], but it is not known which cells are responsible for this IL-6 response [6,14].

Optimal selection of the healthy stem cell donor is essential for outcome after allogeneic stem cell transplantation, and among the well-characterized donor risk factors are major histocompatibility complex mismatches, female donor for male patient, donor age, and Killing Immunoglobulin-like Receptor genotype [15]. As described in a recent article, several studies have now described associations between graft compositions and outcome after ALLO-SCT [16], and the first study of individualized GVHD prophylaxis based on graft composition has already been published [17]. However, several studies have demonstrated that the immunomodulatory effects of G-CSF-induced stem cell mobilization differ between healthy donors [18]. Firstly, the effects of G-CSF on serum levels of a wide range of both pro- and anti-inflammatory cytokines, as well as soluble adhesion molecules and extracellular proteases, differ between healthy donors [9,19]; Secondly, the effect of G-CSF on immunoregulatory metabolites also varies [13]; Thirdly, the numbers of different immunocompetent cell subsets vary between grafts derived from different donors [20]; Finally, a recent study suggests that the responsiveness of immunocompetent cells to G-CSF administration differs between healthy donors, i.e., there are qualitative differences, and not only quantitative differences, between grafts derived from different donors [21]. An important question is therefore whether the G-CSF induced immunomodulation is heterogeneous and whether such differences between donors have an impact on outcome after allotransplantation [16]. The aims of our present study were therefore to investigate whether IL-6 or other IL-6 family cytokines/receptors are influenced by G-CSF therapy and thereby contribute to the heterogeneity of healthy allogeneic stem cell donors, to examine whether this heterogeneity is important for outcome after allogeneic stem cell transplantation, and to elucidate whether G-CSF will alter the release of IL-6 by in vitro cultured monocytes and/or fibroblasts.

IL-6 depends on gp130 for transmembrane signaling, and C-reactive protein (CRP) production is mainly driven by classical IL-6 signaling (dependent on membrane-bound IL-6 receptors) whereas trans-signaling (dependent on soluble IL-6 receptors) seems less important [22]. G-CSF increases IL-6 levels and would therefore be expected to increase the acute phase reaction (including CRP). However, one should emphasize that the final effect of G-CSF on CRP levels depends on the biological context and G-CSF can reduce the acute phase responses after tissue injury [23]. Other cytokines that depend on gp130 for signal transduction (e.g., other IL-6 family members) may then induce an acute phase response in the absence of IL-6 [24,25]. Taken together, these observations suggest that the balance between pro- and anti-inflammatory effects of G-CSF and IL-6 depends on the clinical context. This is also supported by previous studies of post-transplant G-CSF therapy in allotransplant recipients; whether G-CSF therapy will influence post-transplant survival depends on the conditioning therapy and the type of stem cell graft [26]. We have previously reviewed the scientific evidence for a role of IL-6 in the development of immune-mediated complications after allotransplantation [27], and previous studies have also shown that IL-6 serum levels are altered during G-CSF mobilization for a large subsets of healthy stem cell donors [9,19,28]. Even though risk-adapted GVHD prophylaxis based on variations in graft composition is already considered, a better understanding of the mechanisms behind, and the consequences of, donor and graft heterogeneity is needed, including the possible

roles of the IL-6 family and the contribution of G-CSF to the heterogeneity. In our present study; we therefore investigated effects of G-CSF on systemic levels of CRP and IL-6 cytokine family members in healthy stem cell donors.

2. Results

2.1. Healthy Stem Cell Donors Are Heterogeneous with Regard to Ongoing Acute Phase Reaction and the G-CSF Therapy Causes a Further Increase of CRP Levels for a Subset of Donors

Data were available for 39 female and 59 male donors; the clinical characteristics of the recipients and their matched family donors are given in Material and Methods, Section 4.1. The median number of circulating $CD34^+$ cells on the day of stem cell collection was 51.2 $\times$ 10^6/L (range 15.3–160.7). Age was the only factor associated with reduced level of circulating $CD34^+$ cells (Spearman's rho -0.420 $p < 0.01$). Their serum CRP levels were generally low with 75% having CRP level <2 mg/L and 50% below the lower limit of detection (1 mg/L). However, CRP levels were significantly higher (median increase 7 mg/L; median level 9.5 mg/L with range 1 to 49 mg/L, $p < 0.01$) after four days of G-CSF therapy. Those patients with relatively high pretherapy CRP level (i.e., >2 mg/L) also had significantly higher CRP level than the others during G-CSF therapy (Figure 1a).

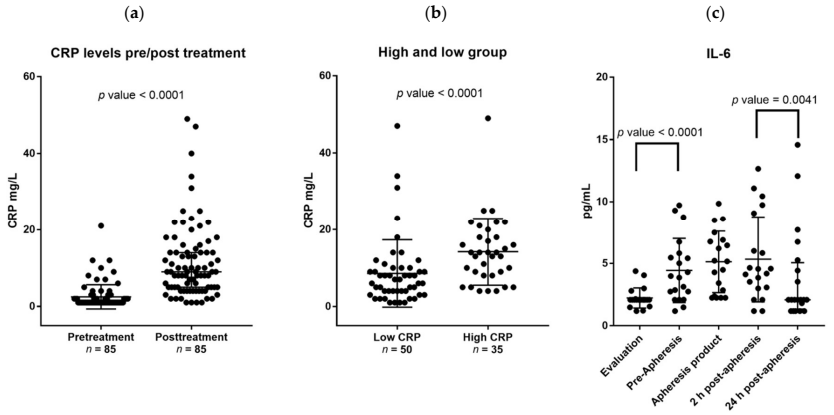

Figure 1. Effects of granulocyte colony-stimulating factor (G-CSF) on C-reactive protein (CRP) and systemic interleukin-6 (IL-6) levels. All results are presented as the levels for individual patients, the median levels and the 75% percentiles. (**a**) This figures shows CRP level prior to (pretreatment) and after four days of G-CSF administration (post-treatment) for all donors with detectable CRP level at these two time points. A significant increase in CRP levels was observed after G-CSF treatment; (**b**) The figure shows a comparison between the differences in CRP levels (i.e., levels during G-CSF minus the pretherapy level; mg/L) for those patients who had low ($\leq$2 mg/L) and high pretherapy CRP level (>2 mg/L); (**c**) This figure presents the variations in serum IL-6 levels (pg/mL) for 20 healthy stem cell donors during mobilization and harvesting of peripheral blood stem cells; each dot represents the observations for one patient at the given time point. Treatment with G-CSF induced a significant increase in systemic IL-6 levels (evaluation versus pre-apheresis levels, *p*-value < 0.0001). This increase was maintained 2 h after apheresis, i.e., the pre-apheresis levels did not differ significantly from 2 h postapheresis levels (*p*-value 0.275). However, the IL-6 levels decreased significantly from 2 h to 24 h postapheresis (2 h postapheresis levels versus 24 h postapheresis levels, *p*-value <0.0041), and this decrease represents a normalization of the systemic IL6 levels during the first 24 h after apheresis (i.e., 24 h postapheresis levels versus pretherapy/evaluation levels, *p*-value 0.123). The median time from donor evaluation (the first sample, also referred to as the pretreatment sample) to start of G-CSF therapy was 16 days. The levels in the graft supernatants (apheresis product, 19 patients tested) are also presented.

The donor heterogeneity with regard to serum CRP levels was maintained during G-CSF therapy (Figure 1b). Furthermore, donors in the fourth age quartile had significantly higher CRP level than the younger donors both prior to G-CSF (median 2 mg/L with range 3 to 21 mg/L versus median 1 mg/L with range 2 to 12 mg/L) and during G-CSF treatment (median 13.3 mg/L with range 1 to 49 versus median 8 mg/L with range 1 to 47 mg/L). Although both age and pretherapy CRP level were associated with higher CRP levels during G-CSF therapy in univariate analyses, age was not significant when corrected for pretreatment CRP levels (Table 1).

Table 1. A summary of the linear regression model of the effects of pre-G-CSF CRP levels and age on CRP levels after granulocyte colony-stimulating factor (G-GSF) administration. Age was initially entered as three different dummy variables corresponding to the second, third, and fourth quartile. Only age above or below 57 years had a significant effect on CRP levels in univariate analysis.

Covariate	Univariate			Multivariate		
	Coefficient	SE [1]	*p*-Value	Coefficient	SE [1]	*p*-Value
Pre G-CSF CRP level	1.48	0.31	<0.01	1.40	0.32	<0.01
Age [2]	5.30	2.45	0.03	2.16	2.39	0.37

[1] Standard error of the mean; [2] Age below or above 57 years of age.

Only pretherapy CRP levels (but not CRP levels during the G-CSF therapy) showed a weak but significant correlation with the levels of circulating $CD34^+$ cells after four days with G-CSF therapy (Spearman's rho −0.21, p-value < 0.03). Finally, the donor CRP levels before and during G-CSF therapy were not associated with risk of acute GVHD or overall survival of the stem cell recipients.

2.2. G-CSF Therapy of Healthy Stem Cell Donors Is Associated with Increased Serum Levels of IL-6 Whereas the Levels of Other IL-6 Family Members Are Not Altered during Stem Cell Mobilization

We investigated the IL-6 cytokine family in more detail for an unselected subset of 20 healthy donors (11 women, nine men). Serum samples were then collected before and during (i.e., immediately before apheresis) G-CSF therapy, immediately after and 24 h after apheresis. Graft supernatants were also analyzed. The levels of IL-6 family members were determined for all samples (Figure 1c). Low IL-6 serum levels were detected in pretherapy samples for all donors, the levels increased significantly during G-CSF treatment ($n = 20$, $p < 0.001$) and were even higher in graft supernatants. However, IL-6 levels normalized within 24 h after apheresis (i.e., 26–30 h after the last G-CSF injection).

As can be seen from Table 2, the sIL-6R levels were not altered by the G-CSF therapy, but the sIL-6R levels were significantly increased in the graft supernatants and in the serum 24 h after stem cell harvesting. Furthermore, the levels of ciliary neutrophilic factor (CNTF), oncostatin M (OSM), and IL-31 showed no variations during stem cell mobilization and collection, but for OSM and IL-31 significantly increased levels were detected in the stem cell grafts compared with the serum levels (Table 2). Finally, leukemia inhibitory factor (LIF) could not be detected in any samples for the 10 patients examined.

Graft levels were significantly higher than the postapheresis peripheral blood levels especially for IL-31 and OSM, whereas the differences between graft and serum levels for sIL-6R and CNTF reached only borderline significance (Table 2). The ratio between serum levels of sIL-6 receptor and sgp130 is termed the IL-6 buffer; this ratio was not altered by G-CSF therapy.

Table 2. Serum levels of IL-6 family cytokines at four different time points during stem cell mobilization and harvesting; the levels in graft supernatants are also included as a comparison. The results for 20 healthy stem cell donors (median age 51 years, range 25–73 years) are summarized, and all the results are presented as the median level and the variation range. All concentrations are given as pg/mL, and statistically significant alterations compared with the pretherapy levels (before G-CSF therapy) are marked in bold (Mann–Whitney *U* test). Graft levels were only available for 19 patients, and statistically significant differences between graft levels and postapheresis levels are indicated in the table (* $p < 0.05$, ** $p < 0.01$).

Mediator	Before G-CSF	During G-CSF (Pre-apheresis)	Graft Supernatant	2 h after Apheresis	24 h after Apheresis
IL-6	2.1 (1.2–4.4)	**3.9 (1.2–9.7)**	**5.2 (2.2–9.9)**	**4.4 (1.2–12.7)**	2.1 (1.2–14.2)
sgp130	19,197 (86–26,942)	17,239 (7004–28,049)	22,985 (7666–36,063)	17,429 (9723–40,714)	18,914 (10,596–32,561)
sIL-6R	4400 (26–6189)	3952 (1932–7938)	**6101 (2103–11,681)**	4401 * (2181–11,843)	4692 (2252–12,936)
IL-31	6.7 (3.6–21.8)	6.4 (3.6–19.5)	**37.8 (5.8–76.8)**	5.3 ** (3.6–15.3)	6.7 ** (3.6–9.8)
OSM	29 (7–214)	31 (8–229)	**94 (11–538)**	32 ** (8–137)	36 ** (10–214)
CNTF	624 (470–1543)	571 (470–2019)	677 (494–2507)	649 (470–1892)	571 * (470–1710)

2.3. CRP Levels during G-CSF Therapy Are Significantly Correlated with the Oncostatin M Serum Levels but There Is No Association with the Corresponding Serum IL-6 Levels

The systemic IL-6 and CRP levels showed a significant correlation before G-CSF therapy (Spearman's rho 0.51, *p*-value = 0.02), but this correlation was absent during G-CSF treatment (Spearman's rho 0.05, *p*-value 0.86) when the CRP levels showed a significant correlation with serum OSM levels (Spearman's rho 0.521, *p*-value 0.022). Finally, age showed a significant association with peripheral blood $CD34^+$ cell level at the time of harvesting, but the $CD34^+$ cell levels did not show significant associations with the levels of any IL-6 family cytokines/receptors at any of the investigated time points.

2.4. Systemic (Serum) Levels of IL-6 Family Cytokines and Especially the Oncostatin M Levels Vary between Donors Both When Tested before and during G-CSF Therapy

Even though IL-6 was the only cytokine that was significantly altered during G-CSF therapy and apheresis, it can be seen from Table 2 that the other IL-6 family cytokines, and especially OSM, showed a considerable variation among donors. Therefore, we did an unsupervised hierarchical clustering analysis of the graft levels immediately after apheresis to further characterize and visualize the overall influence of mobilization and harvesting (Figure 2; 19 patients included, graft levels were not available for patient 6). This analysis identified two main patient clusters; the left cluster included patients that generally showed relatively high levels of OSM and low IL-6 levels, whereas many of the patients in the right cluster showed low OSM levels and higher IL-6 levels. The two clusters did not differ with regard to patient age or gender distribution.

2.5. The Levels of Immunocompetent Cell Subsets in Peripheral Blood and Allogeneic Stem Cell Grafts Vary between Healthy Donors: Studies of Associations between Serum Levels of IL6 Family Cytokines, Circulating Immunocompetent Cells, and Graft Content of Immunocompetent Cells

We investigated the graft composition and the peripheral blood levels of total T cells, $CD4^+$ T cells, $CD8^+$ T cell, B cells, monocytes, and natural killer (NK) cells together with the levels of $CD34^+$ cells for our healthy stem cell donors (Table 3). The peripheral blood levels were determined after four days of G-CSF treatment immediately before stem cell harvesting by leukapheresis. There was a considerable variation between the donors with regard to the peripheral blood levels of all immunocompetent cell subsets; the largest variation being observed for $CD16^+$ NK cells. The number of harvested graft

cells on the first day of apheresis (i.e., after four days of G-CSF treatment) also varied considerably, especially for NK cells, but also for B cells and monocytes.

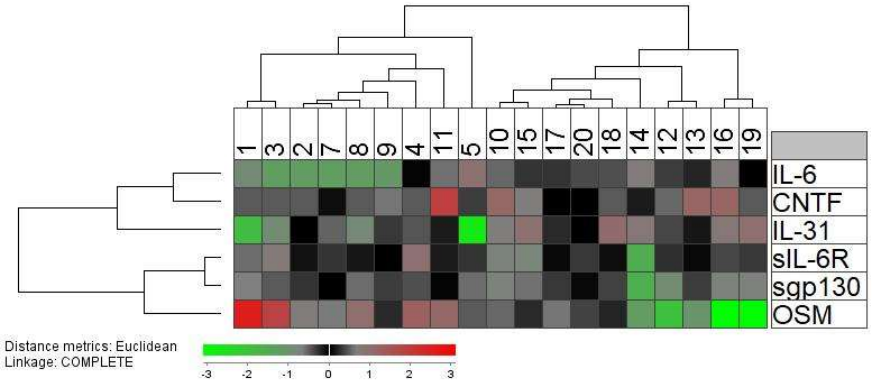

Figure 2. An unsupervised hierarchical clustering analysis of the graft supernatant levels of IL-6 family cytokines/receptors after stem cell mobilization by G-CSF and harvesting by leukapheresis. The analysis included 19 donors because a graft sample was not available for patient 6. The mediator concentrations were normalized to the corresponding median level for each mediator and, thereafter, log2 transformed before an unsupervised hierarchical clustering with Euclidian distance measurement and complete linkage was performed. The color scale thus corresponds to the Euclidian distance from the median since values were normalized to the corresponding median value, i.e., two measurements with the same color show the same distance from the median. The results are presented as dendrograms and a heat map for visualization and interpretation. The individual donors are indicated at the top of the figure whereas the different mediators are presented vertically in the right part of the figure.

Table 3. Serum levels of soluble mediators and their associations with levels of immunocompetent and $CD34^+$ cells in peripheral blood and stem cell grafts. We investigated the levels of six different immunocompetent cell subsets for 20 healthy stem cell donors. Serum levels and levels of circulating cells were determined after four days of G-CSF therapy before apheresis; graft composition was analyzed for the leukapheresis on day 4. For immunocompetent cells the results are presented as the cell number $\times 10^9$/L in peripheral blood/grafts; for $CD34^+$ cells the levels are presented as the number $\times 10^3$/mL in peripheral blood and $\times 10^9$/L in the grafts. Correlation coefficients (Spearman's rho) between serum levels of IL-6 family cytokines/receptors/CRP and immunocompetent cell subsets in the graft and peripheral blood are also presented. Significant correlations are highlighted in bold (* *p*-value between 0.05 and 0.01, ** *p*-value below 0.01).

The Peripheral Blood Levels of Immunocompetent Cell Subsets								
Leukocyte subset	**Peripheral Blood Level** [1]	**IL-6**	**sIL-6R**	**sgp130**	**IL-31**	**OSM**	**CNTF**	**CRP**
T cells, total ($CD3^+$)	3.31 (1.29–4.17)	−0.042	−0.508	**−0.697** **	0.244	−0.511	**0.654** *	0.156
$CD4^+$ T cells	2.54 (0.92–3.47)	0.046	**−0.582** *	**−0.609** *	0.354	−0.495	0.427	0.229
$CD8^+$ T cells	0.60 (0.24–1.08)	−0.135	−0.205	**−0.557** *	0.104	−0.275	0.555 *	0.097
B cells ($CD19^+$)	0.41 (0.21–1.77)	0.289	−0.310	**−0.719** **	0.525	−0.423	0.507	0.384
NK-cells ($CD3^-$ $CD56^+$)	0.30 (0.07–0.77)	−0.449	0.165	0.181	−0.020	0.366	−0.074	−0.249
Total monocytes	2.4 (0.90–3.9)	−0.065	−0.164	−0.296	0.276	−0.046	0.362	0.210
$CD34^+$ cells	40.2 (16.7–148)	−0.21	−0.32	**−0.54** *	0.47	0.37	0.33	0.045
The Graft Composition of Immunocompetent Cell Subsets								
Leukocyte subset	**Graft Level** [1]	**IL-6**	**sIL-6R**	**sgp130**	**IL-31**	**OSM**	**CNTF**	**CRP**
T cells, total ($CD3^+$)	22.78 (8.41–42.81)	−0.088	0.328	0.294	0.097	−0.074	−0.358	−0.539 *
$CD4^+$ T cells	17.55 (6.02–31.66)	0.073	0.459	0.516	−0.162	−0.196	−0.176	−0.444
$CD8^+$ T cells	4.63 (1.30–9.74)	−0.068	0.336	0.204	0.087	−0.007	0.268	**−0.592** *
B cells ($CD19^+$)	3.63 (0.00–12.76)	**0.534** *	0.363	0.169	0.184	0.385	0.277	**−0.622** *
NK-cells ($CD3^-$ $CD56^+$)	1.79 (0.40–5.50)	−0.121	0.253	0.433	0.315	0.415	0.121	−0.407
Total monocytes	12.91 (1.93–25.23)	−0.248	0.071	0.100	0.248	0.324	−0.054	−0.256
$CD34^+$ cells	0.43 (0.085–201)	−0.026	0.319	0.125	−0.258	−0.088	0.400	−0.009

[1] Peripheral blood levels: The vertical column presents the cell subset, the horizontal line the serum soluble mediator.

We investigated the associations between the levels of circulating immunocompetent cells and the systemic (serum) levels of each individual IL-6 family cytokine or CRP (Table 3, upper part). The most striking observations were the inverse correlations between serum sgp130 and the levels of circulating total T cells, $CD4^+$ and $CD8^+$ T cells, B cells, and $CD34^+$ cells; an additional inverse correlation was observed between sIL-6R and circulating $CD4^+$ T cells. Finally, total T cell levels in the blood were also correlated with the CNTF levels. These observations suggest that IL-6 family mediators, and especially gp130/IL-6R, are involved in the trafficking/mobilization of immunocompetent cells during G-CSF therapy of healthy donors.

We also investigated associations between the amounts of harvested immunocompetent cells and serum levels of CRP and IL-6 family cytokines (i.e., graft composition on the first day of apheresis). The graft composition will then reflect an overall effect of G-CSF therapy and the leukapheresis procedure. CRP levels then showed significant inverse correlations with the graft numbers of $CD8^+$ T cells and B cells whereas IL6 was significantly associated with levels of B cell in the graft (Table 3, lower part).

We compared the peripheral blood and graft levels of the various immunocompetent cell subsets for the donor subsets identified in the clustering analysis presented in Figure 2; i.e., whether the levels of immunocompetent cells were dependent on variations in the overall IL-6 family profile. The left cluster showed a lower level of total B cells (Mann–Whitney *U* test; $p = 0.03$); this was the only significant difference that was detected. Finally, the donor age did not show any significant associations with graft or peripheral blood levels of immunocompetent cells.

2.6. G-CSF Can Modulate IL-6 Release by Immunocompetent and Mesenchymal Cells

IL-6 is released by immunocompetent cells and various stromal cells during acute infections in response to danger-associated or pathogen-associated molecular patterns recognized by Toll-like receptors (TLRs) [27,29]. However, a wide range of other endogenous molecules have also been identified as TLR ligands that are able to induce TLR-initiated intracellular signaling, and these observations may suggest that TLRs are important, not only during infections or inflammation, but possibly also for the normal immunological surveillance or homeostasis [30]. Various TLRs are differentially expressed by monocytes, fibroblasts, and mesenchymal stem cells (MSCs) [31], and TLR ligation may therefore influence their functional status in vivo. For these reasons we investigated whether G-CSF can modulate the in vitro release of IL-6 by monocytes, fibroblasts, or mesenchymal stem cells in the presence of various TLR-ligands.

We investigated the effects of G-CSF on the IL-6 release by monocytes in the presence the TLR agonists Pam3CSK4 (TLR1/2), LPS (TLR4) or Flagellin (TLR5), R837 (TLR7 > TLR8), and R848 (TLR7/8). Based on initial dose–response experiments we investigated the G-CSF effects in the presence of two different concentrations for each agonist, both concentrations being lower than the concentrations needed for induction of maximal IL-6 release. These results are summarized in Table 4. Monocytes derived from 10 healthy individuals were investigated. Firstly, the IL-6 release by normal monocytes showed a wide variation between the healthy individuals for all agonists investigated. Secondly, we defined a strong/significant G-CSF effect as at least a twofold alteration. For all agonists a strong G-CSF effect was only observed for a subset of healthy cell donors, i.e., the G-CSF effect differed between individuals, and a strong effect was most common in the presence of the TLR5 agonist Flagellin. The Flagellin 50 ng/mL results are presented in Figure 3a. Finally, the overall results presented in Table 4 showed that G-CSF usually increased the IL-6 levels, but for certain donor/agonist combinations decreased levels were seen.

Table 4. The effect of G-CSF on IL-6 release by monocytes derived from 10 healthy individuals. Enriched monocytes were incubated with various TLR agonists (for each individual two different concentrations were tested), and the IL-6 supernatant levels were compared for cultures with G-CSF and corresponding control cultures without G-CSF. The table presents the median and range of the IL-6 concentrations for all 20 cultures with each agonist (i.e., 10 healthy individuals tested with two concentrations of each agonist); control cultures of monocytes incubated in medium alone showed undetectable IL-6 levels. A significant difference was defined as at least a twofold increase/decrease in the presence of G-CSF—at least 20 pg/mL. Divergent effects between the two concentrations of an agonist were not observed for any agonist/donor combination. The dark color indicates a significant G-CSF induced IL-6 increase for at least one of the two agonist concentrations tested, whereas the bright color indicates a significant decrease. Cultures marked with nt means that these were tested with different LPS concentrations (0.1 and 0.5 ng/mL); none of these alternative LPS/donor combinations showed any significant influence of G-CSF on the IL-6 levels. Monocytes cultured in medium alone without G-CSF/TLR agonists showed undetectable IL-6 levels.

Agonist	Agonist Concentration	IL-6 Supernatant Levels (pg/mL)	Healthy Monocyte Donors									
			1	2	3	4	5	6	7	8	9	10
PAM3CSK4 (TLR1/2)	1 and 5 ng/mL	10.8 (3.1–372)										
LPS (TLR4)	5 and 10 ng/mL	3.1 (3.1–281)				nt	nt	nt	nt	nt	nt	nt
Flagelin (TLR5)	10 and 50 ng/mL	13.9 (3.1–291)										
R848 (TLR7 > TLR8)	50 and 100 ng/mL	3.1 (3.1–180)										
R837 (TLR7/TLR8)	0.5 and 1 mg/mL	188 (3.1–395)										

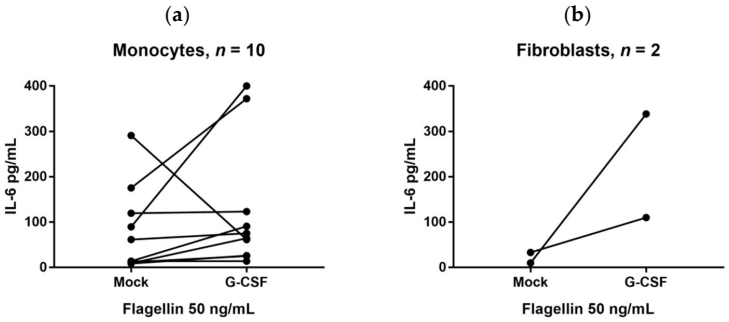

Figure 3. IL-6 release by monocytes derived from 10 healthy individuals (**a**). The cells were cultured with and without exogenous G-CSF 50 ng/mL in the presence of the TLR agonists Flagellin (TLR5) 50 ng/mL. The results are presented as the IL-6 levels in culture supernatants; (**b**) IL-6 release by HFL1 and Hs27 fibroblasts cultured with and without exogenous G-CSF 50 ng/mL in the presence of Flagellin (TLR5) 50 ng/mL; the results from a typical experiment are presented. The results are presented as the IL-6 levels in culture supernatants; the effect of G-CSF on IL-6 release by fibroblasts was detected in six independent experiments.

We also examined the effect of exogenous G-CSF on IL-6 release by two fibroblast cell lines derived from different individuals and tissues and by MSCs derived from a healthy individual. The G-CSF effect was tested in the presence of three TLR agonists: Pam3CSK4 1 ng/mL (TLR1/2 agonist), LPS 5 ng/mL (TLR4 agonist), and Flagellin 10 ng/mL (TLR5 agonist). Both fibroblast cell lines showed increased IL-6 release in the presence of G-CSF. Increased IL-6 release by fibroblasts release was demonstrated in six independent experiments; it was detected early during culture as well as later when cells were close to confluence, and a strong effect was especially seen in the presence of Flagellin (Figure 3b). Finally, enriched MSCs from a healthy donor showed constitutive IL-6 release that was increased in the presence of exogenous G-CSF.

Taken together these results suggest that both immunocompetent and stromal cells contribute to the G-CSF induced IL-6 response in healthy individuals, but their contribution possibly differs between

various individuals and also seems to depend on the microenvironment of the cells as illustrated by the different G-CSF effects in the presence of various TLR agonists.

3. Discussion

Previous studies suggest that healthy stem cell donors are heterogeneous with regard to the effects of G-CSF on donor immunoregulation and the number, as well as the functional status, of immunocompetent graft cells [9,13,15,18–21]. One of these studies even suggests that G-CSF induced donor heterogeneity is important for outcome after allotransplantation [21]. We have previously reviewed and discussed the available evidence for a role of IL-6 in the development of immune-mediated complications after allotransplantation [27]. Previous studies have also shown that systemic IL-6 levels in healthy stem cell donors can be altered by G-CSF therapy; these effects are divergent, and although increased levels are seen for most donors, a minority of them show decreased systemic IL-6 levels in response to G-CSF [9,19,28]. In our present study we observed that healthy donors undergoing G-CSF induced stem cell mobilization and harvesting by leukapheresis are heterogeneous, both with regard to the G-CSF induced acute phase reaction and effects of G-CSF on systemic levels of various IL-6 cytokine/receptor family members.

Several recent studies have described associations between graft composition and post-transplant outcome, e.g., high $CD8^+$ graft cells associated with decreased relapse risk [32] and increased regulatory T cells associated with decreased nonrelapse mortality [33]. The first study investigating individualized risk-adapted prophylaxis against immune-mediated complications based on graft composition has already been published [17]. However, a better understanding of the molecular mechanisms behind, and the consequences for, the recipients of differences in graft composition is needed as a scientific basis for further studies of possible interventions, e.g., in vivo graft manipulation, ex vivo graft manipulation, risk-adapted individualized prophylaxis, or early therapeutic intervention based on biomarker evaluation before clinical signs of complications [16].

Previous studies suggest that G-CSF-induced stem cell mobilization in healthy individuals has a clinically negligible effect on CRP levels with most donors still having CRP levels below 2 mg/L after G-CSF administration [34,35]. In contrast, we observed an increase of at least 9.5 mg/L for a large subset of donors, especially elderly donors. The only other factor predicting this CRP increase was the pretreatment CRP levels, implicating that signs of pretreatment inflammation potentiates the effects of G-CSF on the acute phase reaction.

IL-6 and CRP levels are usually highly correlated [36]; this was also seen for the pretreatment levels for our stem cell donors. However, we did not detect any significant association between CRP and IL-6 levels during G-CSF treatment, but CRP levels were significantly correlated with OSM levels even though the OSM levels did not increase in response to G-CSF. G-CSF itself is not able to induce CRP production in hepatocytes [37]. Taken together, these observations suggest that G-CSF induced CRP release is independent of the IL-6 response and rather caused by a G-CSF induced modulation of OSM effects. Even though tumor necrosis factor alpha (TNF-α) or IL-1 can induce CRP release [38], these two cytokines are less likely to contribute because G-CSF decreases their systemic levels [8]. Finally, the ratio between serum levels of sIL-6 receptor and sgp130 is termed the IL-6 buffer; this buffer regulates the proinflammatory effects of IL-6, including its effects on the acute phase response/CRP levels [39,40]. However, the IL-6 buffer was not altered during G-CSF therapy and therefore is unlikely to be responsible for the increased CRP levels during G-CSF therapy [24,25].

We observed an association between the G-CSF induced acute phase reaction and OSM levels. OSM is released by various immunocompetent cells; it can initiate acute phase reactions and is also involved in tissue repair [41,42]. The OSM receptor uses gp130 as the signaling subunit of the receptor complex; this is similar to the other IL-6 family cytokines, but OSM can also utilize the LIF receptors for signal transduction [27]. OSM seems to have the broadest downstream signaling profile among the IL-6 family members and activates Janus kinase (Jak)/ Signal transducer and activator of transcription (STAT) signaling, the extracellular signal–regulated kinases (ERK) and c-Jun N-terminal

kinase, phosphatidyl-inositole-3-kinase/ Protein Kinase B (Akt) signaling, as well as protein kinase C delta [41,43].

OSM is also regarded as a disruptor of epithelial barrier functions, it is a biomarker for active inflammation in rheumatoid arthritis and increased levels are also reported in allergic rhinitis, psoriasis, and asthma [42]. It seems to have a very complex role in the regulation of inflammation by enhancing the maturation of dendritic cells and thereby increasing their IL-12 release, increasing T cell proliferation, and increasing the release of Interferon-γ [44]. However, it also seems to skew monocyte differentiation into the anti-inflammatory M2 phenotype and does not stimulate development of dendritic cells from monocytes. In vivo studies suggest that OSM has anti-inflammatory effects mediated by inhibition of IL-1 and TNF-α responses, and it seems to suppress inflammation in animal models of autoimmune diseases [45]. OSM does not seem to have direct effects on Th17 cells and regulatory T cells [44]. Taken together, these observations suggest that the predominant effects of OSM depend on the biological context. Our present results suggest that its proinflammatory effects (i.e., the effects on the acute phase reaction) vary between, and thereby contributes to, the heterogeneity of healthy stem cell donors (Table 2, Figure 2), and this variation during G-CSF therapy and in graft supernatants suggests that OSM can alter the functional status of at least certain subsets of graft immunocompetent cells. Even though the possible role of OSM in allotransplant recipients has not been addressed previously, our knowledge about OSM from other studies suggests that it may contribute to the post-transplant outcome (e.g., development of immune-mediated toxicity) in allotransplant recipients through the acute phase reaction, immunoregulatory and proinflammatory effects, modulation of inflammatory resolution and tissue repair after inflammation, or effects on epithelial barrier functions.

The peripheral blood levels and the corresponding graft amounts of immunocompetent cells showed a wide variation between healthy donors (Table 3), and the widest variation in peripheral blood levels was seen for NK cells. The NK cells seem important for outcome after stem cell transplantation [46]. Previous studies have also demonstrated that NK cells show a transient functional alteration following G-CSF mobilization with decreased proliferative capacity; this effect also varies between patients [47,48]. Thus, healthy stem cell donors show both a quantitative and qualitative NK cell heterogeneity after G-CSF mobilization.

The levels of several circulating immunocompetent cell subsets showed an association with the systemic levels of sgp130 that serves as an important modulator of IL-6 signaling through its binding to soluble IL-6R [27]. This observation suggests that IL-6 family cytokines, and especially IL-6, are important for immunocompetent cell mobilization and may contribute to the donor heterogeneity observed during G-CSF therapy. These associations were not detected for the allografts, probably because graft levels also depend on factors related to the apheresis and graft preparation and not only on the G-CSF mobilization [28].

We also investigated whether G-CSF could increase IL-6 release by in vitro cultured cells. IL-6 can be released by several immunocompetent as well as mesenchymal cells [27], and in our present study we included only monocytes together with fibroblasts and normal mesenchymal stem cells. We then used an in vitro model where monocytes and mesenchymal cells were cultured in the presence of TLR agonists; in our opinion this is a more physiological model than culture in medium alone because a wide range of endogenous TLR ligands have now been identified and are expected to be present in the in vivo microenvironments of these cells [49]. A strong/significant alteration of the IL-6 release in the presence of G-CSF was defined as a two-fold alteration. It can be seen from Figure 3 that the in vitro G-CSF effects on the monocyte release of IL-6 differed between healthy individuals (although increased IL-6 levels were most common). Previous in vivo studies also suggest that the effects of G-CSF on IL-6 vary between individuals, i.e., the effect of G-CSF therapy on systemic IL-6 levels of healthy stem cell donors differs and both increased, unaltered, and decreased systemic levels can be seen [9,19].

We investigated monocyte/fibroblast/mesenchymal stem cell release of IL-6 in an experimental model based on serum-free (i.e., possibly suboptimal) culture medium; this was use to minimize the risk of having TLR ligands in the medium. Our model is thus based on the presence of one ligand, whereas we would expect several endogenous TLR ligands to be present during physiological conditions. For these reasons we would emphasize that these results should be interpreted with great care and additional studies in other experimental models are needed to characterize, in greater detail, the effect of G-CSF IL-6 release by such cells.

Fibroblasts express a wide range of TLRs, and we also observed increased IL-6 release for both fibroblast cell lines in the presence of various TLR agonists. The constitutive IL-6 release by MSC was also increased by G-CSF. Taken together these observations suggest that various cells contribute to the IL-6 response during G-CSF therapy. This is similar to the IL-6/CRP responses during infections where both immunocompetent and mesenchymal cells contribute to these responses [49].

Several observations suggest that immunoregulatory events early after stem cell transplantation are important for the outcome after ALLO-SCT, especially the risk of GVHD, for example, the need for early initiation of GVHD prophylaxis and the association between pretransplant conditioning, post-transplant G-CSF therapy, and risk of post-transplant outcome [26]. Furthermore, IL-6 seems important in the development of immune-mediated complications after ALLO-SCT and is regarded a possible therapeutic target in GVHD [27]. However, only future clinical studies can clarify whether G-CSF induced donor heterogeneity, including differences in acute phase reactions and IL-6 family cytokine levels, has any impact on the outcome for the allotransplant recipients.

4. Material and Methods

4.1. Patient Studies and Donor Samples

All studies were approved by the Regional Ethics Committee III, University of Bergen, Norway (REK VEST 2013/634 30 April 2013 and REK VEST 2015/1410, 02 July 2015). Only matched related donors (median age 49 years, range 18–77 years) mobilized with G-CSF 5 μg/kg twice daily were included. The donor and patient characteristics are given in Table 5. These recipients/donors represent an unselected cohort. The routine GVHD prophylaxis was ciclosporin A plus methotrexate. All donors were selected according to the generally accepted suitability criteria [50]. They were all healthy and without any signs of intercurrent disease at the times of evaluation, G-CSF therapy, and stem cell harvesting. Unless otherwise stated samples were collected between 8:00 am and 11:00 am in the morning. Twenty unselected donors were included in the cytokine studies (median age 51 years, range 25–73 years).

Stem cell collection was commenced after four days of G-CSF if the number of circulation $CD34^+$ cells was sufficient. Samples were collected before and after 4 days of G-CSF therapy, immediately after leukapheresis, and approximately 24 h after start of leukapheresis. Graft supernatants were also collected. Samples were centrifuged at $1310\times g$, transferred onto cryotubes within 2 h after sampling, and stored at −70 °C until analyzed. Bio-Plex kits were used to analyze the levels of soluble mediators (Bio-Rad, Hercules, CA, USA), using the Luminex®200™ Bio-Rad platform. CRP was analyzed immediately after sampling by an immunoturbidimetric method (Roche; Basel, Switzerland); the lower detection limit being 1 mg/L.

Table 5. The characteristics of the allotransplant recipients and their donors included in the analysis.

Recipients (n = 85)	Characteristics
Age, median and range (Years)	47 (18–70)
Diagnosis (number)	
AML, de novo	37
AML secondary to myelodysplastic syndrome	17
Myelodysplastic syndrome, high-risk	2
Acute lymphoblastic leukemia	15
Chronic myeloid leukemia	3
Myelofibrosis/Myeloproliferative neoplasia, unspecified	6
Chronic myelomonocytic leukemia	2
Chronic lymphocytic leukemia	2
Hodgkin's lymphoma	1
Leukemia patients not in remission at transplantation	1
aGVHD requiring high dose steroid treatment (number) [1]	38
Conditioning regimes (number)	
Busulfan + cyclophosphamide (myeloablative condition)	66
Fludarabine + busulfan (reduced intensity conditioning)	16
Others	3
Stem cell source (number)	
Peripheral blood mobilized stem cells	85
Bone marrow grafts	0
DONORS (n = 85)	
Sibling/other family donors	78/7
Female/Male	54/31
Age; median (range)	49 (18–77)
Female donor to male recipient	19
Number of CMV positive recipients	60
CMV positive donor to CMV negative recipient	15

[1] The criteria for receiving high-dose systemic steroid treatment were acute GVHD grade II with gastrointestinal involvement or Grade III/IV acute GVHD.

4.2. Flow Cytometric Analysis

Peripheral blood and graft levels of immunocompetent cells were analyzed by flow cytometry. Briefly, peripheral blood mononuclear cells and graft cells were cryopreserved in DMSO and stored in liquid nitrogen until analyzed [21,51]. The cells were thawed and the near-IR fluorescent reactive dye (LIVE/DEAD Fixable Dead Cell Stain Kits, Molecular Probes, Eugene, OR, USA) was used for identification of viable cells. Cells were thereafter stained with CD3-PE-Cy7 (SK7), CD4-PerCP-Cy5.5 (RPA-T4), CD8-V500 (RPA-T8), CD16-Ax647 (3G8), CD19-PerCP-Cy5.5 (SJ25C1), and CD56-PE (B159) (all from Becton Dickinson Biosciences; BD Pharmingen, San Diego, CA, USA). We determined the numbers of $CD3^+$ T cells, $CD4^+$ and $CD8^+$ T cell subsets, B cells ($CD19^+$), and NK cells ($CD16^+CD56^+$) by using a FACS Canto II flow cytometer (Becton Dickinson Biosciences-Immunocytometry Systems; San Jose, CA, USA). The data were analyzed using FlowJo software version 10.2 (FlowJo LLC, Ashland, OR, USA). The monocyte levels were determined by multi-angle polarized scatter separation (MAPSS) optical flow cytometry (Cell-Dyn Sapphire analyzer; Abbot Diagnostics, Santa Clara, CA, USA).

4.3. In Vitro Culture of Monocytes and Fibroblasts

Samples were collected from healthy blood donors at Haukeland University Hospital. Monocytes from healthy donors were isolated from gradient-separated peripheral blood mononuclear cells (PBMCs) by negative selection using the human Monocyte Isolation Kit II (Miltenyi; Bergisch Gladbach, Germany). The isolation was performed according to the manufacturer's instructions. Flow cytometric analysis verified that the purity was $\geq$95%. The Hs27 skin fibroblasts (ATCC CRL1634; Manassas, VA, USA) and HFL1 fetal lung fibroblasts (ATCC CRL153) were also examined together with mesenchymal stem cells (MSC) derived from a healthy individual (Cambrex BioScience; Walkersville, MD, USA).

Cells were cultured with each of the TLR agonists Pam3CSK4 (TLR1/2 agonist; tested at 1 and 5 ng/mL), lipopolysaccharide (LPS) (TLR4 agonist; 0.1, 0.5, 5, and 10 ng/mL), Flagellin (TLR5 agonist;

10 and 50 ng/mL), R837 (TLR7 > TLR8 agonist; 0.5 and 1.0 mg/mL), and R848 (TLR7/8 agonist; 50 and 100 ng/mL) (Invitrogen; San Diego, CA, USA), with and without G-CSF 50 ng/mL (Peprotech; Rocky Hill, NJ, USA). Monocytes (50,000 cells/mL, 1 mL/well; Multiwell™ 48 well culture plates, Falcon, Franklin, NJ, USA) were incubated in RPMI 1640 (Sigma-Aldrich; St. Louis, MO, USA) with TLR-agonists ± G-CSF for 24 h before harvesting of supernatants. Fibroblasts (10,000 cells/mL, 2 mL/well; Nunclon Delta Surface Thermofisher 6-well culture plates; Roskilde, Denmark) were incubated in Dulbecco's Modified Eagle's Medium (Sigma) for 24 h before TLR agonists/G-CSF were added and supernatants harvested 24 h later. MSCs (5000 cells/mL, 2 mL/well; Nunclon Delta Thermo-Fischer 6-well culture plates) were also incubated for 24 h in mesenchymal stem cell medium alone (MSCGM™; Lonza; Basel, Switzerland) for 24 h before TLR-agonists/G-CSF were added and supernatants harvested 24 h later. Cultures were incubated at 37 °C in a humidified atmosphere of 5% CO2. Supernatants were stored at −80 °C until IL-6 analysis (Quantikine ELISA kits; R&D Systems Minneapolis, MN, USA). These mediator analyses were performed in duplicates, and the variation between duplicates was generally less than 10%.

4.4. Statistical Analyses

Statistical analyses of clinical variables were performed using Stata Version 14 (StataCorp. 2009; Stata Statistical Software, College Station, TX, USA) and Graphpad Prism (GraphPad Software, Inc., La Jolla, CA, USA). Differences were regarded as statistically significant when p-values < 0.05.

Author Contributions: T.-H.A.T. and Ø.B. conceived the idea and designed the study and wrote the manuscript. T.-H.A.T. performed the statistical analyses and the laboratory work involving MCSs, fibroblasts, monocytes, and mediator analysis. G.K.M. was responsible for serum sample collection and validation of clinical data. G.T. and A.B.A. collection and validation of clinical data. A.K.B. designed and contributed to the studies on MSCs, fibroblasts, and monocytes. All participants read and accepted the final manuscript.

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Abbreviations

ALLO-SCT	Allogeneic stem cell transplantation
CNTF	Ciliary neutrophilic factor
gp130	Glycoprotein 130
GVHD	Graft-versus-host disease
G-CSF	Granulocyte-colony stimulating factor
IL-31	Interleukin-31
IL-6	Interleukin-6
LPS	Lipopolysaccharide
MCSs	Mesenchymal stem cells
OSM	Oncostatin M
sIL-6R	Soluble IL-6 receptor
TLR	Toll-like receptor.

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