Immunoregulatory networks in healthy adult allogeneic stem cell donors

Studies of peripheral hematopoietic stem cell donors and their recipients

Guro Kristin Melve

Thesis for the degree of Philosophiae Doctor (PhD)
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Scientific environment

This project was conducted in the Leukemia Research Group at the Department of Clincal Science, Faculty of Medicine, University of Bergen and at the Department of Immunology and Transfusion Medicine, Haukeland University Hospital, Bergen. Professor Øystein Bruserud served as the primary supervisor, with Associate Professor Elisabeth Ersvær and Professor Einar Klæboe Kristoffersen as co-supervisors. The PhD work was financed by Haukeland University Hospital, The Norwegian Cancer Association and Helse Vest and also received financial support for reagent costs from the Norwegian Society for Immunology and Transfusion Medicine (Stiftelsen NFITs vitenskapelige fond).

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Abstract

Final outcome after allogeneic peripheral blood stem cell transplantation (PBSCT) is influenced by the high degrees of variation in disease and comorbidities among recipients, as well as the pre-and post-transplant handling of patients. Recent studies suggest that outcome is also influenced by donor heterogeneity, and the impact of G-CSF–induced immunomodulation on graft composition and post-transplant outcome is still not fully understood.

In this exploratory study, we characterized healthy HLA-matched related donors with respect to 27 distinct circulating lymphoid subsets, and systemic levels of 39 soluble mediators and 641 metabolites during hematopoietic stem cell mobilization and collection. A high degree of variation among donors was detected. This heterogeneity was further increased during G-CSF treatment and apheresis through preferential enrichment of certain immune cell subsets, soluble mediators and metabolites both in the donors and the stem cell grafts. Bioinformatics analyses were used to identify donor G-CSF–induced systemic changes and revealed a distinct dichotomy in G-CSF immune cell mobilization response, with potential impacts on recipient outcome. Our findings show that the systemic G-CSF–induced mediator profile predicted stem cell yield, and graft mediator profile was dependent on apheresis device and correlated to graft leukocyte and platelet levels.

Our overall results show that healthy stem cell donors are heterogeneous with regard to immunoregulation, and this heterogeneity is increased by G-CSF treatment and stem cell harvesting. Future clinical studies should further investigate how immunological donor characteristics influence outcome after allotransplantation and the possible implications for hematopoietic stem cell mobilization and collection.

List of publications

Paper I

Peripheral Blood Stem Cell Mobilization in Healthy Donors by Granulocyte Colony-Stimulating Factor Causes Preferential Mobilization of Lymphocyte Subsets.

Melve GK, Ersvaer E, Eide GE, Kristoffersen EK, Bruserud O.

Front Immunol 2018;9: 845.

Paper II

The healthy donor profile of immunoregulatory soluble mediators is altered by stem cell mobilization and apheresis.

Melve GK, Ersvaer E, Rye KP, Ahmed AB, Kristoffersen EK, Hervig T, Reikvam H, Hatfield KJ, Bruserud O.

Cytotherapy 2018;20: 740-54.

Paper III

Immunomodulation Induced by Stem Cell Mobilization and Harvesting in Healthy Donors: Increased Systemic Osteopontin Levels after Treatment with Granulocyte Colony-Stimulating Factor.

Melve GK, Ersvaer E, Akkok CA, Ahmed AB, Kristoffersen EK, Hervig T, Bruserud O.

Int J Mol Sci 2016;17: 1158.

Paper IV

Granulocyte colony-stimulating factor alters the systemic metabolomic profile in healthy donors.

Hatfield KJ, Melve GK, Bruserud O.

Metabolomics 2017;13: 2.

Contents

Abbreviations

AA	Aplastic Anemia
ACD-A	Acid-Citrat-Dextrose Formula A
ADCC	Antibody Dependent Cellular Cytotoxicity
AE	Adverse Event(s)
aGVHD	Acute Graft Versus Host Disease
AHR	Aryl Hydrocarbon Receptor
ALP	Alkaline Phosphatase
ALL	Acute Lymphocytic Leukemia
AML	Acute Myeloid Leukemia
ANC	Absolute Neutrophil Count
APC	Antigen Presenting Cells
ATG	Anti-Thymocyte Globulin
B-ALL	B-Cell Lymphoblastic Leukemia
Bcl-6	B-Cell lymphoma 6 protein
BM	Bone Marrow
BMI	Body Mass Index
BU	Busulfan
CAR T	Antigen-Specific Chimeric Antigen Receptor T cells
Cβc	Common β Chain
Cγc	Common γ Chain
CCL	C-C Motif Chemokine Ligand
CCR	C-C Chemokine Receptor
CD	Cluster of Differentiation
CE	Collection Efficiency
cGVHD	Chronic Graft Versus Host Disease
CLL	Chronic Lymphocytic Leukemia
CML	Chronic Myelogenous Leukemia
CMV	Cytomegalovirus
C3/5	Complement Component 3/5
CSA	Cyclosporine A
CSF3	Colony-Stimulating Factor 3
CSF3R	Colony-Stimulating Factor 3 Receptor
CXCL	C-X-C Motif Chemokine Ligand
CXCR	C-X-C Chemokine Receptor
CY	Cyclofosfamid
DAMP	Danger-Associated Molecular Pattern
DC	Dendritic Cell
DFS	Disease-Free Survival
DLI	Donor Lymphocyte Infusion
DRI	Disease Risk Index
EBMT	European Society for Blood and Marrow Transplantation
ECM	Extracellular Matrix
EDTA	Ethylenediamine Tetraacetic Acid
ELISA	Enzyme-Linked Immunosorbent Assay
ERK	Extracellular Signal-Related Kinases
FACT	The Foundation for the Accreditaion of Cellular Therapy
FOXP3	Forkhead Box P3
GATA	Guanine-Adenosine-Thymidine-Adenosine Recognizer
GC	Gas Chromatography
GC	Germinal Center
G-CSF	Granulocyte Colony-Stimulating Factor
G-CSFR	Granulocyte Colony-Stimulating Factor Receptor

GF	Graft Failure
GM-CSF	Granulocyte-Macrophage-Colony-Stimulating Factor
GPCR	G-Protein-Coupled Receptor
GPFS	GVHD-Free Progression Free Survival
G-Rsp	G-CSF Responsiveness
GVL	Graft Versus Leukemia
HCA	Hierarchical Clustering Analysis
HCT-CI	Hematopoietic Cell Transplant-Comorbidity Index
Hb	Hemoglobin
HGF	Hepatocyte Growth Factor
HLA	Human Leukocyte Antigen
HMG-CoA	3-Hydroxy-3-Methylglutaryl Coenzyme A
HPC	Hematopoietic Progenitor Cells
HPLC	High Performance Liquid Chromatography
HSC	Hematopoietic Stem Cells
HSCT	Hematopoietic Stem Cell Transplantation
HSPC	Hematopoietic Stem And Progenitor Cells
ICAM	Intercellular Adhesion Molecule
IEC	Intestinal Epithelial Cells
IFN-γ	Interferon-γ
IL	Interleukin
IL-1RA	Interleukin 1 Receptor Antagonist
ILC	Innate Lymphoid Cells
IMPDH	Inosine-5'-Monophosphate Dehydrogenase
iNKT	Invariant Natural Killer T Cell
iT_{reg}	Inducible T Regulatory Cell
IRF	Interferon Regulatory Factor
ISCT	The Intenational Society for Cell & Gene Therapy
ISGF3	IFN-Stimulated Gene Factor 3
ISHAGE	International Society of Hematotherapy and Graft Engineering
Iv	Intravenous
JACIE	The Joint Accreditation Committee of ISCT-EBMT
JAK	Janus Kinase
iOPN	Intracellular OPN
kDa	Kilodalton
KIR	Killer Immunoglobulin-Like Receptors
LC	Liquid Chromatography
Lck	Lymphocyte-Specific Protein Tyrosine Kinase
LD	Lactate Dehydrogenase
LFA-1α	Lymfocyte Function-Associated Antigen
LFS	Leukemia Free Survival.
LPS	Lipopolysaccharide
LVL	Large Volume Leukapheresis
MAC	Myeloablative Conditioning
MAIT	Mucosa Associated Invariant T Cells
MAPKs	Mitogen-Activated Protein Kinases
MBAA	Multiplex Bead Array Analysis
MDS	Myelodysplastic Syndrome
MDSC	Myeloid-Derived Suppressor Cells
MEL	Melphalan
Mg	Magnesium
mHAg	Minor Histocompatibility Antigen
MHC	Major Histocompatibility Complex
MMD	Mismatched Donors
MMF	Mycophenolate Mofetil

MMP	Matrix Metalloprotease
MMRD	Mismatched Related Donor
MMUD	Mismatched Unrelated Donor
MNC	Mononuclear Cells
Mo	Monocyte
MΦ	Macrophage
MRD	Matched Related Donor
mRNA	Messenger Ribonucleic Acid
MS	Mass Spectrometry
MSC	Mesenchymal Stroma Cell
MT-MMP	Membrane-Type Matrix Metalloprotease
mTOR	Mechanistic Target of Rapamycin
mTORC	Mechanistic Target of Rapamycin Complex
MTX	Methotrexate
MUD	Matched Unrelated Donor
NBMDR	Norwegian Bone Marrow Donor Registry
NGS	Next Generation Sequencing
NFAT	Nuclear Factor of Activated T Cell Family of Transcription Factors
NK cells	Natural Killer Cells
NMDP	National Marrow Donor Program
NMR	Nuclear Magnetic Resonance
NHL	Non-Hodgkin's Lymphoma
NRM	Non-Relapse Mortality
nT_{reg}	Natural T Regulatoy Cell
OPN	Osteopontin
OS	Overall Survival
P	Potassium
PAMP	Pathogen-Associated Molecular Patterns
PB	Peripheral Blood
PBHCT	Peripheral Blood Hematopoietic Cell Transplantation
PBMC	Peripheral Blood Mononuclear Cells
PBPC	Peripheral Blood Progenitor Cell
PBSC	Peripheral Blood Stem Cells
PBSCT	Peripheral Blood Stem Cell Transplantation
PD-L1	Programmed Death Ligand-1
PI3K	Phosphoinositide 3-Kinases
PC	Platelet Count
PCA	Principal Component Analysis
PMN	Polymorphonuclear Neutrophil
PMP	Platelet-Derived Micro Particles
PMT	Photomultiplier Tube(S)
P.O.	Per Os
PT-CY	Post-Transplant High-Dose Cyclophosphamide
PTLD	Post-Transplant Lymphoproliferative Disorders
RIC	Reduced-Intensity Conditioning
RT-qPCR	Reverse-Transcription Quantitative Polymerase Chain Reaction
RTK	Receptor Tyrosine Kinase
RNA	Ribonucleic Acid
RORγt	Retinoid-Related Orphan Receptor γt
ROS	Reactive Oxygen Species
rhG-CSF	Recombinant human G-CSF
r-metHuG-CSF	Recombinant Methylated Human G-CSF
rhTPO	Recombinant Human Thrombopoietin
SAE	Serious Adverse Advent
SCF	Stem Cell Factor

SDF-1	Stroma-Derived Growth Factor-1
SNP	Single Nucleotide Polymorphism
SOCS3	Suppressor of Cytokine Signaling 3
sOPN	Secreted OPN/ Soluble OPN
SOS	Sinusoidal Obstruction Syndrome
STAT	Signal Transducer and Activator of Transcription
TA-TMA	Transplant-Associated Thrombotic Microangiopathy
T-bet	T-Box Expressed in T Cells
TBI	Total Body Irradiation
TBV	Total Blood Volume
TCD	T Cell Depletion
TCR	T Cell Receptor
TGF-β	Transforming Growth Factor Beta
T_h	T Helper Cell Subset
TIMP	Tissue Inhibitor Of Metalloproteases
TIR	Toll-IL-1-receptor superfamily
TNC	Total Nucleated Cells.
TNFα	Tumor Necrosis Factor-Alpha
TNFRSF	Tumor Necrosis Factor Receptor Superfamily
TPO	Thrombopoietin
Tr1	T Regulatory Cell, Type 1
TRAIL	Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand
T_{reg}	T Regulatory Cell
TRM	Transplant-Related Mortality
UC	Umbilical Cord
UCB	Umbilical Cord Blood
UPLC	Ultrahigh Performance Liquid Chromatography
VCAM	Vascular Cell Adhesion Molecule
V(D)J	Variable (Diversity) Joining Gene Segments
VEGF	Vascular Endothelial Growth Factor
VLA4	Very Late Antigen-4
VOD	Veno Occlusive Disease
VP-16	Etoposide
WBC	White Blood Cell Count
WDMA	World Donor Marrow Association
ZAP-70	Zeta-Chain-Associated Protein Kinase 70

1. Introduction

Hematopoietic stem cell transplantation–a brief historical outline

In the wake of World War II, after radiation from atomic bomb explosions caused lethal bone marrow (BM) injuries, BM infusion was shown to heal radiation-induced murine BM injury, and this discovery paved the way for initial attempts to treat human leukemia with allogeneic BM rescue after sub-lethal radiation therapy and chemotherapy.[1] Until increased knowledge about the human leukocyte antigen (HLA) system allowed the first HLA-matched allogeneic hematopoietic stem cell transplantation (HSCT) in 1968, immune incompatibility between donor and recipient inevitably led to graft rejection or graft versus host disease (GVHD), except after transplantations with syngeneic (from identical twin) and autologous cryopreserved BM.[1] The development of more intensive chemotherapy and discovery of novel immunosuppressive agents allowed significant expansion and diversification of HSCT with respect to indications, conditioning, immunosuppression, donor type and graft source and preparation [1,2] (see Figure 1).

Confirmation of the up to 100-fold increase in circulating hematopoietic progenitor cells (HPC) in response to recombinant human granulocyte-colony stimulating factor (rhG-CSF) allowed the reliable and safe collection of peripheral blood stem cells (PBSC) for sufficient hematopoietic and immunological reconstitution both in the autologous and allogeneic setting.[3,4] Replacement of BM with G-CSF mobilized PBSC as the primary stem cell source led to a less invasive collection procedure, avoided the need for general anesthesia and resulted in shorter time to engraftment and reduction in incidence, severity and duration of donor complications.[5,6]

To date, more than 400,000 allogeneic HSCT have been performed in 75 countries worldwide.[2] The number of annual allotransplantations in Europe currently exceeds 15,000, more than 75% of which are performed with PBSC.[7] In 2017, unrelated donors were used for approximately 60 % of matched PBSCT, and haploidentical transplantations outnumbered umbilical cord (UC) transplants.[7]

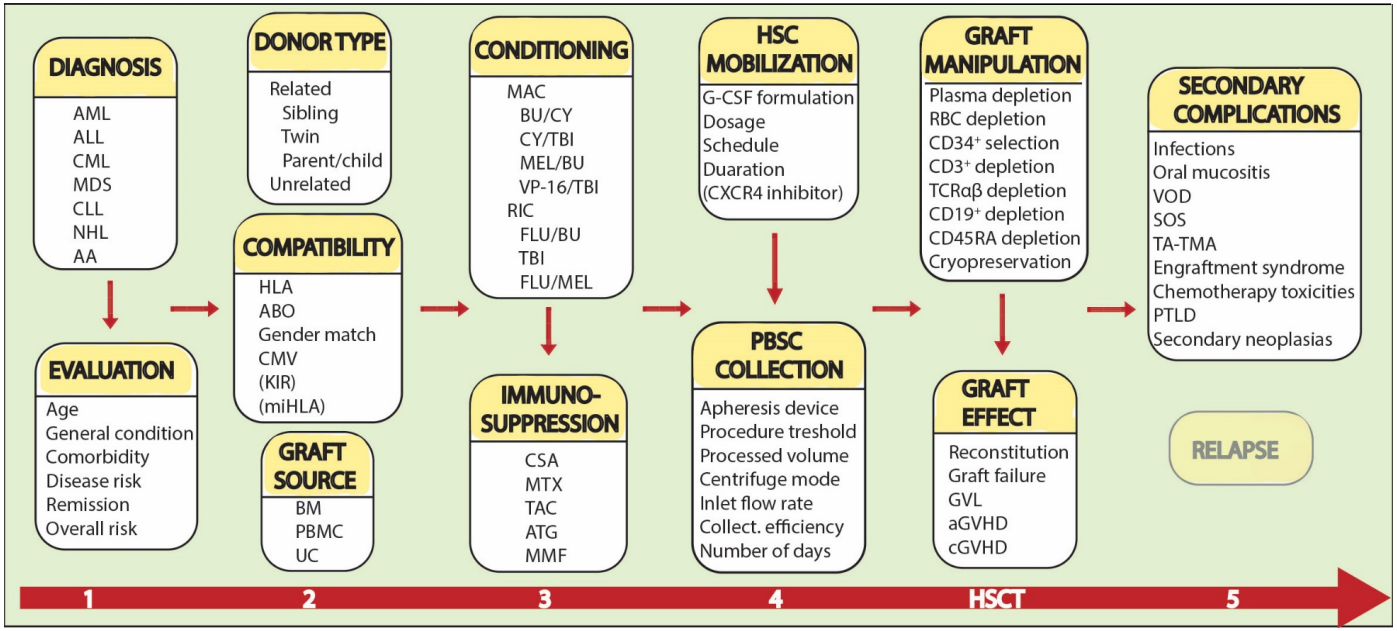

Figure 1. Schematic overview of allogeneic hematopoietic stem cell transplantation (HSCT) showing selected examples of the variability and complexity characterizing the entire process.
1. HSCT candidates are most commonly diagnosed with acute myeloid leukemia (AML), acute lymphocytic leukemia (ALL), chronic lymphocytic leukemia (CLL), chronic myelogenous leukemia (CML), non-Hodgkin's lymphoma (NHL), or aplastic anemia (AA). The indication for HSCT and prognostic evaluation are based on thorough clinical examination and laboratory and radiological investigations mapping general and disease/comorbidity-specific status/stage/risk. Evaluation tools include Karnofsky Performance Score, Disease Risk Index (DRI), and Hematopoietic Cell Transplant-Comorbidity (-Age) Index (HCT-CI).[8-10] Overall risk algorithms predictive of clinical outcome in terms of transplant-related disease-free and overall survival probability (DFS, OS) also include donor type and compatibility, e.g..EBMT risk score.[11] ***2.*** The best available donor is selected based on the evaluation criteria described below (p. 3–5). Choice of graft source depends on a combination of recipient and donor factors and availability. ***3.*** Numerous alternative preparative conditioning regimens are in use, ranging from myeloablative via reduced-intensity conditioning (MAC/RIC) to non-myeloablative based on combinations, types and doses of chemotherapy/irradiation.[12,13] MAC regimens are characterized by high BM toxicity, irreversible cytopenia and mandatory stem cell (SC) support; RIC regimens give intermediate BM toxicity, cytopenia of variable duration and usually require SC support; and non-myeloablative regimens give low BM toxicity and minimal cytopenia without need for SC support.[12] Conditioning aims to (i) eradicate the malignancy and (ii) provide immunosuppression to ensure engraftment and prevent graft failure/GVHD.[13] The choice of regimen depends on the individual risk/tolerability[13]; busulfan (BU), cyclofosfamid (CY), melphalan (MEL), etoposide (VP-16), total body irradiation (TBI). Additional pharmacological immunosuppression is achieved by combinations of e.g. Cyclosporine A (CSA), methotrexate (MTX), tacrolimus (TAC), antithymocyte globulin (ATG) and mycophenolate mofetil (MMF) accustomed to the type of conditioning therapy (Table 8).[14] ***4.*** Mobilization and collection of peripheral blood stem cells (PBSC) is presented on p. 7–15. ***HSCT/ 5.*** The main favorable effects of the graft are (i) complete reconstitution of the blood and immune cell repertoire (p. 18–20) and (ii) malignancy eradication; the graft versus leukemia (GVL) effect, of relatively greater importance following RIC (p. 21–22). Adverse graft effects include graft failure and acute and chronic GVHD (a/cGVHD) (p. 20–22). A few of the numerous other treatment-related complications are mentioned: veno occlusive disease (VOD), sinusoidal obstruction syndrome (SOS), transplant-associated thrombotic microangiopathy (TA-TMA), post-transplant lymphoproliferative disorders (PTLDs).

Selection of donors for allogeneic HSCT

HLA compatibility between donor and recipient is the major selection criterion for allogeneic HSC donation.[15-17] The most relevant HLA genes for donor selection are presented in Table 1 together with the most common secondary donor selection criteria. A matched related donor (MRD) is preferred, but such donors are available for only 30% of patients,[18] whereas the availability of the second choice, a matched unrelated donor (MUD), depends on ethnicity (>60% for Europeans and <20% for Africans).[19] Due to improved treatment protocols, clinical outcomes for MUD transplants are approaching those of MRD transplants,[20] and outcome for transplantation using haploidentical donors has similarly improved.[21] Comparative randomized studies of the clinical outcomes with "last options" haploidentical grafts, mismatched unrelated grafts and umbilical cord blood (UCB) are yet to be published,[18] though haploidentical transplants are now even discussed as commensurable alternatives to MUD and MRD transplants.[22,23] As reduced time to transplant may help avoid relapse and improve overall survival (OS) in high-risk leukaemia,[22] the most readily available donor may be preferable to the most immunocompatible graft.

Age has emerged as the second most important selection criterion for HSCT. Younger donor age has a positive impact on recipient survival, both in unrelated and related HSCT, and an increase of 5.5% in the hazard ratio for each decade of donor age was recorded in a study of 10,000 unrelated transplants.[24-26] The majority of unrelated grafts are now donated by individuals below 30 years of age, and MUDs above 50 years of age are avoided if possible.[24,26] Records for HSCT to high-risk MDS patients over the age of 50 suggest MUDs below 30 years of age may be preferable to older MRDs,[27] and a more careful assessment of risks associated with the increasing use of related donors over 60 and even 70 years of age has been suggested.[28]

ABO compatibility, CMV serocompatibility and gender match between donor and recipient are preferred and may improve post-transplant outcome (Table 1), though different studies have produced conflicting results.[24-26,29,30] The impact of minor histocompatibility antigen (mHAg) incompatibility depends on the mismatched

Table 1. Important donor evaluation/selection criteria with examples of heterogeneity and impact on mobilization/graft characteristics and clinical outcome.

Evaluation/ selection criteria	Examples of variability	Examples of reported effects on mobilization/ graft characteristics/ clinical outcome	Ref.
HLA compatibility	-10/10 or 9/10 MRD or URD -Haploidentical -Other MMD -6/6 or 5/6 matched UCB	Potential increased incidence of GVHD and reduced overall survival from HLA incompatibility, HLA-a*0101 correlated to incidence and severity of cutaneous aGVHD	15,16, 24,31
Stem cell source	-Bone marrow -PBSC -UCB	aGVHD risk: PBSC > BM > UCB / possibly higher incidence of grade III-IV after transplantation with PBSC	14,32
Donor type	-Related -Unrelated	Increased age, comorbidity and adverse reactions and prolonged post-collection recovery in MRD compared to URD	33
Age	7–76	Reduced stem cell mobilization, lower T cell and monocyte graft content and increased graft NK cell concentration in elderly donors, recipient survival negatively affected by advanced donor age	25,26, 34-41
ABO compatibility*	-ABO identical -Major ABO mismatch -Minor ABO mismatch -Bidirectional ABO mismatch	Delayed erythrocyte recovery and engraftment in major ABO-mismatched transplantations, increased risk of hemolytic reactions and after major and minor ABO-mismatched transplantations	30
		Increased requirement of RBC transfusions after ABO-mismatched transplants and of platelet transfusions after major ABO-mismatched transplantations	29
		Increased TRM after major ABO-incompatible transplantation with matched related donors	25
		Increased risk of delayed engraftment and grade II-IV aGVHD in AML patients after haploidentical transplantation with major and bidirectional ABO incompatibility, respectively	42
CMV compatibility	-Donor CMV^+/ recipient CMV^-	Decreased OS in CMV^- recipients after transplantation with CMV^+ MUD grafts	43
	-Donor CMV^-/ recipient CMV^+	Decreased OS in CMV^+ recipients after myeloablative conditioning followed by transplantation with CMV^- MUD grafts	43
Ethnicity	-European -Hispanic -African -Asian	Negative correlation of white ethnicity to stem cell mobilization. Availability of MUD grafts associated to ethnicity	19,35
Gender match	-Male-to-male -Female-to-female -Male-to-female -Female-to male	Inferior stem cell mobilization in females, female-to-male transplantation associated with increased TRM after related and lower relapse risk after unrelated donation Y-encoded SNPs associated with aGVHD in female-to-male transplantation	25,35-37,44, 45
BMI	18–45	Negative correlation of low BMI to stem cell mobilization. Increased donor weight associated with reduced T cell graft content	26,35, 36,39

HLA-matched related and unrelated donors (MRD and MUD) are defined based on compatibility for the six class I HLA-A, -B and -Cw alleles and the four class II HLA-DRB1 and HLA-DQB1 alleles by high-resolution typing, i.e. at the level of the 2nd field (formerly designated 4-digit).[16,17] HLA-DPB1 typing may be included, searching for 12/12 match.[18] Mismatched donors (MMD): two or more allelic disparities between donor and recipient for the abovementioned genes regardless of donor-recipient relationship.[18] Umbilical cord blood (UCB) should be matched 6/6 or 5/6 with the recipient for the HLA-A, –B and –DR.BMI: body mass index. CMV: cytomegalovirus. RBC: red blood cell. SNP: single nucleotide polymorphism. TRM: transplant-related mortality. *Major and minor ABO mismatch are characterized by recipient and donor isohemagglutinins directed against donor and recipient red blood cell antigens, respectively. In bidirectional ABO mismatch both recipient and donor have isohemagglutinins directed against ABO incombatible red blood cells.

mHAg epitope, its distribution, the donor type and clinical setting.[46,47] Mismatches for broadly expressed mHAgs may cause both aGVHD and GVL, whereas hematopoietic system-restricted mHAgs may induce GVL selectively.[46] Furthermore, donor-recipient incompatibility for natural killer (NK) cell killer

immunoglobulin-like receptors (KIR) may reduce relapse after HLA-mismatched and MUD HSCT but seems to increase risk of acute and chronic GVHD, both in HLA-matched and -mis-matched transplants.[48,49] Several cytokine and chemokine gene polymorphisms also impact clinical outcome (see Chapter 1.3).

1.1 Preparation of hematopoietic stem cell allografts

Granulocyte colony-stimulating factor and G-CSF Receptor

Endogenous human G-CSF is a glycoprotein and a cytokine encoded by *the colony-stimulating factor 3 (CSF3) gene* and is produced by various cells including BM stromal cells, monocytes, macrophages, fibroblasts and endothelial cells.[50] G-CSF belongs to the helical cytokine family,[51] and its dominant endogenous form has a molecular weight of 19.6 kilo Dalton (kDa) and 174 amino acids.[52] *In vivo* native G-CSF is the principal lineage-specific growth factor for steady-state granulopoiesis [53] and the dominant cytokine regulator of the stress-induced neutrophil response during infection.[54]

G-CSF acts through homodimerization of the helical Type I cytokine/hematopoietin transmembrane receptor G-CSF receptor (G-CSFR),[55-57] thereby inducing activation of various pathways–the Janus kinase/signal transducer and activator of transcription (JAK/STAT), phosphoinositide 3-kinases (PI3K/AKT) and mitogen-activated protein kinases/extracellular signal-related kinases (MAPK/ERK) pathways–and expression of the suppressor of cytokine signalling 3 (SOCS3).[58] Differential effects on the multitude of kinases downstream of G-CSFR is integrated via mechanisms yet not fully understood to activate target genes, resulting in different cellular responses: proliferation, differentiation, effector functions and/or survival.[57,59] Seven different isoforms of G-CSFR-encoding mRNA have been identified,[60] and a variety of *CSF3R* mutations have been identified in myeloid disorders including severe congenital neutropenia, myelodysplastic syndrome (MDS), acute myeloid leukemia (AML) and atypical chronic myeloid leukemia (CML).[59]

G-CSFR was originally regarded as strictly a myeloid receptor, and G-CSF was supposed to exert a solely indirect effect on lymphocytes via induction of immunomodulatory IL-10 production in monocytes.[61] More recently, time-dependent

induction of G-CSFR in activated CD4 and CD8 T cells during G-CSF treatment has been demonstrated, indicating an additional direct T cell effect.[62-64]

G-CSF-mediated hematopoietic stem cell mobilization

Hematopoietic stem cells (HSC) reside within distinct BM niches that regulate their quiescence and capacity of self-renewal, proliferation and ifferentiation.[65] Physiological release of HSC into peripheral blood displays a circadian rhythm with peak concentrations early in the morning and nadir at night,[66] and release is increased by inflammation, strenuous exercise and tissue injury.[67-69]

Multiple and complex mechanisms have been proposed as contributors to G-CSF–mediated HSC mobilization to peripheral blood. Mobilization does not depend on G-CSFR expression by HSC or stroma cells, and G-CSF is thought to act on several mature hematopoietic cells, including neutrophils, monocytes, T cells and B cells, to induce the mobilization process through bone remodeling and suppression of osteoblasts.[70-72] The constitutively expressed potent HSC attractant chemokine C-X-C motif ligand 12 (CXCL12) together with its main receptor C-X-C chemokine receptor 4 (CXCR4) represent the major components in BM retention and quiescence of HSC.[73] G-CSF-induced HSC mobilization is facilitated through several CXCL12/CXCR4 axis-suppressing mechanisms: (i) reduced BM stroma CXCL12 production[74]; (ii) apoptosis and inhibition of differentiation of CXCL12-producing osteoblasts[75]; (iii) cleavage of $CD34^+$ HSC CXCR4[76]; (iv) proteolytic cleaving of CXCL12 by carboxypeptidase M or dipeptidyl peptidase 4 (CD26) or other similar enzymes[77,78]; (v) reduced HSC and stroma cell expression of platelet endothelial cell adhesion molecule-1, potentially modulating HSC migration in response to CXCL12[79]; and (vi) enhanced HSC hepatocyte growth factor/c-Met signaling, potentially inhibiting HSC responsiveness to CXCL12.[80] The sympathetic nervous system is probably also important for the G-CSF induced osteoblast suppression and CXCL12 downregulation.[81]

During G-CSF treatment, the BM neutrophil population is significantly expanded through enhanced release of neutrophil serine proteinases and metalloproteinases, including neutrophil elastase, cathepsin G and Matrix metalloprotease 9 (MMP-9).[82]

In addition to their cleaving of CXCL12,[83] their proteolysis of vascular cell adhesion molecule (VCAM) [82] and the receptor tyrosine kinase c-kit impedes the anchoring and quiescence of HSC in the BM niche, potentially enhancing their release into peripheral blood.[84]

Other parts of the innate immune system also participate in HSC mobilization. Mobilizing agent-induced sterile inflammation through the release of endogenous danger-associated-molecular pattern (DAMP) molecules, reactive oxygen species (ROS) and proteolytic and lipolytic enzymes is thought to trigger activation of and crosstalk between the complement and coagulation cascades.[85,86] Induction of the complement component fragments C3a and C5a modifies HSC retention and contributes negatively and positively to mobilization, respectively.[87,88] Finally, the role of several bioactive metabolites, such as sphingosine-1-phosphate, adenosine and phospholipase C β2, is being elucidated.[89,90]

G-CSF-induced immune cell mobilization and immunomodulation

After 4–6 days of G-CSF administration, peripheral blood white blood cell count (WBC) averages 40 × 10^9/L, corresponding to an approximately six-fold increase over baseline concentrations, with significant individual variation (approximate range: 5–120 x 10^9/L).[91-95] A wide range of myeloid and lymphoid cell subsets are mobilized to peripheral blood along with neutrophils and hematopoietic progenitor cells.[91,96,97] Innate and adaptive immune cells and hematopoietic stem and progenitor cells (HSPC) are thought to rely on many of the same retention factors in the BM.[98,99] The mobilization of immune cells may therefore at least partly depend on the same mechanisms (see p. 6–7). The G-CSF–induced egress of various immune cell subsets from the BM or other lymphoid organs can potentially differ and depend on the expression of various retention factors. Different mobilization of distinct immune cell subsets may thus represent one immunomodulatory effect of G-CSF. Reported effects of G-CSF on different parts of the immune system are presented in Table 2. G-CSF produced *in vivo* essentially serves as a pro-inflammatory mediator during the innate immune response to infections, and G-CSF production is induced by bacterial components like lipopolysaccharide (LPS) and inflammatory mediators (interleukin 1

(IL-1), tumor necrosis factor-alpha (TNFα), IL-17.[100-103] In the course of an infection, however, endogenous G-CSF has been suggested to regulate inflammation by paradoxical reduction of neutrophil mobilization.[104]

Table 2. Examples of reported G-CSF effects on the innate and adaptive immune systems

Innate immunity			
Immune function	**Subset/ factor**	**Effect**	**Ref.**
Physical barriers	IEC	Decreased apoptosis of intestinal epithelial cells	105
Phagocytosis	Neutrophils	Increased bactericidal capacity	106
	Mo/MΦ	Increased bactericidal capacity	107
Cytotoxicity	NK cells	Reduced unstimulated and IL-2–stimulated cytotoxicity	108
		Reduced proliferation	108
Complement system activation	C3 and C5	Classical immunoglobulin-dependent complement cascade activation	109
Cytokine production	Mo/MΦ	Decreased M1/M2 ratio*	110
Adaptive immunity			
Antigen presentation	DC§	Selective increase of pDC (former DC2) over cDC, (former DC1) potentially skewing T cell differentiation towards Th2/T_{reg}	111,112
	pDC§	Reduced expression of costimulatory molecules CD40, CD80, CD86 and CD123	111
		Downregulated CD62L with assumed reduced migration to secondary lymphoid organs (of $CCR7^+$ cells)	112
T cell activation	$CD3^+$ T cells	Upregulated mRNA for activation markers CD69 and CD53	64
		Reduced T cell activation level based on CD25, CD95 and HLA-DR expression	96
		Downregulated mRNA expression of costimulatory and adhesion molecules CD5, CD44, LFA-1α	64
B cell activation	$CD19^+$ B cells	Increased expression of activation markers CD23 and CD25	113
T helper cell differentiation†	$CD4^+$ T cells	‡Increased/decreased expression of T cell-specific Th2 master transcription factor GATA-3	64,114
		Downregulated transcriptional regulator complex ISGF3	64
		‡Increased/decreased IL-4 secretion and decreased IL-2 secretion	64,114,115
		Increased IL-10 secretion	114
		‡Increased Th17 differentiation/ Decreased level of Th17 cells and Th17-specific transcription factor RORgγt	115,116
T cell proliferation	$CD3^+$ T cells	Upregulated mRNA expression of the proliferation promoter STAT5	64
	$CD4^+$ T cells	Reduced proliferative capacity	114
T cell-mediated cytotoxicity	$CD8^+$ T cells	Reduced alloresponse through up-regulated inhibitory NK receptor CD94/NKG2A expression	117
Induction of suppressor cells	T_{reg} cells	‡Reduced /increased/ unchanged proportion in PBSC compared to BM grafts. Increased proportions of Vδ1, $CD27^+$Vδ1 and $CD25^+$Vδ1T_{regs}	118-122
		Reduced CD62 expression indicating poor suppressive effect	118
	MDSC	Increased proportion in PB	123,124
	$CD34^+$ Mo	Increased proportion in PB	125

CCR: C-C chemokine receptor. Dendritic cells are currently classified into conventional and plasmacytoid subsets: cDC/pDC and cDCs subdivided into DC1 and DC2 subsets.[126] §: DCs are categorized as an innate subset, but link innate and adaptive immunity through antigen presentation to T cells. GATA: guanine-adenosine-thymidine-adenosine recognizer. IEC: intestinal epithelial cells. ISGF3: IFN-stimulated gene factor 3. LFA-1α: lymfocyte function-associated antigen 1. MDSC: myeloid-derived suppressor cells. Mo/MΦ: monocyte/macrophage. *Macrophages can be classified into the subsets M1 and M2, producing pro-inflammatory and anti-inflammatory cytokines, respectively.[110] mRNA: messenger RNA. NK: natural killer. RORγt: retinoid-related orphan receptor γt. T reg: T regulatory. ‡ contradictory results.

Knowledge of the effects of G-CSF on adaptive immune response (Table 2) is derived from studies of recombinant human G-CSF (rhG-CSF) administration

(Table 3). Extensive immunosuppressive effects, involving the entire repertoire of immune cell subsets have been reported with (i) generally reduced cellular activation, expression of costimulatory molecules, migration, proliferation, antigen presentation and cytotoxicity [64,96,111,112,117]; (ii) polarization towards production of anti-inflammatory cytokines and T helper cell subset 2 (Th2) phenotype [64]; and (iii) induction of various suppressor cells.[119,120,123,125] This trend is nuanced by several contradictive results regarding, in particular, T cell differentiation and regulatory T cell induction or enrichment.[114,118,121] In general, these studies are small and very heterogeneous with respect to study objects (animals, hematological and oncological patients, healthy donors), G-CSF administration (formulation, dose, schedule, duration, combination with chemotherapy), sample collection (peripheral blood, BM, splenocytes, *in vitro* cultures) and study conditions (stem cell mobilization, transplant models, infection/cancer/autoimmune disease models, *in vitro* stimulation).

Table 3. Typical administration characteristics of rhG-CSF analogs and biosimilars

G-CSF administration	Standard procedure alternatives	Formulation/variation range	Important effects on mobilization/ graft characteristics
G-CSF analog	Filgrastim [127]	Non-glycosylated r-met-HuG-CSF	Superior stem cell mobilizing with lenograstim compared to filgrastim in some studies
	Lenograstim [4,37]	Thr-133 glycosylated r-met-HuG-CSF	
	Pegfilgrastim [128]	Pegylated* non-glycosylated r-met-HuG-CSF	Slower pharmaceutical degradation*
	Zarzio/Tevagrastim/ Ratiograstim,/Nivestim/ Grastofil/Accofil/ Filgrastim Hexal [129-133]	Biosimilars**	Significant differences compared with originator drugs have not been reported
Dose [35-37,95,127,134-138]	10–12 (16) µg/kg/24 h	3–24 µg/kg/24 h	Association of total G-CSF dose given to stem cell yield and to adverse effects
Schedule [95,137-139]	One single dose or twice daily	One single dose or twice daily	Improved stem cell yield after G-CSF administration twice daily
Duration [33,34,44,92,135,138]	4–5 d	4–9 d	Harvest on day 4 of G-CSF administration associated with lower stem cell yield compared to day 5

*covalent attached polyethylene glycole molecules increases the molecule size to reduce renal clearance, can be administered as a single dose for allogeneic stem cell mobilization[128,140] ** Biosimilars are formulations that are biological equivalent but not identical to the originator drug due to differences in cell lines and production/purification technology.

Collection of mobilized hematopoietic stem cells by leukapheresis

Stem cell collection usually starts after 4–6 days of rhG-CSF treatment (Table 3). The main principle of PBMC apheresis is separation of whole blood components by individual density using specialized collection kits within a closed tubing system.[141] (Table 4). Two alternative mechanisms for cell separation are used; while continuous

flow devices use centrifugation alone, intermittent flow devices combine centrifugation with enrichment in a separation chamber, resulting in cyclic phase cell

Table 4. Important characteristics of stem cell collection procedures with examples of variability

Apheresis variables	Common alternatives/values	Graft characteristics/reported donor and recipient outcome (examples)
Apheresis device	Terumo BCT Spectra Optia Terumo BCT Cobe Spectra Fenwal Amicus Haemonetics MCS+ Baxter CS 3000 Plus Fresenius AS104	Grafts from Cobe Spectra, Amicus and Haemonetics MCS+ show differences in product volumes, WBC and $CD34^+$ counts. Grafts harvested with Spectra Optia are characterized by larger volumes and higher concentrations of platelets and neutrophils but lower lymphocyte and red blood cell platelet content compared to Cobe Spectra. Lower incidence of aGVHD in recipients of grafts from Spectra Optia was recently reported in a small study[142-146]
Threshold for apheresis	(8)15–20 x 10^3 $CD34^+$ cells per L PB pre-collection*	Pre-apheresis PB $CD34^+$ count is significantly correlated to administrated G-CSF dose and to stem cell yield [36,135,147,148]
Processed blood volume	Normal volume apheresis (2–3 x TBV) Large volume apheresis (3–6 x TBV)	Processed blood volume is significantly correlated to stem cell yield. Large volume apheresis reduces collection efficiency [148,149]
Centrifugation technique	Continuous flow Intermittent flow	Centrifugation technique dependent differences in graft volume and RBC and platelet contamination are reported [142,150,151]
Inlet flow rate	5–140 ml/min	Inlet flow rate is negatively correlated to stem cell yield [36]
Collection efficiency	5–120%	CE potentially influences graft purity and volume and need for repeated apheresis [93]
Number of collections	1–5	The number of collections potentially influences the ratio between progenitor and differentiated graft cells, total nucleated cell dose and infused plasma volume [33,44,92]
Technical dysfunction	Return problems Hemolysis Leakage Clotting	Technical dysfunctions may lead to need for repeated procedures and potentially lead to volume, electrolyte and coagulation disturbances in the donor and influence graft volume and composition [44,93,152]

Collection efficiency (CE): Percentage collected of processed $CD34^+$ cells = $(CD34^+_{graft}/(CD34^+_{pre} + CD34^+_{post})/2)$ * (Processed volume–AC volume))*100 [153] *evaluated using ISHAGE (International Society of Hematotherapy and Graft Engineering) "single platform" technique.[147] TBV: total blood volume.

separation and collection.[141,154] The HSC rich buffy coat is directed into the product bag, while uncollected blood components are returned to the donor.[141,154] Semi-automated apheresis devices use optical sensor systems and procedure specific computer controlled programs for interphase control.[93]

The device-dependent inlet flow necessary for PBMC apheresis is normally obtained through peripheral vein access using the antecubital veins.[93] Less than 20% of PBSC donors require central venous catheters placed in the internal jugular, subclavian or femoral veins.[44,93,155-157] In the extracorporeal apheresis circuit, the foreign surface of the tubing set represents a potent pro-coagulative factor, leading to platelet activation and contact-mediated activation of the hemostatic system.[158] The preferred anticoagulant for extra-corporeal circuits is the divalent cation-chelator acid-citrate-dextrose formula A (ACD-A), which inhibits hemostasis by reduction of the co-factor function of ionized calcium in phospholipid-dependent tenase and

prothrombinase complex assembly.[158,159] Further technical details are presented in Tables 4 and 5.

The aim of the apheresis procedure is to optimize yield with minimal risks to the donor and recipient. Individualized procedure settings are based on a total assessment of stem cell mobilization, hematology count, collection efficiency depending on yield prediction and the donor's individual age and health-related risk.[160,161] Stem cell mobilizing capacity shows great variability among healthy donors and, together with apheresis dependent variables, contributes to variations in infused stem cell doses (Table 1 and 4). Donor age is the most important dose-predictive factor, but several other donor and G-CSF administration characteristics, including gender, BMI, baseline platelet count and several genetic polymorphisms, show associations to HSC mobilizing ability (Table 1, [162]). A stem cell graft containing a minimum of (4–)5 x 10^6 $CD34^+$ PBPC per kg recipient weight is associated with reduced relapse and improved OS/DFS in MUD and MRD transplants and is achieved by one apheresis in 63–89% of collections.[38,93,94,163,164] Suboptimal mobilization with an achieved total dose below 4 x 10^6 $CD34^+$ cells/kg or mobilization failure with a yield below 2 x 10^6 $CD34^+$ cells/kg after up to three aphereses is reported in 2–5% and <0.5% of allogeneic donations, respectively.[44,93-95,135]In case of poor mobilization, a dose down to 3 x 10^6 $CD34^+$ cells per kg recipient weight is normally accepted, and less than 5% of donors need three aphereses or more.[38,93,135]

Common strategies to improve suboptimal donor mobilization are increased G-CSF doses, prolonged administration or salvage BM harvest.[165] The use of the CXCR4 antagonist plerixafor is increasing.[166-171] The direct antagonism of plerixafor with CXCR4/CXCL12 leads to more rapid HSC mobilization with lower toxicity compared to G-CSF, and the modest mobilization effect of single dosage can be overcome by increased doses, intravenous administration or combination therapy.[166,167,169,171,172] Other potential interventions for poor mobilization include treatment with non-steroidal anti-inflammatory drugs,[173] pre-harvesting exercise,[174] customizing harvest hours to circadian mobilization rhythms[175] and modulation of sympathetic activation.[176,177] Standardized algorithms for early identification and follow-up of poor mobilizers based on defined donor characteristics, and for

controlled studies of the effect and risk of alternative salvage strategies for both donor and recipient are needed.

Combined adverse effects of G-CSF and leukapheresis

The most important adverse effects of HSC mobilization and leukapheresis are presented in Table 5. Even after a single subcutaneous injection of rhG-CSF, healthy donor G-CSF plasma levels exceed peak physiological concentrations typically reached in hematological malignancies or in sepsis by more than 20– or 5-fold, respectively.[178-180] However, the treatment is relatively well tolerated,[92,181,182] with dose-dependent but usually mild and transient adverse effects (Table 5). During stem cell collection, symptoms and adverse effects can arise from both G-CSF administration and apheresis, and synergistic effects are possible; hence most studies do not distinguish between adverse events caused by G-CSF and apheresis.

Table 5. The most common adverse effects of G-CSF treatment and apheresis in healthy HSC donors

Adverse effects (AE)	Frequency	Cause
Immunohematological disturbances[44,92,93,134,155,183,184]		
Leukocytosis (mild/moderate: WBC > 50 x 10^9/L, severe: WBC> 100 x 10^9/L)	20–30%	G-CSF
Thrombocytopenia (PC <100 × 10^9/L)	20–30%	Combined
Neutropenia		Apheresis
Anemia (Hb <8 g/dL)	<1%	Combined
Hypercoagulability/thromboembolism	<1%	Combined
Hemorrhage§		Combined
General symptoms[44,92,95,127,134,155,185]		
Skeletal pain	70–95%	G-CSF
Fatigue	20–60%	Combined
Headache/ muscle pain/ flu like symptoms/fever/insomnia/spleen enlargement	20–60%	G-CSF
Infections/nausea/emesis/anorexia/ dizziness	< 10–20%	Combined
Hemodynamic‡/cardiovascular disturbances[44,186]		
Hypotension/vasovagal reactions/syncope/over-hydration	20%	Apheresis
Access problems [44,92,93]		
Local pain/skin rash/vascular damage/hematoma	20–60%	Apheresis
Nerve injury/arterial puncture/air embolism/pneumo- /haemothorax/cardiac perforation	<1%	Apheresis
Biochemical effects[127,134,158,185,186]		
Hypocalcemia †	-	Apheresis
Hypomagnesemia/hypopotassemia †	-	Combined
Increased PB [ALP], [AST], [GGT], [LD] and [UA]	-	G-CSF

‡ Normally, extracorporeal blood volume amounts to <15% of TBV.[186] † $Ca^{2+}$and Mg^{2+}ions are chelated by ACD-A, whereas hepatic citrate metabolism leads to metabolic acidosis counteracted by renal bicarbonate excretion, potentially leading to hypopotassemia.[158] Various electrolyte disturbances give similar symptoms: paresthesia, muscle cramps, nausea, vomiting, abdominal pain, chills, and fever (moderately reduced electrolyte levels) or (rarely) spasm, tetany, seizures or arrhythmia.[44,186] Electrolyte disturbances are prevented by oral or intravenous supplementation with calcium gluconate/chloride, calcium carbonate, magnesium sulfate and/or potassium.[158,186] §. Decreased platelet counts due to combined effects of G-CSF and apheresis combined with anticoagulation results in post-donation bleeding in up to 5 - 10% of healthy donors,[152] usually clinically insignificant.[44] ALP: alkaline phosphatase, AST: aspartate aminotransferase, GGT: gamma-glutamyltranspeptidase, Hb: hemoglobin, IV: intravenous, LD: lactate dehydrogenase, P: potassium, PB: peripheral blood, PC: platelet count. P.O.: per os. Mg: magnesium, UA: uric acid, WBC: white blood cell count.

Combined and partially synergistic effects of G-CSF and apheresis influence the peripheral blood cell counts and the coagulation and immune systems during stem

cell donations. Not only will pre-apheresis WBC count show great variability among healthy donors (see p. 7), the decrease in peripheral blood WBC and concentrations of lymphocytes and neutrophils also vary between 20 and 75% during apheresis,[44,92,134,155,183] reflecting heterogeneity in G-CSF response and apheresis procedure settings (Tables 3 and 4). G-CSF administration for allogeneic stem cell mobilization leads to a modest but significant decrease in platelet count (about 10 x 10^9/L)[44,92,134,155,183] and hemoglobin level (approximately 0.2 g/L).[44,92,155] Leukapheresis leads to further decline in platelet concentration by approximately 20–50%.[44,92,93,183,186-188]

A minority of donors experience absolute neutropenia for up to four months after stem cell collection,[183] and protracted decreases in peripheral blood counts is not uncommon. Follow up of approximately 4000 healthy donors showed slightly but significantly reduced median WBC and neutrophil counts compared to baseline levels five years post-donation.[44] Neutrophil counts normalize within 2 years in the majority of donors but in a subpopulation, remain reduced for more than 4 years.[183] Monocyte and lymphocyte levels may be reduced for at least one year and up to two years, respectively,[44,183,184] whereas platelet level and hemoglobin concentration are usually normalized within 6–12 months.[44,189] Processing of a large blood volume, several consecutive apheresis procedures, younger age and female gender have been shown to predict pronounced cytopenia after apheresis.[44,92,93,134,183] Isolated severe neutropenia is observed more often after G-CSF treatment lasting more than 5 days, and platelet reduction is correlated to G-CSF dose.[183] Healthy, untreated platelet donors return to baseline platelet counts more quickly after donation compared to healthy stem cell donors mobilized with G-CSF and thereafter have a more pronounced rebound increase in platelet counts,[190,191] further substantiating the importance of G-CSF as a cause of cytopenia.

Activation of the coagulation system is a part of the general systemic response to G-CSF, leading to increased platelet activation, ADP-induced platelet aggregation and elevated thrombogeneic plasma factors like von Willebrand factor, D-dimer and FVIII.[192-194] Apheresis also leads to platelet activation, but the hypercoagulability is counterbalanced by anticoagulant effects. While partial thromboplastin time is

decreased after G-CSF treatment, it is transiently prolonged during apheresis, and platelet counts are decreased due to combined effects from G-CSF, apheresis and anticoagulation.[38,152,194] Stem cell collection therefore infrequently leads to severe thromboembolic complications and results in post-donation bleeding, usually moderate, in up to 5 - 10% of healthy donors.[152,195,44]

The immunoregulatory effects of G-CSF have been summarized in Table 2. Apheresis also has several immunomodulatory effects, and the term "extracorporeal immunomodulation" has been used to describe the removal of plasma proteins and cellular immune components by various plasmapheresis, lymphocytapheresis or granulo-cytapheresis techniques tried in the treatment of certain autoimmune, neurological and hematological diseases.[196] Furthermore, due to exposure to high centrifugal forces and artificial surfaces during apheresis, various blood and immune cells undergo stress-induced activation or fragmentation with increased release of soluble mediators into the apheresis product.[197-199] Plasma proteins like immune-globulins, cytokines and soluble HLA Class I molecules (sHLA-I) can potentially bind to graft cells, plastic surfaces and cells in the donor and graft recipient.[200-202] Whether such potential immunomodulation caused by apheresis may result in significant clinical effects for patient or donor in the setting of allogeneic HSCT is not currently known.

Typically, the mild to moderate side effects of G-CSF and apheresis resolve within 1–4 weeks and are relieved with non-steroid analgesics, which may even enhance HSC mobilization.[93,155,173] Less than 1% of donations are complicated with severe adverse events, and the total fatality rate including both G-CSF treatment and apheresis has been estimated at 1:10,000 donations.[92,195,203] The role of *CSF3R* mutations in myeloid malignancies,[59] epigenetic and chromosomal alterations in donor lymphocytes [204] and development of myeloid malignancy in three healthy family donors 1–5 years post-donation [205,206] have raised concerns of potential severe long term adverse effects of G-CSF. However, persistent chromosomal aberrations in donors have not been confirmed five years after mobilization,[207] and despite follow-up time being too short to draw final conclusions, several prospective register studies

of more than 50,000 healthy unrelated donors, with a median follow up of 3–5 years, have not revealed increased development of malignancy or autoimmunity.[207,208]

1.2 The cellular compostion and functions of the HSC graft

In a traditional view of hematopoiesis, self-renewal and differentiation represent inversely proportional traits of HSC, with hierarchical development of mature blood and immune cells through a process from multipotent stem cells via oligopotent and unipotent progenitors. Recently, this perspective has been challenged due to the detection of a very low proportion of oligopotent progenitors in adult BM, indicating multipotent and lineage restricted progenitors as the functionally predominant subsets in adult hematopoiesis.[209] A few small studies compare the infused doses of progenitors and mature immune effector cells from allogeneic PBPC grafts and BM grafts; the results are summarized in Table 6. The transplanted doses to recipients of PBSC grafts show, on average, a 1.5–30–fold increase of progenitor and immune cell subsets over corresponding doses from BM grafts.

Table 6. Summary of seven studies comparing BM and PBPC grafts from HLA-matched related allogeneic donors with respect to infused doses of progenitor and mature immune cells to the recipients

Progenitor/ immune cell subset	Immunophenotype	Fold increase PBPC/BM	Range BM (10^6/kg)	Range PBPC (10^6/kg)
TNC	-	3.9–4.6 (132) [91,96]	2–3860 (285)	150–3270 (244) [91,210,211]
HSC (total)	$CD34^+$	1.4–3.7 (178) [91,96,212]	0.5–154 (199)	0.7–68.3 (235) [91,211,213]
HSC (multipotent)	$CD34^+CD38^-(CD90^+45RA^-)$	-	0.02–0.1 (13)	0.07–0.2 (25) [212]
T progenitor	$CD34^+CD2^+$	-	0.04–0.2 (13)	0.04–0.2 (25) [212]
	$CD34^+CD7^+$	-	0.03–0.1 (13)	0.05–0.2 (25) [212]
B progenitor	$CD34^+CD10^+$	-	0.3–1.3 (13)	0.06–0.2 (25) [212]
	$CD34^+CD19^+$	-	0.2–0.9 (13)	0.04–0.1(25) [212]
	$CD34^+CD20^+$	-	0.04–0.2 (13)	0.02–0.08 (25) [212]
Myeloid progenitor	$CD34^+CD13^+$	-	0.6–2.8 (13)	1.8–6.7 (25) [212]
	$CD34^+CD33^+$	-	0.2–19.1(26)	0.4–18.0 (31) [213]
T cells	$CD3^+$	4.9–16.1(198) [91,96,214]	3.6–1699 (326)	15.6–2123(336) [91,210-214]
T_h cells	$CD3^+CD4^+$	13.1–15 (152) [91,96,214]	9–51 (75)	50–663 (75) [91,212,214]
T_c cells	$CD3^+CD8^+$	8.3–27.4 (152) [91,96,214]	1.7–40 (75)	20–472 (75) [91,212,214]
αβT cells	$CD3^+TCR\alpha\beta^+$	16 (20) [214]	18–86 (10)	240–1064 (10) [214]
γδT cells	$CD3^+TCR\gamma\delta^+$	13 (20) [214]	0–6 (10)	6–85 (10) [214]
Naïve Th	$CD3^+CD4^+45RA^+$	11.3–17 (67) [96,214]	44–280 (10)	4–24 (10) [214]
Memory Th	$CD3^+CD4^+45RO^+$	9.8–17 (67) [96,214]	1–22 (10)	18–296 (10) [214]
B cells	$CD19^+$	6–11 (152) [91,96,214]	1.8–47 (79)	2.9–193 (82) [91,213,214]
Monocytes	$CD14^+$	24–29.3 (67) [96,214]	2.1–60.7 (36)	27–1053 (41) [213,214]
NK cells	$CD56^+CD16^+$	7.8–19.4(152) [91,96,214]	0–154 (194)	0–665 (214) [91,211,212,214]

BM: bone marrow. HSC: hematopoietic stem cell. NK: natural killer. PBPC: peripheral blood progenitor cells. TNC: total nucleated cells. TCR: T cell receptor. T_h: T helper. T_c: T cytotoxic. The number of participants in the various studies are given in paretheses.

The graft target doses for differentiated immune cell subsets are less precisely defined than for HSC. Similar to HSC, the peripheral blood levels of T cells, B cells

and NK cells may show circadian variations,[215] and the distribution of subsets varies between compartments. Whereas approximately 50% of the total neutrophil population is normally located in peripheral blood [216] the mononuclear fraction is located in primary, secondary and tertiary lymphoid organs to a higher degree, and only about 2% of body total lymphocytes are found in the peripheral blood of healthy individuals.[217]

Table 7. Summary of donor and manufacturing process heterogeneity

Donor selection	G-CSF treatment	Stem cell collection	Graft preparation
Age	Formulation	Apheresis device	Plasma removal*
Weight	Dose	Procedure length	Red blood cell depletion*
Comorbidity	Schedule	Centrifugation technique	Immune cell depletion
Ethnisity	Duration	Processed blood volume	Cryopreservation
Gender match		Number of collections	Additive solutions
HLA compatibility			
ABO compatibility			

*Plasma removal and RBC depletion are performed in minor and major ABO incompatibility, respectively, to avoid hemolysis of recipient or donor RBCs.

As presented in the previous sections and summarized in Table 7, each step of the multi-stage donor selection and graft manufacturing process adds variability to the grafts. Despite strict guidelines for the preparation of allogeneic stem cell grafts, lack of agreement on concise specifications and the inherent heterogeneity of donors, recipients and procedures leads to more pronounced dose differences within the PBPC group than between PBPC and BM grafts. Infused peripheral blood graft

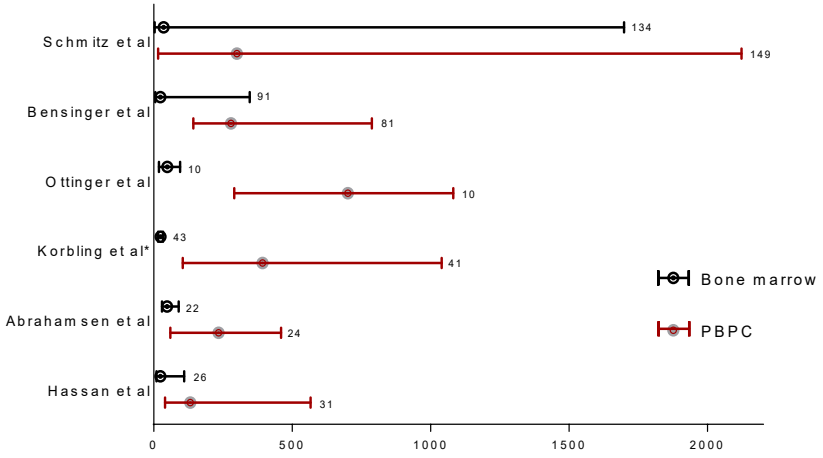

Figure 2. Comparison of BM and PBMC graft CD3⁺ doses.[91,210-214] Median (mean[214]) infused BM or PBMC CD3⁺ doses (10^6/kg) with variation ranges and number of study participants are presented, also see Table 6.

immune subset doses show more than a 100-fold variation between recipients (Figure 2).

Post-transplant immunosuppression

Post-transplant immunocompetence is further modulated by the donor-recipient histocompatibility and the individual recipient immune profile. G-CSF–treated donor progenitor and immune cells evolve in a host environment shaped by the primary disease and burden of comorbidity, previously performed chemo- and radiation therapy, ongoing immunosuppression and the residual host immune system (Figure 1, p. 2). The overall imprint of these factors is decisive for quantitative and qualitative recipient immunity in terms of absolute and relative concentrations, distribution and activation levels of various immune cell subsets and soluble mediators. Due to the chemotherapy-specific toxicity profile against different cells (myeloablative/lymphotoxic), combinations with TBI or inclusion of two or more agents is usually preferred for conditioning; after RIC and mismatched/haploidentical HSCT, additional immunosuppressive treatment to enable engraftment and protect against graft rejection and GVHD is needed.[13] To alleviate harmful effects from both donor and recipient T cells, several alternative combinations of immunosuppressive agents directed against different molecular and cellular targets are used (Table 8). The immunomodulatory effects of TBI are also highly variable as the practice is currently not standardized and the techniques and radiation doses vary among centers.[218]

Table 8. Examples of immunosuppressiva used as prophylaxis in HSCT recipients [14,219-222]

Drug class	Drug	Mechanism of immunosuppression
Calcineurin inhibitors	Cyclosporine A Tacrolimus	Inhibits activation of NFAT leading to reduced IL-2 transcription/T cell activation
Antimetabolites	Methotrexate (Folate antagonist) MMF (IMPDH inhibitor)	Prevents T cell proliferation by inhibition of purine/purine nucleotide synthesis
mTOR inhibitors	Sirolimus	Prevents intracellular signal transduction (more efficiently in conventional T cells compared to T regs)
Antibodies	ATG (polyclonal)	Modulates multiple key immune response, adhesion and cell migration surface molecules resulting in complement-related lysis and/or apoptosis of conventional T cells with spared or expanded T_{reg} cells and reduced DC functionality. Contradictory reports with respect to the effect on NK, B and iNKT cells
	Alemtuzumab (monoclonal anti-CD52)	Eliminates mature lymphocytes and monocytes by antibody-dependent cell-mediated cytotoxicity
Alkylating agents	(PT)-CY	*Promotes n vivo* TCD by DNA alkylating of proliferating cells

ATG: anti-thymocyte globulin. IMPDH: inosine-5'-monophosphate dehydrogenase. MMF: mycophenolate mofetil. mTOR: mechanistic target of rapamycin. NFAT: nuclear factor of activated T cell family of transcription factors. PT-CY: post-transplant high-dose cyclophosphamide.

Immune reconstitution after PBSCT

The overall post-transplant goal is hematological and immunological recovery, combining eradication of malignant cells with tolerance by the host. Neutrophil engraftment, the first of three consecutive days with absolute neutrophil count (ANC) $\geq 0.5 \times 10^9$/L, and platelet engraftment, platelet count $\geq 20 \times 10^9$/L without platelet transfusions,[223] are achieved significantly faster after PBPCT than after BMT.[32] Reported times to reconstitution of other immune cell subsets after allogeneic PBPC transplantation are summarized in Table 9.

Table 9. Approximate time to reconstitution of various immune cell subsets after PBSCT (examples)

Immune cell subset	Immuno-phenotype	Time to recovery	Examples of reported associations to clinical outcome
Neutrophils	$CD15^+16^+$	2–4 w [224,225]	Rapid PMN engraftment strongly correlated to improved OS/NRM [226]
DC1	$CD11c^+CD123^{lo}$†	4 w [227]	Slow DC engraftment associated with increased incidence of relapse, aGVHD and inferior survival [228]
DC2	$CD11c^-CD123^+$†	> 6 m[227]	
Mo/MΦ	$CD14^+16^{lo}14^{lo}16^+$	4 w [214,225]	Early recovery of Mo associated with improved OS [229]
NK			
cytokine producing	$CD56^{++}16^+$	1–> 6 m [225,227]	Delayed reconstitution of NK cells, especially immature $CD56^{++}$ associated with increased incidence of aGVHD [230]
cytotoxic	$CD56^+16^{++}$	1–> 6 m [214,225,227]	Reconstitution time of NK cells/$CD56^{dim}$ subset negatively associated to aGVHD, cGVHD and CMV reactivation [212,231]
T cells (total)	$CD3^+$	1–1.5 y [212,225,232]	Lower T_{reg}/ $CD3^+$ 9 m post-transplant associated to increased cGVHD risk [232]
T_h	$CD4^+$	>2 y [232]	Early $CD4^+$ recovery/ high 9 m percentage associated with low TRM and improved OS [233,234]/ increased cGVHD risk [232]
Naïve	$CD45RA^+CCR7^+$	>2 y [232]	Higher 3 m naïve $CD4^+$ percentage in patients with cGVHD [232]
CM	$CD45RA^-CCR7^+$	>2 y [232]	Increased $CD4^+$ CM/EM 3 m post transplant in cGVHD patients [232]
EM	$CD45RA^-CCR7^-$	1.5 y [232]	
T_c	$CD8^+$	1 m–1 y [212,232,235]	Lower T_{reg} / T_c 9 m post-transplant/enhanced $CD8^+$ associated to higher cGVHD risk [212,232]
Naïve	$CD45RA^+CCR7^+$	1.5 y [232]	Higher 3 m naïve $CD8^+$ percentage in patients with cGVHD [232]
CM	$CD45RA^-CCR7^+$	>2 y [232]	Higher 3 m $CD8^+$ CM percentage in patients with cGVHD [232]
EM	$CD45RA^-CCR7^-$	1–1.5 y [232]	Lower 3 m $CD8^+$EM percentage in patients with cGVHD [232]
TEMRA	$CD45RA^+CCR7^-$	9 m [232]	Lower 3 m TEMRA percentage in patients with cGVHD [232]
Naïve T_{reg}	$CD4^+FOXP3^+45RA^+CD62L^+$	>2 y [232]	Increased 6 m naïve T_{reg} percentage in patients without cGVHD [232]
$T_{reg\ CM}$	$CD4^+FOXP3^+45RA^-CD62L^+$	1.5 y [232]	Increased T_{reg} CM/EM 3 m post transplant in cGVHD patients [232]
$T_{reg\ EM}$	$CD4^+FOXP3^+45RA^-CD62L^-$	>2 y [232]	
γδ T	$CD3^+4^-8^-\gamma\delta^+$	1 m [236]	
iNKT	$CD3^+V\alpha24^+$.	1 m [237]	
Naïve B	$CD19^+IgD^+$	1 y [235]	Delayed reconstitution of B cells is associated with increased incidence of aGHVD /cGVHD irrespective of graft source [212,238]
Memory B	$CD19^+IgD^-$	>1–2 y [235]	

CM: central memory. † DC1/DC2 dendritic cell immunophenotype defined by the authors [227], update in [126]. EM: effector memory. iNKT: invariant natural killer T cells. Mo/MΦ: monocyte/macrophage. NRM: non-relapse mortality. T_c T cytotoxic. TEMRA: T effector memory RA (terminally differentiated cytotoxic effector cells). T_h: T helper. T_{reg}: T regulatory. TRM: transplant-related mortality. 2–4 w: 2–4 weeks. 3/6/9 m: 3/6/9 months. 1.5 y: 1.5 years.

Earlier immune recovery post transplant using PBPC has been attributed to homeostatic expansion of mature immune cells transferred with the graft.[212,214] Thereafter, de novo differentiation of engrafted donor progenitor cells is anticipated

to dominate the process of reconstitution. Soluble mediators from the graft and growth factors produced by transplanted progenitors and immune cells potentially influence the differentiation of the reconstituted immune system along with soluble mediators from recipient stroma and residual immune cells [239] (see also chapter 1.3).

Immune reconstitution is a selective process typically recapitulating immune ontogeny.[227,235] Recovery of the innate immune system proceeds the adaptive, and preferential reconstitution of certain subpopulations in advance of others leads to increased DC1/DC2, $CD56^{++}16^{+}/CD56^{+}16^{++}$ NK cell and naïve/memory B cell ratios early post transplant.[227,235] In contrast, T cell recovery does not parallel ontogenesis; T_c regenerate faster than T_h and memory T cells before naïve.[232] Analysis of recent thymic emigrants show selectively reduced thymic production of $CD4^{+}$ T regulatory cells (T_{regs}) at least 2 years post transplant.[232] However, post-transplant immune subset counts to a limited degree reflect TCR diversity, and $CD4^{+}$ TCR diversity has been reported to be approximately 50 times higher compared to $CD8^{+}$ diversity during the first year post transplant.[240]

The term "immune reconstitution" is poorly defined in published literature. Studies of reconstitution are, in general, biased with variability of target values for recovery of various immune cell subsets and heterogeneity with respect to donor type and stem cell source. The reconstitution times after PBSCT given in Table 9 are therefore approximate, and the results are conflicting for several immune cell subsets. Some studies report delayed recovery of NK cells compared to BMT, with both quantitatively deficient and functionally impaired NK cells for at least 6–12 months post transplant.[225] Immune recovery may be influenced by donor type, and reconstitution of $CD4^{+}$ T cells has been suggested to be faster in MRD than in MUD transplants.[234] Time to reconstitution is also highly influenced by host pre–, peri– and post-transplant clinical and therapeutical characteristics.

The existing knowledge of post-transplant recovery of immune function is mainly based on studies of BM transplantation. Data on the influence of PBPC graft progenitor and immune cell content on quantitative and qualitative recovery are too scarce and conflicting to support final conclusions.[91,241-248] *In vitro* studies have demonstrated reduced T cell proliferation and NK cell cytotoxicity for more than one

year post transplant.[225,227] PBPC grafts contain relatively low amounts of B cell progenitors and no plasma cells,[97,212] and B lymphocytes may reconstitute faster after BMT than after PBSCT.[235] However, due to less pronounced $CD4^+$ T cell deficiency after PBSCT,[235] a higher frequency of somatic hypermutation may, in theory, reduce long-term impairment of humoral immunity.

Graft failure

The incidence of primary and secondary graft failure (GF), defined as ANC below 0.5×10^9/L by day 28 post transplant or recurrence of this ANC level after initial engraftment, respectively,[249] is approximately 5–10% after allogeneic HSCT.[249,250] Donor T and NK cells and both recipient and donor T_{reg} cells are thought to facilitate immune reconstitution, and immunological rejection of donor HPC by recipient T cells and possibly NK cells is considered the primary mechanism of GF, whereas antibody-mediated rejection is more controversial.[250,251] GF can also be caused by non-immunological mechanisms (e.g. drug toxicity, viral infections, sepsis).[265,266] RIC, HLA incompatibility, *ex vivo* T cell depletion (TCD), transplanted TNC $\leq 2.5 \times 10^8$/kg and non-malignant disorder are the most important risk factors for GF; graft source (UCB/BM) and major ABO-incompatibility are weakly and indirectly associated factors.[250-252]

Graft versus leukemia and graft versus host effects

Post-transplant immunity is composed of (i) expanded mature graft immune cells, (ii) graft progenitor cells matured in the recipient and (iii) residual recipient immune cells. The interplay between donor and recipient cells is crucial for both reconstitution, graft rejection, GVHD and GVL effects. The complex cooperation of various donor and recipient subsets in development of aGVHD is not fully elucidated, and existing knowledge is primarily based on murine models.[14,253] The pathological process includes three stages. (i) Conditioning-induced tissue damage leads to activation of host antigen-presenting cells (APCs), induced by damage-associated molecular patterns (DAMP) and pathogen-associated molecular patterns (PAMP), with increased production of pro-inflammatory cytokines (see Chapter 1.3).[14,253] (ii) Donor T cell are activated, primarily in recipient secondary lymphoid organs, where

$CD4^+$ and $CD8^+$ TCR recognize non-self MHC class II and I antigens presented by recipient APCs or mHAgs presented by either recipient or donor APC (primary activation signal) [14,46,253]; APCs further provide costimulatory (secondary) and cytokine (tertiary) signals, leading to T cell differentiation and proliferation (see chapter 1.3, Table 11 and 13).[14,46,253] (iii) A cytolytic response that acts preferentially against recipient skin, gastrointestinal tract and liver tissues is effectuated by a complex network of reactions involving numerous cellular and soluble mediators.[14,253] Based on the severity of skin reactions, cholestasis and gastrointestinal dysfunction, aGVHD is clinically classified into grades I–IV.[253]

Closely associated mechanisms underlie the GVL effect; recipient APCs present mHAgs and MHC-dependent and leukemia-associated antigens to donor $CD4^+$ and $CD8^+$ cells, resulting in production of pro-inflammatory cytokines and cytotoxicity through the Fas and perforin pathways.[254,255] Less is known about the pathophysiological mechanisms of cGVHD, but differentiation of donor naïve T cells towards Th17 and Tfh phenotype (detailed in chapter 1.3, Table 13), impaired germinal center B cell maturation and abnormal activation and differentiation of T and B cells generating auto– and allo-antibodies are recently proposed pathogenic factors leading to pathogenic M2 differentiation of macrophages, thymic damage, disturbed T_{reg} generation and homeostasis and multi-organ tissue fibrosis.[256] While cGVHD diagnostic criteria traditionally included temporal relation to HSCT with occurrence >100 days post-transplant, clinical organo-specific features are currently considered decisive.[257] The pathogenesis of cGVHD is supposed to be linked both to aGVHD, GVL and autoimmune disease.[256]

The importance of graft composition for clinical outcome

In the complex post-transplant *in vivo* setting, the functionality of single reconstituted immune cell subsets is hard to characterize accurately. Intricate interactions of multiple host and donor factors and microorganisms decide the individual balance between immune reconstitution, graft rejection, infection, relapse, cancer cell eradication and attack on normal host cells. Several of these processes are mutually dependent. A recent study confirmed a complex diversity of reconstitution

dynamics dependent on both conditioning regimens and graft compositions; NK and B cells reconstituted faster and $CD4^+$ slower after $CD34^+$-selected transplantation, and RIC promoted faster lymphocyte and T cell recovery.[245] aGVHD and/or cGVHD delay the reconstitution of both NK, Tc, DC1 and B cells.[212,214,230,231]

The incidence and severity of post-transplant infections are indicators of immunological functionality. For example, higher lymphocyte counts and especially naïve $CD4^+$ counts in PBPC compared to BM recipients are associated with decreased incidence of bacterial and fungal infections in the first year post transplant.[235] Another example is the impact of CMV reactivation on post-transplant evolvement of the entire T cell repertoire and NK cell repertoire.[258,259] More refined immune repertoire analyses, called next generation sequencing (NGS), provide comprehensive lymphocyte repertoire diversity analysis with definition of individual T and B cell clones and possibilities for simultaneous phenotypic and functional assessment of multiple populations, increasing the resolving power of reconstitution dynamics diagnostics.[260,261] For example, NGS studies of γδ TCR repertoires during reconstitution after PBSCT and CMV reactivation provide novel insights in γδ T cell activation mechanisms, substantiating an adaptive, clonal γδ T cell response in viral reactivation.[236]

The importance of different donor T cell subsets as well as donor and recipient immune cell subsets beyond the T cell compartment for post-transplant immunity is gaining attention. NK cells are classified as innate lymphoid cells (ILCs) group 1 and, based on recognition of their novel functions in self-tolerance, regulation of adaptive immune reactions and memory, are considered to bridge innate and adaptive immunity in addition to serving their classical role as innate players.[262,263] Donor and recipient NK cells may promote engraftment and graft rejection, respectively.[262,263] Donor NK cells are presumed to exert GVL effects through recognition of malignant cells by (i) HLA-mismatched inhibitory KIRs or (ii) HLA-matched activating KIRs and to prevent GVHD by cytolytic elimination of host DC or activated donor T cells by similar receptor-mediated mechanisms.[262,263] However, their role in GVHD is still controversial, with the potentially aggravating effect of NK release of pro-

inflammatory cytokines.[263] NK cells are also vulnerable to aGVHD with increased risk of impaired maturation and functionality.[264]

The γδ T cell subset is another with well-documented HLA independent anti-leukemia effects; however, the associated mechanisms have not been definitively established.[265,266] γδ T cells are characterized by great diversity both phenotypically and functionally.[265,266] Plasticity is a basic featureof these cells, and still new effector and regulatory subsets are discovered with context-dependent potential to influence innate and adoptive immune responses via multiple mechanisms, including antibody-dependent cellular cytotoxicity (ADCC), perforin-granzyme and TNF-related apoptosis-inducing ligand (TRAIL) pathways, cytokine production and antigen presentation.[265,266] This diversity may explain the conflicting views of the importance of γδ T cells in aGVHD development.[265,267,268]

Table 10 provides examples of reported effects of donor graft content of various T, B and NK subsets. The results from pre-clinical trials are, to variable degrees, confirmed in humans. In general, the human studies are small and the interpretation of the results confounded by the extensive heterogeneity in transplantation protocols, making assessment of the relative importance of single immune cell subsets for the complex *in vivo* human post-transplant immunity difficult. Studies claiming to have identified the only or the most important subset for a given post-transplant endpoint tend to reach different conclusions. For example, different groups report the concentration each of $CD3^+$,[269] $CD8^+$,[270] T_{regs},[271] iNKT cells,[272,273] as the only graft mature immune cell parameter predictive of aGVHD.

Table 10. Donor-derived lymphocyte subsets that are important for post-transplant immune functions–examples selected with focus on graft composition studies when available

Lymphocyte subset	Post-transplant donor-derived immunity (examples from pre-clinical studies and bone marrow transplantation)	Subset specific clinical experience or applications (PBSC, examples)
T	*Ex vivo* BM TCD leads to *1)* reduced risk of GVHD, *2)* slower immune recovery, *3)* higher incidence of graft rejection, relapse and PTLD (M/H, multiple studies) [274]	$CD3^+$ doses above 8 x 10^6/kg after RIC lead to increased aGVHD risk without significant changed engraftment rate or relapse incidence in AML (MUD PBSC, n = 203) [269]
T_h	*In vivo* depletion of donor-derived $CD4^+$ cells prevents aGVHD with sustained GVL effect (M) [275]	High graft CD4/CD8 ratio is associated with increased risk of aGVHD (MRD/URD PBSCT, n = 299) [276]; PBSC $CD4^+$ dose correlated to cGVHD using MRD but not alternative donors (n = 2350) [277]
T_c	Upregulated expression of PD-L1 in GVHD target tissues and $CD8^+$ cells after i*n vivo* $CD4^+$depletion leads to reduced GVHD with sustained GVL effect (M) [275]	$CD8^+$ dose associated to aGHVD risk: Contradictory,possibly conditioning/donor-dependent results [242,244,246,270] $CD8^+$ 2-log depletion fails to reduce the incidence of GVHD (MRD/URD PBSCT, n = 41) [278]. High $CD8^+$ doses correlate to lower relapse risk and improved OS (MRD/MUD/MMRD/MMUD PBSCT, n = 499) [276,279]
naïve*	$CD62L^+CD44^-(CD25^-)$ T_{Ns} can induce both GVHD and GVL (M) [280,281]	$CD45RA^+$ depleted PBSCT associated with rapid T cell recovery, reduced cGVHD and similar rates of aGVHD, infections, relapse and 78% 2 y OS in high-risk leukemia (n = 35) [282]
memory*	$CD4^+CD62L^-CD44^+CD25^-$ TEM mediates GVL without GVHD (M) [280] $CD8^+CD62L^+CD44^+$ TCM induce both GVL and GVHD (M) [281]	Enriched $CD8^+45RA^-RO^+$ memory cells from the original MRD are tried to treat relapse after allo-HSCT (Phase I, n = 15) [283]
$Foxp3^+$ T reg**	$Foxp3^+$ T_{regs} suppress GVHD, contradictory results regarding GVL suppression (M), [284,285] *in vitro*-induced $CD8^+$ T regs show $Foxp3^+$ instability *in vivo* and may exacerbate GVHD (M) [285]	Systematic review including eight studies of MRD/Haplo/MUD/ MMUD PBSCT for hematological malignancies: High graft T_{reg} content is associated with reduced aGVHD risk (6 studies, n = 408), reduced NRM and improved OS (2 studies, n = 168) [271]
Tr1	Tr1 is the most abundant post-HSCT regulatory cell and is essential for GVHD prevention (M) [286]	Allo-Ag-hyporesponsive Tr1 is tried infused after TCD haplo-HSCT for high-risk leukemia (Phase I/II, n = 12) [287]
αβ T	Exerts GVL, GVHD and anti-infectious effects; corresponds to $CD4^+$ and $CD8^+$ cells (see above)	αβ-T/B cell depleted haplo-HSCT leads to *1)* shorter neutrophil/platelet recovery, *2)* lower incidence of aGVHD and cGVHD and *3)* incidence of LFS similar to MUD transplantations (n = 98) [247]
γδ T	Different subsets exists, including Vδ1 and Vδ2; context-dependent effector or regulator functions; GVL and anti-infectious effects, time dependent exacerbation or attenuation of GVHD (M, human *in vitro* studies) [288,289]	High content of γδ T cells in MUD unmanipulated grafts increases the risk of aGVHD (n = 63) [267]; high content of γδ T cells in TCD grafts from partially MMRDs is associated to superior 5 y LFS without increased risk of aGVHD (n = 153) [290]
iNKT	Low iNKT numbers observed in cGVHD; iNKT infusion prevents and ameliorates cGVHD in a IL-4 and T_{reg} dependent manner (M) [291]	Low content and low expansion capacity of iNKT cells in MRD/MUD PBSC grafts predicts aGHVD (n = 141) [272,273]; high PBSC graft content of iNKT improves GPFS (MRD/MUD/MMUD, n = 80) [292]
NK	Haploidentical KIR incompatible/alloreactive NK cells eradicate AML, and eliminate GVHD by killing of host APC (M) [293]; NK cells retain GVL effect and reduce GVHD by inhibition of donor T cell proliferation, IFNγ production and T cell lysis (M) [294]; pre-activated NK cells may contribute to GVHD (M, H) [295,296]	GVH KIR incompatibility and *in vitro* NK alloreactivity predict low incidence of rejection and GVHD in high-risk leukemia (n=112) and favorable DFS in AML (n = 57) [293]; higher NK cell dose in MRD PBSCT is associated with shorter immune recovery, lower incidence of infections and lower NRM (n = 61) [248]; naïve $NKG2A^+KIR^-CD57^-$ NK cell repertoire is associated with reduced incidence of relapse and increased incidence of infections after MRD/MUD/MMUD PBSCT (n = 106) [259]. CD56-enriched DLI following PTCy RIC haplo-HSCT result in rapid Th, NK and T_{reg} reconstitution and low frequency of GVHD, viral reactivation and NRM (n = 10) [297]
B	Experience from BMT: *In vivo* TCD, especially selective ATG-based TCD, is strongly associated to PTLD risk (n = 26 901) [298]	High $CD19^+$ dose leads to increased risk of aGVHD (MRD/URD PBSCT, n = 299) [276]; *ex vivo* Alemtuzumab-based T- and B cell reduced grafts is more favorable than pre-transplant *in vivo* recipient treatment with respect to early EBV reactivation (MRD/MMRD/ MUD/ MMUD, PBSCT = 105, BMT = 5, PBSCT/BMT = 1) [299]

* $CD4^+$ and/or $CD8^+$ subsets. ** In addition to the original described $CD4^+CD25^{hi}Foxp3^+$ T_{reg} (T regulatory cell) phenotype multiple regulatory cell lines have been identified and a simplified classification of $Foxp3^+$ T regs has been proposed[300]. αβ T: The most common variant of T cell receptor (TCR) contains an α and a β chain and is MHC restricted. A minor subset of CD3+ cells express non–MHC-restricted TCR containing a γ and a δ chain. ATG: anti-thymocyte globulin. B: $CD19^+$B cell subset. BM: bone marrow. EBV: Epstein-Barr virus. GPFS: GVHD-free progression free survival. H: human. iNKT: Invariant Natural Killer T Cell. LFS: leukemia-free survival.M: murine. MMUD: mismatched unrelated donor. MRD: matched related donor. MUD: matched unrelated donor. NK: $CD16^+$Natural Killer Cell subset. NRM: non-relapse mortality. OS: overall survival. PD-L1: programmed death ligand-1. PTCy: post-transplant cyclophosphamide. PTLD: post-transplant lymphoproliferative disorders.T: $CD3^+$T cell subset, T_c: $CD8^+$cytotoxic cell subset, T_h: $CD4^+$ helper cell subset. Tr1: T regulatory cell type 1. TCD: *ex vivo.* T cell depletion..

Graft manipulation and adoptive transfer of cells

T cell depletion (TCD) can be performed *ex vivo* or *in vivo.* Post-transplant *in vivo* TCD with cyclophosphamide has recently been established as a superior alternative to conventional *ex vivo* $CD3^+$ depletion with respect to recipient outcome.[301] A recent retrospective study of recipients of MMUD grafts also reported better outcome after post-transplant *in vivo* TCD with cyclophosphamide compared to ATG.[302]

The following discussion is confined to manipulation of the graft with various *ex vivo* depletion and enrichment techniques that alter the balance between immune cell subsets. In haploidentical HSCT, graft manipulation is a prerequisite to reduce severe graft versus host and host versus graft immune reactions caused by bidirectional alloreactivity.[287,297,303] Various physical and immunological techniques have been developed, from the original soybean lectin agglutination and rosette depletion via monoclonal antibody methods to the use of semi-automated devices for positive and negative selection, culture and differentiation within closed systems.[287,297,303]

Documented experience with graft manipulation, ranging from $CD34^+$ selection to depletion of the entire T cell compartment or selected subsets like αβ or naïve $CD3^+$, possibly combined with B cell depletion, provides a basis for the clinical importance of single subset concentrations and the balance between various cellular factors (Table 10).[287,297,304,305] Results from clinical studies using depleted grafts potentially provide information about the functions both of the remaining subsets in the graft and of the depleted graft cells, but discrimination between these two factors is not always clearly discussed in the interpretation of results. Neither are the subdivisions and phenotypes of immune cells unambiguously defined across studies. To mention a few examples, naïve T cells are sometimes treated as a uniform population, independent of their $CD4^+$/$CD8^+$ origins, and the definition of central and effector memory cells and various regulatory subsets varies between studies (Table 10).

The balance between different immune cell subsets may also be changed by pre- or post-transplant adoptive transfer of effector or regulatory cells to improve immune reconstitution and to prevent relapse or aGVHD, respectively. Prophylactic post-transplant donor lymphocyte infusion (DLI) of unmanipulated $CD3^+$ cells may improve OS in high-risk leukemia.[306] Several strategies are used to overcome the high

aGVHD risk associated with conventional T cell infusion: (i) delayed post-transplant infusion awaiting recipient donor cell tolerance,[255] (ii) escalated doses to facilitate tolerance induction,[255] (iii) depletion of $CD8^+$ or naïve subsets,[307] (iv) combination with T_{reg} cells,[308] (v) suicide gene modification to allow *in vivo* elimination in the event of aGVHD,[309] (vi) selection and expansion of tumor-specific subsets,[254,310] (vii) G-CSFmodified DLI,[311] (viii) post-transplant infusion of genetic modified and expanded $CD3^+$ cells with antigen-specific chimeric antigen receptor (CAR T),[312] and (ix) pre-transplant infusion of allogeneic universal gene-edited TCR– and CD52-deficient CAR T cells.[313] Furthermore, several small studies report beneficial effects of pre– or post-HSCT infusion of NK cells, but timing and cellular activation may be of importance; increased incidence and severity of aGVHD have been observed after delayed infusion and IL-15 activation.[263,264,296] Finally, pre– or post-transplant T_{reg} infusion, possibly enhanced with *ex vivo* antigen stimulation and expansion or *in vivo* IL-2 activation, may improve engraftment, prevent aGVHD and possibly replace pharmaceutical immunosuppression.[264,314,315] However, the relative importance of different T_{reg} subsets including thymic natural nT_{regs}, $CD4^+$ and $CD8^+$ inducible iT_{regs} and $FoxP3^-$ T_{regs} type 1 (Tr1) for attenuation of aGVHD or GVL has not been established.[264]

The importance of several other immune cell subsets including non-NK ILC, mesenchymal stroma cells (MSC), myeloid-derived suppressor cells (MDSC) and mucosa-associated invariant T cells (MAIT) as allogeneic HSCT graft and adoptive immune therapy components is currently under investigation.[264] The complexity in the field is further increased by new pharmaceutical approaches to achieving donor cell tolerance with sustained GVL effect, e.g.: (i) inhibition of T cell costimulatory signals (CTLA-4 Ig, CD28 antagonists), (ii) reduced migration of pro-inflammatory immune cells by chemokine receptor antagonists, (iii) inhibition of mediator-induced immune cell activation and proliferation by blocking JAK signaling, (iv) altered epigenetic regulation of transcription by histone deacetylase inhibitors, and (v) reduction of post-transplant inflammatory responses by treatment with the acute phase reactant alpha-1-antitrypsin.[316]

1.3 Soluble mediators in allogeneic HSCT

As illustrated in Tables 11 and 12, a wide range of soluble mediators play central roles throughout the HSC mobilization and transplantation processes. The classification of mediators and their receptors/substrates may differ depending on structural/phylogenetic or functional criteria.[317,318,319] Furthermore, as pleiotropy (multiple biological functions) and redundancy (shared biological functions) are fundamental characteristics of most mediators, divisions between the listed groups may fluctuate.[320] Numerous mechanisms for pleiotropy and redundancy are described, including (i) receptor distribution on various cell lines, (ii) receptor promiscuity, (iii) sharing of receptor components, and (iv) signal pathway trans-activation/crosstalk.[320] These mechanisms contribute to the complexity and fine-tuning of the network of interacting soluble mediators in homeostasis and disease, for which information about the level of a single mediator is of little value without knowledge of its biological context and the clinical setting. For example, G-CSF functions both as a pro-inflammatory cytokine and a growth factor as well as a strong chemotactic agent for HPC during mobilization.[321] Moreover, response to G-CSF is context dependent: while immunosuppression of the graft is considered the primary effect of HSC mobilization (see Chapter 1.1, Table 2), post-transplant administration of G-CSF has been suggested as a possible factor in higher incidences of aGVHD/cGVHD and higher transplant-related mortality.[322,323]

G-CSF is one of several cytokines known to induce the release of both progenitor cells and more differentiated immune cells into peripheral blood through several direct and indirect mechanisms, and the pre-treatment levels of TNFα and IL-6 have been reported to predict HSC-mobilizing capacity.[324] As detailed on pages 6–7, the mobilization process comprises complex interactions of interdependent immunoregulatory cytokines, interleukins, chemokines, growth factors, adhesion molecules and matrix metalloproteases. Furthermore, $CD34^+$ cell expression of VCAM-1, CD44, VLA-4 and integrinα is correlated to stem cell yield.[325]

Table 11. Selected examples of cytokines with importance for allogeneic stem cell mobilization and transplantation

Selected cytokines	Important sources [50,239,317-319,326-332]	Receptor* [317,319,328,333-337]	Important general immune functions [317,319,326,336-339]	Examples of reported effects/functions in allogeneic HSC mobilization and HSCT
Immunomodulatory cytokines				
IFNγ	NK, iNKT, MΦ, Mo, Th1, $CD8^+$, BC	IFN /*CII*	PI. Th1 differentiation. Cytotoxicity. MHC cl I/II upregulation. Cell growth inhibition. Pro-apoptotic.	CT amplifies secretion.[253,340] G-CSF decreases secretion.[341] aGVHD: effector/context dependent,[14,253] associated to aGVHD onset and severity, upregulation of chemokines and -receptors.[340]
CD40L	$CD4^+$, EC, P	TNF/ *TNFRSF*	PI. BC/Mo/TC /P activation. Ig class switching/GC formulation. AM expression, cytokine release.	Post-transplant levels associated to transfused and engrafted platelets rather than to aGVHD.[327]
TNFα	MΦ, Mo,$CD4^+$, BC, PMN, NK	TNF/ *TNFRSF*	PI (/AI). II mediator (pyogenesis, acute phase response). AI regulator. Chemotaxis inducer. Limits extent/duration of inflammation.	Pre-G-CSF level correlated to pre-apheresis $CD34^+$ count.[324] G-CSF decreases secretion.[324,341] CT amplifies secretion.[253,340] Effector in aGVHD,[253,342] associated to aGVHD onset and severity.[340]
TGFβ	EPC, EC, MΦ, T_{reg}, HSC, $CD8^+$, $CD4^+$, NK, PMN	TGF/*TGFβ*	AI. Regulates Th diff. T_{reg} induction. Decreases cell growth.	Conflicting results regarding G-CSF effect.[114,341] Donor TC TGFβ induces IL-10 production, attenuates aGVHD, impairs GVL and mediates cGVHD.[14,253]
Interleukins				
IL-1β	MΦ, Mo, DC	IL-1/*TIR*	PI. Th17 and HSC myeloid differentiation. CXC inducer.	CT amplifies secretion.[253,340] TC activator /aGVHD effector.[253,342]
IL-1 RA	Mo, Φ, FB, PMN.	IL-1/*TIR*	AI. IL-1α/β inhibitor.	Patient and/or donor polymorphisms of *IL1RN* associated to plasma concentration of IL-1 RA and predictive of aGVHD in some studies (contradictory results).[342]
IL-2	$CD4^+$, $CD8^+$, DC, NK, iNKT	G4(cγc)/*CI*	TC activation.TC/BC cell growth/clonal expansion. T_{reg}/NK differentiation. and proliferation.	G-CSF decreases secretion.[341] aGVHD: T cell activator/effector.[14,253] Dose-dependent promoter of (i) early T_{reg} reconstitution tried in aGVHD prophylaxis.[343] (ii) $CD56^{bright}$ NK cell differentiation with potential use in cGVHD therapy.[344]
IL-4	Th2, ILC2, γδ TC, iNKT	G4(cγc)/ *CI*	AI. Th2/Th9/BC differentiation. VCAM-1 inducer.	Conflicting results regarding G-CSF effect.[341] Promotes aGHVD tolerance.[253] IL-4 induced Th2 diff. associated to pulmonal, hepatic and cutaneous aGVHD.[340]
IL-6	Mo, MΦ, PMN, TC, BC, HSC, OB	G2(gp130)/*CI*	PI. Th17/22//BC differentiation. Antibody-production. Leukocyte migration-control via CXC/AM expression.	Heterogeneous G-CSF response.[324] CT amplifies secretion.[253,340] Shortens time to engraftment.[345] TC activator and an effector in aGVHD.[253,342]
IL7	MSC, DC, BC, MΦ, Mo	G4(cγc)/ *CI*	PI. BC/TC/ILC development.V(D)J recombination.	CT amplifies secretion.[342] Negatively respectively posetively correlated to TC and BC reconstitution.[346] TC activator in aGVHD.[342,346] Associated to increased aGVHD risk and mortality (contradictory results).[346,347]
IL-8/ CXCL8	MΦ, Mo, Ly, PMN, EC, EPC, FB, MC, DC	CXCR1/2/ *GPCR*	PI. PMN/NK/TC/ chemotaxis/degranulation. Pro-angiogenetic.	G-CSF decreases secretion.[324] Induces HSC mobilization.[341] Included in diagnostic aGVHD biomarker panel.[348] Reduces cGVHD in children.[349]
IL-9	Th2, Th9, ILC	G4(cγc)/ *CI*	TC growth. $CD8^+$ proliferation. Th1 inhibition. Th2 inflammatory reactions.	Low post-transplant level correlated to aGVHD incidence.[350]
IL-10	Mo, T reg> Th2>Th1, BC, MΦ, DC	IL-10/*CII*	AI. Inhibits inflamm. cytokine prod./Th17 proliferation. Enhance BC/T_{reg} survival and BC differentiation/ isotype switch.	G-CSF effect: conflicting results.[341] TBI increases B cell production.[253] High post-transplant levels promotes tolerance against aGVHD/conflicting results.[253,347] Early reconstitution of IL-10 producing NK inversely correlated to aGHVD incidence.[351]
IL-11	MSC	G2(gp130)/*CI*	Hematopoiesis. Tissue protection after chemical/radiation damage. TIMP-1 inducer.	Promotes tolerance against aGHVD/conflicting results.[14,253]
IL-12	Mo, MΦ,PMN, DC, BC	G2(gp130)/*CI*	PI. Enhances cytotoxic factors. DC maturation. TC proliferation. Th1 differentiation.	CT amplifies secretion.[340] The majority of studies report association to aGHVD.[14,352]
IL-15	MSC, EPC, Stroma, Mo, MΦ, DC, $CD4^+$	G4(cγc)/*CI*	PI:TC activation. NK proliferation/cytotoxicity. γδ TC differentiation. CD8+ memory/NK/iNKT homeostasis.	Enhances immune reconstitution.[353] High post-transplant level correlated to aGVHD incidence and predicts cGVHD.[14,354]
IL-17A	Th17. ($CD8^+$, $CD4^+$,γδ TC, NK, PMN, iNKT, ILC)	IL-17/*CI*	PI. Induces secretion of IL-6, IL-8, GM-CSF, G-CSF, chemokines and MMPs. PMN recruitment/activation.	Suggested to influence aGVHD severity, role not finally established.[253,340] Associated with cutaneous and chronic GVHD via CSF-1 dependent macrophage accumulation leading to fibrosis.[340]
IL-18	MΦ, DC, OB	IL-1/*TIR*	PI: Enhances NK cell function/Th1 differentiation.	Inconsistent reports of importance for aGVHD.[14,253]
IL-22	Th22, Th1, Th17. (γδ TC, NK, ILC)	IL-10/*CII*	PI. Increase acute phase response. Tissue repair/regeneration.	Contribution in aGVHD depends on donor/recipient origin, target organ and timing.[355]

Chemokines				
CXCL1-2	MC, MΦ, Mo, DC, PMN, EPC	CXCR2/ *GPCR*	I. Neutrophil activation and migration.	Induces HSC mobilization.[341]
CXCL5	DC, EPC	CXCR2/ *GPCR*	I. Neutrophil migration.	Shown to ameliorate aGHVD in combination with anti-CCL24 in animal model.[356]
CXCL10	MΦ, Mo,,MC, DC, $CD4^+$	CXCR3/ *GPCR*	D. Th1 response.	Attraction of Th1, Th17, $CD8^+$ and NK to the skin, liver and intestinal mucosa in aGVHD[341,357]. Included in diagnostic aGVHD biomarker panel.[348]
CXCL11	MΦ, Mo, MC, DC, $CD4^+$	CXCR3/ *GPCR*	I. Th1 response.	Promotes infiltration of Th1, Th17, $CD8^+$ and NK in liver and intestinal mucosa during aGVHD[341,357]. Upregulated conjunctival gene expression in cGVHD.[358]
CXCL12	RC, DC, OB, MSC	CXCR4/ *GPCR*	H. BM homing of HSC and immune cells. Myelopoiesis. B. lymphopoiesis.	G-CSF decreases secretion, leading to HSC and immune cell mobilization.[324] Homing of graft cells to BM (Suppressor).[341]
CCL1	TC, MC	CCR8/*GPCR*	I. Th2/T_{reg} migration.	Downregulated PBMC gene expression in aGVHD.[358]
CCL2	MΦ, MC, TC, NK, EPC	CCR2/*GPCR*	I. Migration of inflammatory monocytes.	Upregulated conjunctival gene expression in cGVHD.[358] Chemotaxis to aGVHD target organs.[358]
CCL3	MΦ, MC, DC, TC, BC, NK, EPC	CCR1/5/ *GPCR*	I. Mo, MΦ /T/NKC migration.	Induces HSC mobilization.[341] Attraction of Th1, Th17 and $CD8^+$to the skin, liver and intestinal mucosa in aGVHD.[341]
CCL4	MΦ, MC, DC, TC, BC, NK, EPC, HSC	CCR5/ *GPCR*	I. TC-DC interactions.	Attraction of Th1, Th17 and $CD8^+$to the skin and liver in aGVHD.[341]
CCL5	MΦ, MC, DC, TC, NK, EPC	CCR1/3/5/ *GPCR*	I. Innate/adaptive immunity.	Attraction of Th1, Th17 and $CD8^+$to the skin and liver in aGVHD.[341] T cell recruitment/correlates to severity.[14,341]
CCL8	Mo, MΦ, BC	CCR1/2/3/5/*GPCR*	I. Th2 response. Homing to skin.	Infiltration of Th1, Th17 and $CD8^+$ in skin a, liver and intestinal mucosa during aGVHD.[341] Correlated to aGVHD severity.[14]
CCL9-10	MΦ, Mo, DC, PMN, TC	-	-	Upregulated skin gene expression in murine aGVHD.[358]
CCL11	MC, MΦ, Mo, DC	CCR3/*GPCR*	D. Th2 response. Eosinophil/basophil migration.	Upregulated skin gene expression in murine aGVHD.[358]
CCL25	DC, EPC	CCR9/*GPCR*	H. T memory cell homing to gut. Thymocyte migration.	Infiltration of Th1, Th17 and $CD8^+$ in intestinal mucosa during aGVHD.[341]
CCL27	PMN, DC	CCR10/ *GPCR*	H. T cell homing to skin.	Attraction of Th1, Th17 and $CD8^+$to the skin in aGVHD.[341]
Hematopoietic growth factors				
TPO	HC, MSC, OB	G1/*CI*	Regulates HSC→ platelet differentiation/expansion.	rhTPO tried in treatment of post-transplant thrombocytopenia.[359]
VEGF	MΦ, Mo, AC, AD-MSC, HSC	CIV/*RTK*	Stimulator of cell survival, proliferation, migration and adhesion. Regulates angiogenesis/permeability.	Heterogeneous G-CSF response[324]. Suppresses HSC mobilization.[332] Protects against aGVHD [14,360].
HGF	MSC, MΦ, Mo, AC, OB, HSC	CVIII/*RTK*	Regulator of cell growth, motility and morphogenesis. Contributes to angiogenesis, organ regeneration and wound healing.	G-CSF increases secretion, leading to upregulated PMN MMP9-release.[361] Correlated to incidence and severity of aGVHD and included in diagnostic aGVHD biomarker panel.[348,362]
Leptin	AC, MΦ, Mo, MSC	G2/*CI*	PI. Activator of II and AI. Promotes production of inflammatory cytokines and Th17 differentiation.	Low pre-conditioning level associated to increased risk of early relapse.[363] High post-transplant level correlated to cGVHD incidence and persistent anorexia.[364,365]
G-CSF	MSC, MΦ, Mo, EC, FB, OB	G2(gp130)?/*CI*	Promotes PMN expansion, differentiation, migration and phagocytosis.	HSC mobilization (p. 6). G-SCF primed BMT enhances engraftment and reduces aGVHD.[366] G-CSF-mediated graft Th2 polarization reduces and post-transplant G-CSF administration increases aGVHD risk, respectively.[253]
GM-CSF	MSC, MΦ, Mo, TC, BC, EC	G4(cβc)/*CI*	Promotes PMN expansion, migration, FcγR1, CR-1, CR-3 and AR expression and phagocytosis. PI cytokine inducer.	Induces HSC mobilization.[341] Correlates to incidence of aGVHD in children.[349]

*Receptor family, alternatively receptor (for chemokines), and receptor superfamily is presented. AC: adipocyte. AI: adaptive immunity. ^A^: animal model. AM: adhesion molecule. AR: adhesion receptor. BC: B cell. CCL: C-C motif chemokine ligand. CCR: C-C chemokine receptor. CI: Class I cytokine receptors. CII: Class II cytokine receptors. CR: complement receptor. CT: conditioning therapy. Cβc: common β chain ligand. Ctx: chemotaxis. Cγc: common γ chain ligand. CXC: C-X-C motif chemokine. CXCL: C-X-C motif chemokine ligand. DC: dendritic cell. D: dual function. EC: endothelial cells. EPC: epithelial cells. GC: germinal center. GM-CSF: granulocyte-macrophage-colony-stimulating factor. GPCR: G-protein-coupled receptor. H: homeostatic. HC: hepatocyte. HGF: hepatocyte growth factor. I: inflammatory. II: innate immunity. ILC2: innate lymphoid cells type 2.Ly: lymphocyte. Mo: monocyte. MΦ: macrophage. MSC: mesenchymal stroma cell. IL-1RA: interleukin 1 receptor antagonist. OB: osteoblast. P: platelets. PI: pro-inflammatory. PMN: polymorhponuclear neutrophil. RC: reticular cells. RTK: receptor tyrosine kinase. TC: T cell. Th: T helper subset. TNFRSF: tumor necrosis factor receptor superfamily. TIR: Toll-IL-1-receptor superfamily. rhTPO: recombinant human thrombopoietin. V(D)J: Variable(Diversity)Joining gene segments. VEGF: Vascular endothelial growth factor.

Table 12. Examples of adhesion molecules, matrix metalloproteases and matrix metalloprotease inhibitors in allogeneic HSC mobilization/HSCT

Selected mediators	Important sources [332,367-371]	Important functions* [367,369-371]	Examples of reported effects/ functions in allogeneic stem cell mobilization and transplantation
Adhesion molecules			
P-Selectin	EC, P	WBC rolling	Facilitates rolling of HSC in homing and engraftment [361]
E-Selectin	EC	WBC rolling	Expression increased by G-CSF [324]; facilitates rolling of HSC in homing and engraftment [361].
VCAM-1	EC, OB	WBC adhesion/ transmigration	Expression increased by G-CSF [324]; constitutively expressed on BM endothelium/stroma, contributes to retention of VLA-4$^+$ HSC; cleaved by MMPs during mobilization [361]; biomarker for diagnosis and prognosis of post-transplant hepatic SOS [348]
ICAM-1	EC	WBC adhesion/ transmigration	Expression increased by G-CSF [324]; regulates HSC homeostasis in the BM niche [372]
Matrix metalloproteases			
MMP-1	MΦ	Collagenase.	Overexpressed in intestinal GVHD lesions [373]
MMP-2	MSC, EC, EPC, HSC, TC, MΦ	Gelatinase	Secretion from stroma cells and HSC increased by G-CSF [361]; mediates HSC mobilization, proMMP-2 is activated by MT1-MMP cleavage of CXCL12, further activates MMP-9 and -13 contributes to general proteolysis (see MMP-9) [361]
MMP-3	MΦ	Stromelysin	Included in diagnostic cGVHD biomarker panel and associated to post-transplant bronchiolitis obliterans [348,374]
MMP-7	EPC, MSC, MΦ	Matrilysin	
MMP-8	PMN, EPC	Collagenase	Secretion from neutrophils increased by G-CSF [375]; mediator HSC mobilization through proteolysis of CXCL12 [375]
MMP-9	PMN, HSC, TC, MΦ	Gelatinase	Secretion from neutrophils and HSC increased by G-CSF [324,361]; mediates HSC mobilization through proteolysis of CXCL12 and HSC retention and quiescence factors (VCAM, ECM-components, c-kit) [341,361]; associated to aGVHD incidence and severity [376]
MMP-12	MΦ	Elastase	Host-derived MMP-12 suggested to limit development of post-transplant idiopathic pneumonia syndrome (murine transplant model) [377]
MMP-13	EPC, MSC, MΦ	Collagenase	Mediates HSC mobilization through ECM proteolysis [361]
Metalloprotease inhibitors			
TIMP-1	MΦ, EC, MSC	Cell growth regulator	Involved in HSC migration in mobilization and homing through interaction with chemokines, cytokines and MMPs [378]; reduced post-transplant levels of pro-fibrotic TIMP-1 in primary myelofibrosis [379]; increased levels in patients diagnosed with aGHVD [380]
TIMP-2	EC, MSC	Cell growth regulator	Involved in HSC migration in mobilization and homing through interaction with chemokines, cytokines and MMPs [378]
TIMP-3	EC, MSC	Apoptosis inductor	Expression decreased by G-CSF [381]; thought to regulate HSC proliferation and trafficking [381]; TGFβ-induced TIMP-3overexpressed in intestinal and cutaneous aGVHD, possibly contributing to apoptosis [373]
TIMP-4	P, MSC	P aggregation regulator	

*In addition to the listed functions, all MMPs share shedddase activity and all TIMPs inhibit MMPs. MMPs have both pro- and anti-angiogenetic functions whereas TIMPs are mainly inhibitors of angiogenesis.[369] MMP substrate specificity: collagenases: collagen type I-III, stromelysins: laminin, gelatinases: type IV collagen, elastase: low substrate specificity.[367] EC: endothelial cells. ECM: extracellular matrix. EPC: epithelial cells. ICAM: intercellular adhesion molecule. MΦ: macrophage. MMP: matrix metalloprotease. MSC: mesenchymal stroma cell. MT-MMP: membrane-type matrix metalloprotease. P: platelets. PMN: polymorhponuclear neutrophil. SOS: sinusoidal obstruction syndrome. TC: T cell. TIMP: tissue inhibitor of metalloproteases. VCAM: vascular cell adhesion molecule. VLA: very late antigen.

In addition to G-CSF, granulocyte-macrophage-colony-stimulating factor (GM-CSF), stem cell factor (ancestim), IL-8/CXCL8 and the CXCR4 antagonist plerixafor have been used for HSC mobilization as monotherapies or in combination with G-CSF and led to different immunoregulatory profiles for the grafts.[169,332,382]Grafts mobilized with GM-CSF contain lower and plerixafor-mobilized grafts contain higher concentrations of T and B cells compared to G-CSF-mobilized grafts.[169,382]

Two small studies recorded higher concentrations but similar proportions of different T cell subsets in plerixafor-mobilized compared to G-CSF–mobilized grafts.[169,382] Plerixafor has been observed to preferentially mobilize plasmacytoid dendritic cells and $CD56^{++}$ NK cells, which may reduce the incidence of GVHD.[172,383] G-CSF treatment also affects levels of other mediators (Tables 11 and 12), but data are relatively limited and heterogeneous. One study, which compares a limited number of interleukins and immunoregulatory cytokines in BM and PBSC grafts, shows generally heterogeneous mediator levels in both types of grafts, with higher levels of IFN-γ and IL-10 but lower levels of TGF-β1in PBSC than in BM.[384]

The CXCL12-CXCR4 axis; cytokines like GM-CSF, HGF, IL-3, IL-6, SCF and Flt3-ligand: adhesion molecules and proteases also participate in the post-transplant homing of the HSC to BM.[385] Mediators in the stem cell graft supernatant including VCAM-1 and platelet-derived micro particles (PMP) are thought to increase HSC chemotactic responsiveness towards a CXCL12 gradient, contributing to the rapid engraftment of PBSC.[385] The reconstitutive capacity of PBSC may be further increased by combining G-CSF mobilization with blockade of the adhesion molecule CD44 or CD49.[386] Combined mobilization treatment with CXCR4- and CXCR2-antagonists gives swift MMP-9–dependent mobilization of highly engraftable HSC, potentially reducing time spent on donation and adverse effects.[387] CXCL12 is thought to facilitate reconstitution through induction of adhesion molecules, retaining HSC in the BM niche and thereby allowing differentiation and expansion of CD8α DC and subsequently $CD4^{+}$ cells.[388] GM-CSF and Flt3 ligand contribute to progenitor and DC expansion, and IL-15 and especially IL-7 are important for the reconstitution of T cells and IL-2 for NK-cells.[388,389] The same mediators may also contribute to aGVHD, and the dynamics over time in absolute and relative mediator levels seem to be crucial predictors of favorable or pathological post-transplant courses.[347,388,389,380]

The traditional dichotomous view of cytokines as pro– or anti-inflammatory, with differentiation of CD4+ T cells into Th1 or Th2 subsets, has been nuanced by the discovery of several new transcription factors and differentiation pathways, which are summarized in Table 13. Various factors including (i) the timing and sequence of cytokine actions; (ii) the nature and the origin of target cells; (iii) the characteristics

of the activating signals and signaling pathways; and (iv) genetic heterogeneity(single nucleotide polymorphisms, SNPs, in cytokine and cytokine receptor genes) decide the effect and specific role of a cytokine in a given clinical setting, allowing a high degree of plasticity and complexity.[340]

Table 13. Differentiation scheme of naïve CD4$^+$ T cells[390]

CD4$^+$ subset	Master cytokines	Master transcription factors	Cytokine release induced after differentiation
Th1	IL-12, IFN-γ	T-bet (STAT4)	IL-2, IFN-γ, TNF-α
Th2	IL-4, IL-33	GATA3 (STAT6)	IL-4, IL-5, IL-9, IL-10, IL-13
Th9	IL-4, TGF-β	PU-1 (IRF4)	IL-9, IL-10, IL-21
Th17	TGF-β, IL-6, IL-23	RORγt (STAT3)	IL-17A, IL-17F, IL-22, IL-6
Th22	IL-6, TNF-α	AHR	TNF-α, IL-22
T_{reg}	TGF-β	Foxp3	IL-10, IL-35, TGF-β
Tfh	IL-6, IL-21	Bcl-6	IL-4, IL-21

AHR: Aryl hydrocarbon receptor Bcl-6: B-cell lymphoma 6 protein. IRF: Interferon regulatory factor. T-bet: T-box expressed in T cells.

Variation in allotransplant setting further increases complexity, as the effects of a given cytokine differs depending on the origin of donor or host cytokine-producing cells/target cells.[340] Given this variability, the traditional view of G-CSF–induced HSC mobilization as an anti-inflammatory process (see Table 3), and the classification of meditators in aGHVD into tertiary signals for T cell activation and inflammatory mediators [253] (see p. 21 and Table 11) may need modification. First, G-CSF mobilization in healthy donors is associated with increased expression of genes encoding pro-inflammatory mediators [391,392]. Second, the cytokine storm induced by conditioning chemo- and irradiation therapy may be modified by several factors:

- diverse and individual PAMPs and DAMPs of the gastrointestinal microbiota,[340]
- differences in immunosuppressive therapy,[340] and
- diverse conditioning regimens, RIC showing unique cytokine patterns and distinctive associations to aGVHD compared with myeloablative regimens.[340,393]

Third, the examples of contradictions and context-dependent adaptions of single mediator functions are numerous:

- The role of IFNɣ in GVHD is complex. IFNɣ is released during conditioning and has traditionally a proinflammatory function and a central role in controlling the expansion of alloreactive T cells.[253] However, high IFNɣ levels lead to

apoptosis of donor cells and reduced incidence of GVHD, and IFNγ-knockout $CD8^+$ donor T cells induce more severe GVHD.[394,395]

- The role of IL-10 in aGVHD pathogenesis is also controversial. Preparative IL-10 treatment is associated with low incidence of aGVHD,[396] and low IL-10 levels are observed in patients with early donor chimerism and aGVHD.[397] In contrast, in an animal model, IL-10 caused exacerbated aGVHD due to induction of T cell expansion.[398]
- The presumed pro-inflammatory cytokines IL-12 and 1L-18 may also have unexpected protective effects against GVHD due to apoptosis of donor T cells.[399-401]
- IL-2 is thought to promote or protect against aGVHD depending on dose, IL-22 depending on cell of origin (Th17 or ILC) and IL-6 depending on mode of signaling (i.e. trans-signaling through soluble IL-6R complex versus classical signaling through membrane-bound IL-6R).[340]

Finally, G-CSF–induced skewing of T cell differentiation from Th1 to Th2 dominance possibly represents greater immuno-regulatory complexity than a purely immunosuppressive effect. Based on animal studies, Th1 seems to preferentially induce aGVHD in the gastrointestinal tract whereas Th2 and Th17 seem to be encourage GVHD development in lungs, liver and skin.[402-404] This organ-dependent infiltration of different lymphocyte subsets is regulated by interactions between chemokines and their receptors (Table 11).

Beyond the knowledge of expressed mediators in allogeneic HSCT, numerous recipient and donor genetic polymorphisms of adhesion molecules, cytokines, chemokines and their receptors affect both HSC mobilization and post-transplant outcome. Among others, CXCL12, CXCR4, VCAM1 and CD44 polymorphisms are associated with the HSC mobilization response after G-CSF dministration [162] and recipient/donor SNPs of TNFα, IL-6, IL-10, IL-6R, IL-7Rα, IL-23R, CCL5 and CCR9 with aGVHD or/and cGVHD (reviewed in [405]).

To summarize, a wide range of interacting immunoregulatory cytokines, interleukins, adhesion molecules and matrix metalloproteinases as well as proteinase inhibitors regulate both HSC mobilization and post-transplant outcome. Due to a high

degree of crosstalk and context dependency, investigation of mediator profiles is preferable to analyses of single mediator levels.

1.4 Osteopontin in immunoregulation

Osteopontin (OPN) is an extensively glycosylated and phosphorylated non-collagenous protein in the small integrin binding ligand N-linked glycoprotein ("SIBLING") family.[406] Alternative splicing, post-translational modifications and several cleaving sites for thrombin and matrix metalloproteases, including MMP-3 and MMP-7, provide structural and functional diversity to the molecule. OPN is involved in a wide range of homeostatic mechanisms as a structural molecule of mineralized tissues, an extracellular soluble factor and an intracellular molecule.[407]

Soluble OPN (sOPN) is secreted from osteoblasts, osteocytes and osteoclasts along with a wide spectrum of immune cells, endothelial cells, smooth muscle and epithelial cells.[408] sOPN is ligand for several integrins including $\alpha_v\beta_{1,3,5,6}$, $\alpha_4\beta_7$ and $\alpha_{4,5,8,9}\beta_1$ [409,410] and for the CD44 isoforms CD44v6 and CD44v7.[411] Intracellular OPN (iOPN) is a shorter isoform originating from alternative translation and binds to the CD44-ezrin/radixin/moesin complex.[412] OPN has also been identified in the cell nucleus.[413] Intracellular and intranuclear OPN participate in cell duplication, cytoskeletal rearrangement and cell migration and contribute to innate immune receptor signal transduction.[413,414]

OPN is upregulated and maintains and rearranges cells and tissues in response to mechanical, oxidative and physical stress as well as through various physiological and pathological inflammatory processes including bone remodeling and wound healing, normal hematopoiesis and angiogenesis, immune responses during infection and development of atherosclerosis.[410,415] Its involvement in cell signaling, proliferation and motility, regulation of apoptosis and survival makes OPN important for normal immune regulation as well as in autoimmune diseases and carcinogenesis.[416]

OPN contributes to the migration of HSC towards the endosteal surface of the stem cell niche and is a quiescence factor, limiting the proliferation and differentiation of HSC and the size of the stem cell pool.[417,418] HSCs can adhere to OPN, and after

peripheral stem cell infusion, OPN is important for the homing of HSC to BM.[418] G-CSF treatment reduces the concentration of OPN in the stem cell niche, which may contribute to stem cell mobilization.[419] Stroma-derived OPN attenuates the HSC phenotypes associated with aging.[420]

Interactions between OPN and CD44 receptors are important for migration of macrophages, neutrophils, dendritic cells, T cells and NK cells.[421-424] T cell migration is dependent on OPN concentration; in an *in vitro* chemotaxis model, human T cell movement was determined by the OPN concentration gradient, and T cell activation was required for adhesion of T cells to surfaces coated with OPN.[425] It is likely that OPN-CD44 interactions contribute to G-CSF–induced mobilization of immune cells (see above). OPN is a central regulator of innate and adaptive immunity, and it is involved in the regulation of T cell differentiation as well as T cell proliferation.[426,427]

OPN secretion by activated T cells dependens on the master Th1 transcription factor T-bet (Table 13) and is an essential early step in type 1 immune responses by (i) potentiating the IL-12 response and inhibiting the IL-10 response in macrophages and plasmacytoid dendritic cells (pDC) and (ii) skewing T helper cells and cytotoxic T cells towards the Th1 and Tc1 phenotypes, respectively.[426,428] In conventional dendritic cells (cDCs), iOPN expression is thought to promote Th17 T cell differentiation through suppressed IL-27 production,[429,430] and in autoimmune disease models, differential regulation of DC IL-27 and OPN expression has been shown to suppress inflammation.[431] Inhibition of IL-10 response is mediated via OPN-CD44 ligation, and CD44-deficiency has been reported to enhance Th2 differentiation.[432]

In B cells, OPN is secreted after IL-4–induced activation of the alternate B cell receptor pathway in combination with the classical pathway.[433] iOPN support for differentiation of T follicular helper (Tfh) cells and T follicular regulatory (Tfr) cells is crucial for normal B cell development in germinal centers in response to antigenic challenge.[434] OPN enhances polyclonal B cell activation and increases immunoglobulin production,[435] and overexpression of OPN in B cells is associated with B-cellmediated autoimmunity.[436]

OPN is important for NK cell differentiation from hematopoietic stem cells and also for homeostasis and functional response in normal NK cells.[437] NK cells can be

directly activated by OPN to contribute to inflammatory responses, and iOPN contributes to the development of long-lived memory-like NK cells.[424]

Binding of sOPN to CD44 protects lymphocytes from activation-induced cell death, thereby prolonging inflammation, a mechanism that ensures that the normal immune response is completed.[415] However, the anti-apoptotic effects of OPN can also extend the life span of pathogenic immune cells and cancer cells, thereby aggravating autoimmune disease and cancer.[415] The majority of OPN studies describe pro-inflammatory characteristics for the molecule; but anti-inflammatory effects have also been reported in colitis, sepsis, wound healing and autoimmune diseases.[410] Researchers have tried to explain this dichotomous presentation of OPN though time-dependent functions of the mediator in early and late phases of immune responses, and concentration-dependent and isoform-dependent effects represent other potential mechanisms through which OPN influences health and disease.[438,439]

The complex and context-dependent role of OPN in inflammation and cell survival is also emphasized by the apparently contradictory results from two studies of murine aGVHD models. In the first model, OPN blockade attenuated GVHD through reduced migration of immune cells to target organs and reduced activity and viability of $CD8^+$ T cells.[440] In the second model, OPN knockout mice were more susceptible to GVHD, and the disease was characterized by more severe gastrointestinal inflammation and increased epithelial apoptosis.[441] OPN is also a biomarker for cGVHD.[374]

To summarize, OPN is important in the regulation of numerous fundamental cellular processes. It can be released by a wide range of cells, including many immunocompetent cells, and its role in immunoregulation depends on the biological context. Evidently, OPN can exert a wide spectrum of effects in response to the overall challenges and available resources of an organism, and by that, on the total immunoregulatory profile. G-CSF–induced reduction of OPN levels in the stem cell niche contributes to HSC and possibly to immune cell mobilization. The diverse effects of OPN may also explain why its impacts seems to differ in various experimental models of allogeneic stem cell transplantations and GVHD.

1.5 Metabolomics and immunoregulation are closely connected

Metabolomics can be defined as the systematic identification, quantification and analysis of the highest possible number of low-molecular weight metabolic products and intermediates of a biological system (the metabolome).[442] The metabolomic profile represents a "functional readout" of an organism and reflects the dynamic response to physiological, pathophysiological and developmental stimuli and to genetic and environmental modulation.[443] Both the levels of single metabolites and the overall metabolomic profile are important for immunoregulation.[444] The interaction between metabolic pathways and the immune system is regulated by genetic and nutritional factors and by the intestinal genome.[445] Metabolism provides substrates for Adenosine triphosphate (ATP) synthesis and building blocks for synthesis of macromolecules and fuels development, differentiation, proliferation and effector functions of all cells and tissues, including the immune system.[446] By alteration of histone– and DNA-modifying enzyme activity and by supplying substrates, metabolism can directly modify epigenetic signatures of immune cells.[447]

The interface between metabolism and the immune system seems to represent a dynamic equilibrium optimizing the cellular response to a wide range of external stimuli and bioenergetic demands.[444] Several metabolic switches control the intensity and duration of innate and adaptive immune activation.[446] The most important metabolic checkpoint kinases, mechanistic target of rapamycin complex 1 and 2 (mTORC1 and mTORC2) and 5´-AMP-activated kinase (AMPK), sense and integrate extrinsic and intrinsic signals including (i) immunocompetent cell activation, (ii) growth factors and immunoregulatory factors, (iii) nutritional status, (iv) positive and negative feedback from downstream effector pathways.[444,448,449] This signal integration leads to metabolic reprogramming of cells to adapt to the actual immunological challenges and bioenergetic potential.[444,446,448,449] In metabolic reprogramming of T cells, induction of the transcription factors MYC and hypoxia inducible factor 1 α (HIF-1α) is especially important.[450,451]

The metabolic pathways most commonly used by T cells have different intracellular locations (Figure 3) and depend on cellular activation and differentiation status, and

may also vary between T cell subsets. Studies have shown that naïve T cells primarily use oxidative phosphorylation for energy generation, cellular housekeeping functions and survival.[452] T cell activation is however characterized by metabolic reprogramming to aerobic glycolysis (Warburg metabolism) and induction of the pentose phosphate pathway. This leads to less bioenergetic efficiency, but higher abundance of metabolic intermediates that are needed for anabolic processes important for cell growth and proliferation and for maintenance of the cellular redox balance.[453,454] Enhanced glycolysis can also supply the tricarboxylic acid (TCA) cycle with lactate, recently demonstrated as an important fuel for mitochondrial metabolism.[455] Aerobic glycolysis seems to be required for normal effector cell cytokine mRNA translation.[456] Lactate produced during glycolysis controls T cell motility and migration, skews pro-inflammatory cytokine generation towards IL-17 production and inhibits $CD8^+$ cytolytic function.[457] T cell activation is enhanced when glucose uptake is increased and suppressed in malnutrition or when glucose metabolism is inhibited.[458] Activated T cells also upregulate glutamine consumption through the TCA.[450]

$CD4^+$ T cell differentiation is critically dependent on metabolism. Glycolysis is the preferred metabolic pathway of Th1, Th2 and Th17 cells, whereas T_{regs} rely on mitochondrial fatty acid oxidation.[452] Glycolysis controls the induction and suppressive function of iT_{reg} cells.[459] In contrast to T_{regs}, Th17 cells do not take up exogenous fatty acids for synthesis of cellular membranes, but rather use de novo acetyl-CoA carboxylase-mediated phospholipid synthesis from glucose.[460] Interestingly, decreased availability of alpha-ketoglutarate due to glutamine-deprivation has been suggested to shift the balance between Th1 and T_{reg} generation towards a T_{reg} phenotype.[461]

In vitro studies of T cells suggest that human $CD8^+$ cells are less glycolytic than $CD4^+$ cells both during quiescence and upon stimulation, and more reliant on oxidative phosphorylation for cytokine production.[462] Following the primary immune response, metabolic reprogramming from glycolysis to fatty acid oxidation is

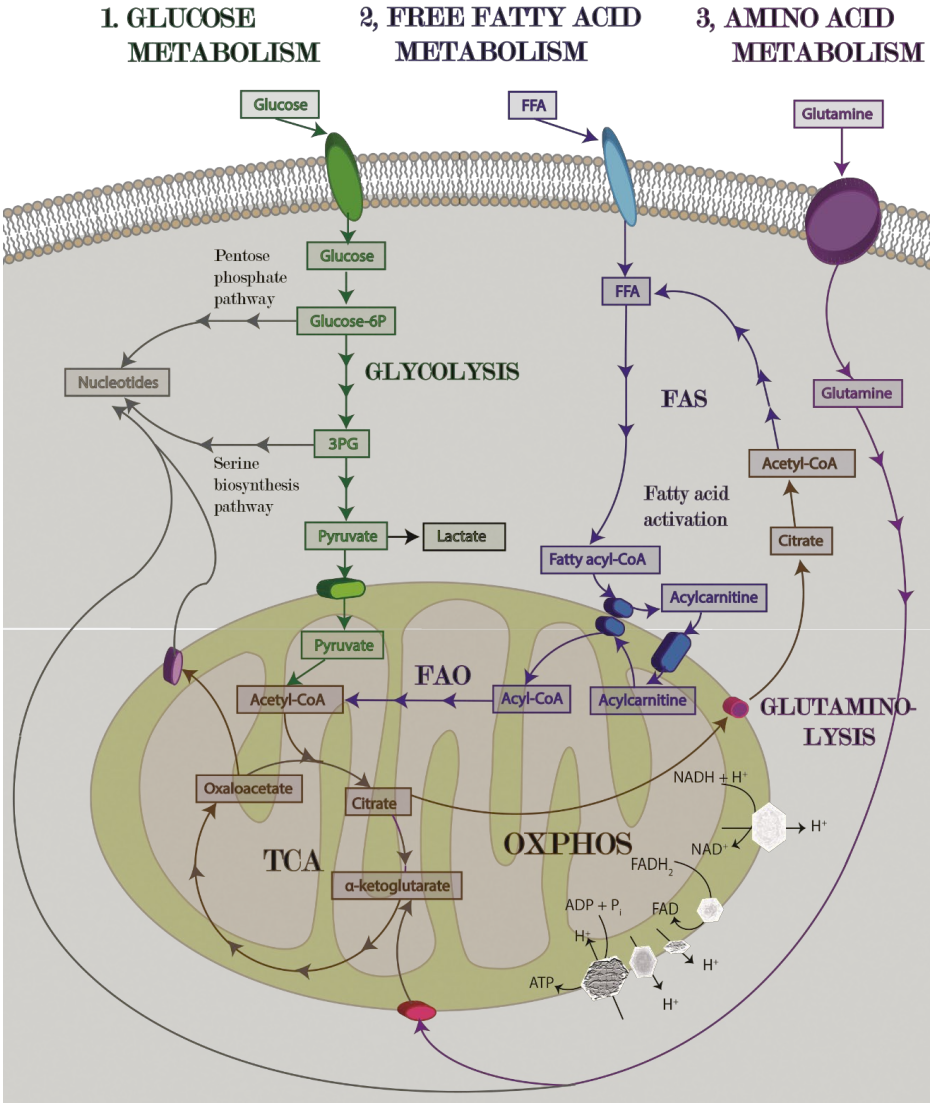

Figure 3. Overview of important T cell metabolic pathways.[463] **1. GLUCOSE METABOLISM.** After entering the cell by facilitated diffusion, glucose may, dependent on the needs of the organism, undergo **glycolysis** to pyruvate or be used for nucleotide synthesis by oxidation in the **pentose phosphate pathway or** by shuttling of the intermediate 3-phosphoglycerate (3PG) into the **serine biosynthesis pathway**. Pyruvate is decarboxylated to acetyl-coenzyme A (acetyl-CoA) that enters the tricarboxylic acid circle (**TCA**), or is alternatively transformed to lactate under anaerobic conditions. **2. FREE FATTY ACID (FFA) METABOLISM.** FFA enter the cell through specific transport proteins or is synthesized in cytosol (fatty acid synthesis–**FAS**). After activation in cytoplasma, the fatty acid intermediates are transported across the mitochondrial membrane for **β-oxidation/fatty acid oxidation (FAO)** to acetyl-CoA that enters the **TCA**. **3. AMINO ACID METABOLISM.** Excess amino acids from the diet are taken up by the cell by membrane-bound transporter proteins that are upregulated in activated T cells. Glutamine is important for activation and differentiation of T cells. Through **glutaminolysis**, glutamine is hydrolyzed to glutamate, that is either used for protein synthesis, entering the **TCA** after metabolization to α-ketoglutaratoxaloacetate or used for nucleotide synthesis. **TCA** and oxidative phosphorylation **(OXPHOS)** take place in the mitochondrion and involve release of the energy from sugar, fatty acid and amino acid derivatives through oxidation of acetyl-CoA to oxaloacetate before high-energy electrons from the TCA are passed through the mitochondrial electron transport chain with oxygen as the final electron acceptor, creating a proton gradient for synthesis of adenosine triphosphate (ATP).

essential for $CD8^+$ memory T cell development and survival,[464] and compared to naïve cells, the mitochondria in memory T cells are larger with increased respiratory capacity.[465]

Crosstalk between metabolism and the immune system during the humoral immune response has not been characterized in detail. However, transition from the naïve quiescent state to B cell proliferation depends upon metabolic reprogramming, and B cell activation leads to increased uptake of glucose that is mainly used for nucleotide biosynthesis.[466]in addition, efficient glycolysis is necessary for antibody production, and mitochondrial remodeling during B cell activation lead to augmented oxidative phosphorylation.[466-468]

Recent studies have shown that OPN may also function as a metabolic regulator, at least for certain types of cells,[414,469] but it is not known whether modulation of immunocompetent cell metabolism is important for the immunoregulatory effects of OPN. Furthermore, several metabolites including RBC and platelet-derived sphingosine-1-phosphate, ceramide-1-phosphate, adenosine triphosphate, uridine triphosphate and uridine diphosphate-glucose are thought to participate in HSC mobilization and homing.[332]

The possible importance of T cell metabolism for allogeneic HSCT

The results are conflicting regarding T cell post-transplant metabolic adaptions. Some animal models suggest upregulated glycolysis in donor-derived $CD4^+$ T cells, especially early post-transplant,[470] whereas other studies report that chronic stimulation of $CD4^+$ cells is reliant on oxidative phosphorylation and shows failure to engage glycolysis for effector function.[471,472] Allotransplanted murine T cells show hyperpolarized mitochondrial membrane potential and increased oxidative stress,[473] upregulation of the pentose phosphate pathway,[470,472] increased glutamine uptake and glutaminolysis [470] and accumulation of fatty acids.[470] Upregulation of fatty acid oxidation has been demonstrated for T cells during GVHD, but not in T effector cells activated under other conditions,[474] while another study reported T cell upregulation of both glycolysis and oxidative phosphorylation in GVHD.[473] Intestinal microbial

metabolites have also been shown to be important for T cell homeostasis and influence GVHD pathogenesis and severity.[475]

Targeting of metabolic pathways is an emerging therapeutic option for cancer and autoimmune disease [476,477] and may potentially be used in the assessment of graft function and prevention of graft rejection after solid organ transplantation.[478,479] Malignant transformation of tumor cells and clonal expansion of T effector cells share similarities in metabolic reprogramming towards Warburg metabolism.[454,480] As inhibition of glycolytic activity in $CD8^+$ T cells is thought to increase their antitumor efficacy,[481] glycolysis inhibitors may attenuate GVHD-inducing T cells without inhibition of graft versus leukemia effect.[482] In murine GVHD models, several potential metabolic checkpoints and pathways have been targeted, including glycolysis via mTORC1 or 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3,[470] fatty acid oxidation,[474] fatty acid synthesis through acetyl-CoA carboxylase-1,[483] mitochondrial F_1F_0-ATPase,[473] sphingolipid biosynthesis via ceramide synthase-6 [484] and replenishment of intestinal microbiome-derived butyrate.[475]

In a small study of healthy allogeneic stem cell donors, G-CSF was suggested as a factor in impairing lymphocyte mitochondrial function leading to increased generation of ROS, increased activation-induced apoptosis and cell cycle arrest.[485] Recipient pre-transplant metabolic distress has been suggested as a predictor of relapse in another recent study.[486] More detailed metabolic characteristics of human T cells during HSC mobilization, collection and allotransplantation remain to be elucidated in clinical trials. Targeting of T cell metabolic pathways may have a therapeutic potential when used post transplant to prevent or treat GVHD and may potentially be used pre-transplant to optimize donor and graft T cell metabolism.

2. Scientific question and aims

A major challenge in studying allogeneic HSCT is capturing the complexity of interactions between donor-derived and recipient immunity. While extensive recipient variability with respect to disease, comorbidity and treatment protocols is a well-known confounding factor, less is known about the importance of donor heterogeneity. Our main scientific question was the following:

- How is the balance between different allogeneic donor immune cell subsets and immunoregulatory soluble mediators altered during hematopoietic stem cell mobilization and collection, and how is this reflected in the graft?

In this study, we aimed to map central donor immunological and metabolomic parameters including selected lymphocyte subsets, cytokines, soluble adhesion molecules, matrix metalloproteases and metabolites. Cell and mediator profiles were analyzed prior to, during, and after stem cell mobilization and apheresis and in the stem cell graft in order to

- characterize the immunomodulatory and metabolomic effects of G-CSF treatment and leukapheresis on the donor and on the stem cell graft and thereby describe donor and graft heterogeneity;
- explore potential associations between immunological donor profiles and mobilization response; and
- examine possible associations between donor and graft immunological profiles and clinical outcome.

3. Summary of papers

3.1 Paper I:

Peripheral Blood Stem Cell Mobilization in Healthy Donors by Granulocyte Colony-Stimulating Factor Causes Preferential Mobilization of Lymphocyte Subsets.

The literature is contradictory regarding the immunomodulatory effects of G-CSF during hematopoietic stem cell mobilization and the importance of graft composition for clinical outcome after allogeneic HSCT.

A detailed multicolour flow cytometry-based characterization of 27 donor T, B and NK cell subsets showed skewed phenotype proportions during G-CSF treatment with preferential mobilization of naïve T and T regulatory subsets, mature and memory B cells and reduced fractions of NK and γδ T cells. Apheresis further skewed the distributions, with notable enrichment of the B cell fraction in the allogeneic stem cell graft. Bioinformatics analyses revealed extensive donor variability in the G-CSF immune cell mobilizing response and suggested slower platelet engraftment and increased risk of aGVHD in recipients of grafts from donors with strong G-CSF responses.

Our results confirm allogeneic hematopoietic stem cell mobilization and collection as a heterogeneous process with regard to effects on various immunocompetent cell populations in healthy donors as well as in the stem cell graft. Both G-CSF treatment and apheresis contribute to extensive redistribution of the lymphoid compartment, representing an immunomodulatory potential of the graft. Healthy donor G-CSF immune cell mobilization response seems to have a stronger impact on clinical outcome than infused cell doses, but this hypothesis should be tested in larger studies sufficiently powered to enable meaningful sub-group analyses based on diagnosis, conditioning, immunosuppression and donor type.

3.2 Paper II:

The healthy donor profile of immunoregulatory soluble mediators is altered by stem cell mobilization and apheresis.

Soluble plasma mediators including immunoregulatory cytokines, chemokines, growth factors, adhesion molecules, proteases and protease inhibitors are regulators of innate and adaptive immunity and are important for hematopoietic stem cell mobilization and post-transplant immune reconstitution.

Donor and recipient plasma levels of 38 mediators were monitored by Luminex quantification, and bioinformatics analyses used to identify individual profiles. Healthy donors generally displayed substantial heterogeneity in plasma mediator levels. G-CSF treatment and apheresis led to altered donor mediator profiles, mainly through increased levels of most mediators, and the donor profile during G-CSF administration was associated with the stem cell yield. Grafts harvested with Cobe Spectra showed significantly higher content of neutrophils, lymphocytes, platelets and red blood cells and significantly different mediator profiles compared to Spectra Optia. Recipient pre-transplant mediator profiles showed associations to the Hematopoietic Cell Transplant (HCT) Comorbidity Index. Graft infusion led to relatively small changes in single mediator concentrations, but the overall mediator profiles were altered, allowing separation of the patients into two subsets with different prognoses independent of comorbidity.

Individual donor heterogeneity in plasma mediator levels before mobilization seemed to have more impact on the subsequent donor and graft mediator profiles at the time of harvest than G-CSF and apheresis induced changes during stem cell mobilization and collection. G-CSF–induced systemic donor profiles may predict stem cell yield. Graft mediator profiles seemed to be apheresis device dependent and associated with leukocyte and platelet levels in the graft. Recipient mediator profiles changed during HSCT, and the possible predictive impact of early post-transplant mediator profiles for clinical outcome should be further investigated in future studies.

3.3 Paper III:

Immunomodulation Induced by Stem Cell Mobilization and Harvesting in Healthy Donors: Increased Systemic Osteopontin Levels After Treatment With Granulocyte Colony-Stimulating Factor.

The phosphoprotein osteopontin has complex functions that are dependent on concentration, time and context, including hematopoietic stem cell mobilization and differentiation. Osteopontin also regulates the migration of several leukocyte subsets and the differentiation of T cells through interaction with CD44; this may influence graft composition and immunomodulation.

Healthy donor systemic osteopontin plasma concentration was quantified during hematopoietic stem cell mobilization and collection. The effect of G-CSF treatment was rather weak, with a moderate total increase of osteopontin concentration, and an inverse correlation between systemic osteopontin levels and neutrophil concentrations. CD44 expression was relatively stable during G-CSF administration and was generally stronger in T cells compared to B cells ,with particularly high CD44 expression in T memory and Tr1 cells. Allogeneic stem cell recipients showed high levels of osteopontin compared to healthy stem cell donors, platelet donors and myeloma patients, and these levels were stable during the first week post transplant.

Stem cell mobilization with G-CSF influenced healthy donor systemic osteopontin levels. Whether the sustained rise in osteopontin levels during apheresis can be attributed to combined effects of G-CSF and apheresis is currently not clear. Altered levels of osteopontin during stem cell mobilization and collection have potential immunomodulatory effects on the graft cells and the recipient and/or may be a biomarker of altered immunoregulation.

3.4 Paper IV:

Granulocyte Colony-Stimulating Factor Alters the Systemic Metabolomic Profile in Healthy Donors.

Metabolic pathways interact with innate and adaptive immune activation and T cell differentiation, migration and memory. The healthy donor metabolomic profile prior to and during G-CSF treatment may be reflected in the stem cell graft and possibly influence post-transplant donor-derived immunity.

We applied an untargeted metabolomics approach for identification of the global serum metabolomic profile of 15 healthy stem cell donors. G-CSF treatment altered the concentration of 239 out of 641 metabolites, as documented using ultrahigh performance liquid chromatography–tandem mass spectrometry (UPLC-MC) and gas chromatography/mass spectrometry (GC-MS). Despite heterogeneous individual distribution of metabolites with partially overlapping groups in principle component analysis prior to and after G-SCF treatment, G-CSF–treated and pre-treatment samples could be distinguished with 97% predictive accuracy by random forest classification based on the overall metabolomic profile. The levels of numerous long-chain fatty acids and carnitine-conjugated lipids were significantly increased during G-CSF administration, whereas high proportions of branched and aromatic amino acids displayed reduced concentrations. Metabolomic pathway enrichment analysis revealed glycogen and pyrimidine metabolism as the most important pathways augmented during G-CSF treatment.

Our study is the first to explore the short-term systemic metabolomic effects of G-CSF and suggest significantly altered healthy donor global metabolomics profiles during HSC mobilization, but this finding should be confirmed by additional studies that include larger sets of donors and long-term analyses. Several of the G-CSF modified metabolites have established immunoregulatory and angioregulatory effects and may influence epigenetic regulation, and the potential impacts on donor management, graft characteristics and recipient outcome need to be clarified.

4. Methodological considerations

The following discussion is limited to aspects of the underlying methodological not already covered in the thesis and of particular importance for the reliability and interpretation of the results of the present project.

4.1 Considerations in choice of study design and analytes

As emphasized through the previous chapters, during the last decade, numerous reports of the effects of single immune cell subsets and soluble mediators have been published, often with contradictory conclusions. To avoid oversimplification of the complex biological context generated by the fusion of the immune systems from two different individuals, our priority was to focus on broad panels of biomarkers rather than single analytes. In retrospect, based on recent research, the selection of biomarkers for analysis could have been improved. In particular, we acknowledge the importance of IL-7, IL-12, IL-15, CCL3 and CCL8 for the process of allogeneic HSCT (Table 11). However, the capture of all relevant variables is not achievable, and the advantage of knowledge and experience using reagents and techniques that are well established in our laboratory should not be underestimated.

Limitations related to the sample size are discussed in Chapter 5, p. 65.

4.2 Preanalytical aspects: variability of sample handling

Sample collection and anticoagulation

Despite its ready availability as a specimen that reflects systemic immunological and metabolic status, sampled peripheral blood represents a “snapshot” of the overall biological status of the organism at the time of sampling, with limited resolution of the contributions from different organs and processes. Moreover, the blood sampling and preparation process introduces risk of bias generated by variability in blood drawing technique, choice of anticoagulant and processing time and methods. Pre-analytical variability has been estimated to account for more than 90% of errors occurring throughout laboratory test-based diagnostic processes.[487]

In order to minimize pre-analytical errors in the current project, we standardized the handling of samples. Samples were drawn at the same time of the day to avoid diurnal variations and processed immediately to prevent overall or selective loss or alterations of cellular and soluble analytes. Anticoagulation agents with ACD, ethylene diamine tetraacetic acid (EDTA) and heparin are reported to maintain similar PBMC viability [488] but may result in skewed reported distributions of different immune cell subsets.[489] We chose ACD due to presumed superior preservation of T cell functionality compared to EDTA.[488,490] We rejected heparin due to its platelet activating effect [491] that would have confounded the known effects of G-CSF and apheresis in platelet activation (see Chapter 1.1), with potential implications for mediator analyses with increased levels of platelet-derived cytokines. Likewise, we preferred plasma to serum for mediator analyses to avoid the effects of *ex vivo* sample preparation on the levels of platelet-derived cytokines and potentially reduced levels of other mediators due to clotting-induced degradation.[492,493] The impact of different anticoagulants differs between mediators, but a recent study from our group indicated ACD is preferable to EDTA and represents the best compromise in assessment of a broad panel of mediators including matrix metalloproteinases, tissue inhibitors of metalloproteinases and adhesion molecules.[493]

Finally, the reproducibility of metabolite analyses has been shown to be good in both serum and plasma analyses.[494,495] Compared to plasma, serum has higher metabolite concentrations, which increases sensitivity [494] and was therefore preferable for the current project. However, higher metabolite concentrations in serum may be partly caused by blood cell metabolic activity during the coagulation process,[495] which represents a potential source of error in Paper IV.

Sample processing, cryopreservation and storage

Gradient separation of whole blood with Ficoll-Hypaque to isolate the mononuclear white blood cell fraction potentially leads to loss and skewed distributions of immune cell populations. The efficiency of the method is influenced by several factors including cell size and concentration.[496,497] Consequently, the yield of various immune cell subsets, and thus the results in Paper I, may have been influenced both

by individual variations in peripheral blood immune cell concentrations and by the general increase of virtually all immune cell subsets during G-CSF treatment (Figure 1, Paper I). Increased cell concentration generally improves the separation and yield of different subsets,[497] and identical dilution of whole blood samples with saline prior to and after G-CSF treatment may lead to overestimation of G-CSF–induced concentration increases. Furthermore, separation of peripheral blood using the apheresis technique leads to a different distribution in the stem cell graft that is not directly comparable to Ficoll-Hypaque separated samples from peripheral blood. This is an important caveat to the reported correlations between peripheral blood and graft immune cell concentrations (Table 3, Paper I).

Plasma samples were diluted prior to Luminex mediator analyses. Due to a high degree of heterogeneity between donors in mediator levels, it was challenging to find the optimal sample dilution to keep the results within the standard range of the assay. As values below or above the standard range were set to a fixed value, the variation of mediator levels between donors was even greater than reported in Paper II.

Cryopreservation of PBMC samples enables efficient flow analyses, improving intra- and inter-individual comparisons of samples from different time points by minimizing the day-to-day variation of reagents and instrument performance.[498] The cost of this standardization is cryopreservation-induced alterations of immune cell phenotypes.[499] T cell populations are especially vulnerable to cryopreservation, in particular, central memory cells and certain subpopulations of the Th17 and T_{reg} subsets.[499] Furthermore, cord blood hematopoietic cells show altered expression of cell surface molecules (e.g. cytokine receptors, adhesion molecules) after cryopreservation, with functional consequences for migration and proliferation potential.[500,501]

The impact of cryopreservation on T cell characteristics has implications not only for the validity and interpretation of flow analyses, but also for the use of cryopreserved HSC grafts for allogeneic transplantation. Preconditioning cryopreservation of allografts have hitherto been used primarily when there was doubt about the reliability and availability of the donor. Cryopreserved allogeneic PBSC grafts show inferior *in vitro* growth potential despite normal $CD34^+$ numbers

and may increase the risk of impaired engraftment compared to fresh PBSC grafts,[502] although these results may be biased by delayed cryopreservation after transport. Furthermore, T cells and other immunocompetent components of the graft show lower recovery after cryopreservation than HPCs, and the tolerance to freezing seems to differ among different T cell subpopulations.[503-505] The potential implications for functionality and clinical outcome remain to be addressed. Although graft T cells are very important for the antileukemic effect of allotransplantation, beyond simple quantification there is no routinely performed assessment of graft T cell function.

Generally, reduced graft viability due to long-time storage is associated with increased incidence of aGVHD and TRM.[506] Cryopreservation leads to reduced viability, but the impact of cryopreservation on aGVHD and engraftment is contradictory.[507-509] Cryopreservation of human T_{regs} leads to reduced L-selectin expression, and in animal models, this results in impaired homing to secondary lymphoid organs and reduced ability of T_{regs} to protect from aGVHD.[510] However, in cryopreserved stem cell grafts, such effects may be counteracted by attenuation of adhesion molecules on progenitor cells and aGVHD-inducing cells, and the decrease in L-selectin expression may be reversible during thawing.[511] Available studies are too small to offer definitive conclusions regarding possible consequences of cryopreserved grafts on clinical outcome, and there is a need for larger prospective, randomized studies to provide clarification.

In the current project, a minority of the HSC grafts were cryopreserved prior to transplantation. The numbers were too small for meaningful statistical evaluation of the clinical effect, but an influence from graft cryopreservation on clinical outcome is hard to exclude.

Finally, even though the half-life of soluble mediators is short and mediators may be released and degraded during storage, most cytokines show stable levels for at least 2 years at -80 °C.[512] For P Papers II–IV, we endeavored to implement immediate cryopreservation of samples, maintain similar storage time within groups of compared individuals and avoid multiple freeze-thaw cycles to minimize pre-analytical errors.

4.3 Analysis aspects

Multiparameter flow cytometry

In contrast to methods that measure average cellular responses, including reverse-transcription quantitative polymerase chain reaction (RT-qPCR) and common cell proliferation and cytotoxicity assays, flow cytometry enables high-resolution single-cell analysis of surface-expressed and intracellular markers.[513] It can be used for quantification of absolute numbers and relative proportions of different immune cell subsets as well as functional assessment of characteristics such as activation and differentiation state, secretory and migratory capacity, proliferative and cytolytic potential.[513] Advancements in multiparameter/polychromatic flow cytometry include broadened availability of fluorochrome conjugates, upgraded instrumentation with enhancement of lasers, dichroic filters and photomultiplier tubes (PMT) as well as more refined software compensation and analysis modalities.[513] These developments provided unique opportunities to capture cellular heterogeneity, including detection and in-depth characterization of rare subsets, and gain insight into the complex cellular interactions characteristic of immunological processes. However, a side effect of more complex methodology is increased risk for bias and reduced capability to detect it.

Standardization of flow cytometry is limited by the vast availability of instruments, reagents and analysis software. Virtually inevitable variability in reagents, instrument settings and performance must be accounted for in the interpretation of every flow cytometry-based study, and particularly in comparison studies. Of particular importance are discrepancies in antibody characteristics based on lot, vendor, choice of conjugated fluorochrome and tandem conjugate instability, fluctuations of PMT voltage and inherent inaccuracy of signal log amplification.[514] Autofluorescence of cells, non-specific binding of antibodies and variability in mathematic software compensation of spectral overlap between fluorochromes are further complicating factors, together with subjective visualization in sequential manual gating.[498] All these factors make distinction between different subpopulations and distinction between antigen-positive and –negative cells difficult.

These technical challenges are exacerbated by biological heterogeneity in antigen expression and inconsistent definitions of immune cell phenotypes between studies, leading to a virtually infinite number of potential subsets separated by indistinct boundaries.[515] For example, to varying degrees, studies distinguish between $CD4^+$ and $CD8^+$ naïve T cells and between effector and central memory T cells (Table 10), and there are no consensus definitions for the increasing number of reported regulatory T cell subsets.[516] T cell versatility also contributes to the fluctuating boundaries between subsets; for example, T_{regs} may be converted to Th17 cells under inflammatory conditions.[516] Finally, the interplay between donor and residual recipient immune cells after allotransplantation cannot readily be defined by flow cytometry, and the importance of species-dependent phenotype, level of antigen expression and immune cell distribution may be underestimated in comparison with murine and human studies.

In the current project, the reagent panels were carefully constructed to match marker expression with the conjugated antibody fluorescence intensity in accordance with the optical setup of our flow cytometer.[517] The antibodies were precisely titrated to optimize the resolution of antigens and cellular subsets, and we used antibody cocktails to reduce pipetting errors.[517] PMT voltages were adapted to the cells of interest, and the chosen fluorochromes and laser alignment and instrument performance were monitored daily. We used bi-exponential log transformation, and acquisition of cells was performed at the slowest speed possible in order to increase sensitivity with higher resolution of weakly expressed markers and unstained populations.[518] Fluorescence minus one (FMO) controls ensured proper adjustment of the results for unspecific staining.[514] We used template gating with copy and reuse of the template set of gates to all samples with minimal manual individual adjustment in order to achieve unbiased comparison across samples.[519] However, we observed considerable variability in biomarker expression between individuals.[520] Consequently, the biological heterogeneity of healthy donor immune cells is probably even greater than what is reported in Paper I.

For the most advanced flow cytometers, which use up to five lasers, the number of parameters that can be investicated per sample is approaching that of mass cytometry.

Beyond its superiority in multidimensional approach, the advantages of mass cytometry are the lack of need for spectral compensation and the absence of autofluorescence bias. However, the sensitivity and acquisition rate tend to be lower for mass cytometry compared to flow cytometry, and flow cytometry offers greater ability to analyze live cells. These two complementary single-cell analysis methods may advantageously be combined in transplant immunology studies.[521,522]

Multiplex bead array analysis and ELISA

Methodological choices in soluble mediator quantification are influenced not only by test performance characteristics (reviewed in [523]) but also by practical and economical limitations. Enzyme-linked immunosorbent assay (ELISA) [524] and multiplex bead array analysis (MBAA) [525,526] can both use the "sandwich antibody technique," show a high degree of correlation in cytokine quantification and have in common high sensitivity, specificity, precision, accuracy and reproducibility.[524-528] In contrast to bioassays, the two methods are not particularly informative with respect to the biological activity of the analytes, but they have wider analytical ranges, higher specificity and precision and are less time-consuming and labor intensive. Based on required sample volume per analyte, protocol simplicity and efficiency, instrumentation needs, reagent cost, capacity of multianalyte detection and availability of antibodies with the required specificities, we chose ELISA for quantification of single analytes (Paper III) and MBAA for simultaneous measurement of multiple mediators (Paper II).

Besides the challenges associated with statistical evaluation of multiplex data sets discussed in Chapter 4.4, multiplex technology is encumbered by the "matrix effect," i.e. unwanted cross-reactivity between the components within the assay, reducing the reliability of the results.[526-528] We used standardized kits carefully optimized and validated by the vendor in order to minimize such errors.

In addition to the soluble mediator quantification in Paper II, the intracellular concentration of several of the same cytokines in various immune cell subsets was analyzed in Paper I. In retrospect, the possibilities for exploring intracellular and

soluble mediators in context at the individual level during the mobilization and stem cell collection process might have been better exploited.

Metabolomics analytic platforms

To investigate the metabolomic response during G-CSF administration, we chose an explorative approach using untargeted global metabolomics, i.e. explorative analysis of a huge number of metabolites. This approach, often with limited preliminary knowledge of the investigated issue, was not based on a specific hypothesis, but was rather intended to be hypothesis generating.[529]

The power of metabolomics platforms in high-throughput, multiplex quantification of metabolites is explained by the combination of the highly sensitive and accurate analytical tools mass spectrometry (MS) or nuclear magnetic resonance (NMR), with preparative separation of metabolites by gas, liquid, high performance liquid or ultrahigh performance liquid chromatography (GC, LC, HPLC, UPLC). MS acquires molecule-specific spectra expressed as mass-to-charge ratios (m/z) after initial ionization of the sample by electrospray or atmospheric pressure, and tandem MS (MS/MS) further increases resolution by fractionating the spectral data from two connected instrument components in time or space.[530] Chromatic separation of compounds by GC, LC, HPLC and UPLC prior to MS or MS/MS leads to method-dependent dynamic ranges. In order to maximize the total analytical range and capture as much of the complexity of the mixture as possible, four different platform approaches were used, as described in Paper III.

Apart from the high costs and the statistical challenges connected to analyses of huge data sets that are discussed in the next section, the untargeted metabolomics approach has several limitations that should be kept in mind when interpreting our results. To mention a few of the most important, only about 25% of compounds are identified properly during metabolomics analyses.[531,532] Instrument-dependent MS spectra, differences in compound definitions between the numerous established in-house and public spectral libraries and shortage of pure synthetic reference standards are still obstacles to highly specific metabolomics analyses producing results that are comparable across studies.[533-537] The sum of metabolic compounds in an organism is

countably infinite, and despite improved heuristic algorithms for compound characterization and discrimination, molecular formulas are not entirely unambiguous.[538] Moreover, metabolic intermediates participating in dynamic biological processes tend to be versatile, with each compound often playing more than one role in several disparate pathways, and diversion of metabolic pathways is regulated by the physiological context, further complicating the contextualization and interpretation of metabolomics data.[529]

4.4 Post-analytical aspects: handling multivariate data sets

During the last decades, the classical conflict of a reductionist versus a holistic approach to understanding biological science has been actualized by in-depth characterization of the human genome, transcriptome, proteome and metabolome and the identification of multiple new interconnections between the different biological systems. These developments have caused the pendulum to swing back from confirmatory testing of strictly pre-defined hypotheses towards a more explorative and broader perspective encompassing analyses of systems biology and a personalized diagnostic and therapeutic approach. Using multivariate data sets, the number of variables typically exceeds the number of participants, and personalized clinical trials in the purest form will move against n = 1.[539] The huge increase of available data has lead to a higher level of knowledge, but also to analytical challenges and increased risk of bias.

Powerful biostatistical tools and a critical attitude towards the methodological pitfalls and limitations are both equally important for correct interpretation of multivariate data. In the first two papers we used hierarchical clustering analysis (HCA), with unsupervised classification and subgrouping based on data similarity in order to extract the underlying structure of the data sets.[540-542] In HCA, the similarity between data is evaluated semi-quantitatively as the proximity of different observations using distance measures including Euclidean and Pearson Chi-Square distances.[540-542] The first step of the hierarchical agglomerative bottom-up clustering algorithm is construction of a list of inter-pattern distances for all unordered pairs of observations.[540,543] We used the complete-linkage clustering algorithm, estimating the

proximity of observations as the maximum distance between all pairwise observations.[540,543] The list of pairwise distances is then sorted ascendingly until a graph of the dissimilarity values is formed, shaped as a nested hierarchy of clusters and subclusters.[540,543]

The HCA method provides a visual intuitive categorization of complex data and is easily performed by specialized software,[543] but it cannot handle missing data. Furthermore, the data must usually be transformed (e. g. median normalization, $\log_2$ transformation) prior to analysis to achieve meaningful comparisons between individuals.[541,542] Transformation may alter the relationship between data and thus bias the results. In the current project, the median normalization of the data prior to biostatistical analyses partially masked donor heterogeneity. Several different approaches are used both for data transformation, distance measure and alternative clustering methods, and there is no clear guidelines for choice of methods to use on different data sets.[541,542] Consequently, each data set may be interpreted in various ways, which allows flexibility and individual exploration of data, but at the cost of unambiguity, exactness and comparability between studies. Overfitting of the statistical models to fulfill underlying or analysis-generated hypotheses is perhaps the greatest potential pitfall of the method.[541,542]

Although visually appealing and intuitive understandable through pattern recognition, the two-dimensionality of HCA is a limiting factor for the presentation of highly complex data.[541,542] Principal component analysis (PCA) was used in Paper IV and potentially captured a higher degree of the variability of multidimentional data sets by decomposition of the variance into vectors with subsequent three-dimensional comparison of groups in an orthogonal coordinate system.[529] The method may have revealed outlayers and hidden bias in the data set, but it is inferior to hierarchical clustering analyses with respect to detection of non-linear data trends.[529] Correlation-based network analysis is another approach for analysis of multidimensional data, emphasizing the importance of networks rather than pathways.[529] In retrospect, due to a high degree of overlap of participants between our four papers, we might have considered evaluating correlations across the datasets to examine relationships between the sets of immune cells, soluble immune mediators and metabolites.

Prior to the overarching bioinformatics analyses, we performed simple statistic tests evaluating single analyte changes during HSCT mobilization and collection. Correct handling of multivariate data requires a strategy for multiple testing to optimize the significance level and minimize the sum of type I and type II errors (false positive and negative results, respectively [529]). The Bonferroni correction, involving division of the overall significance level for the study by the number of simultaneously performed comparisons, is considered the most conservative approach and will, in practice, eliminate the risk of type I errors.[529,541] However, this method leads to a high risk of type II errors. As the opposite extreme, no correction for multiplicity has been proposed, a rationale that prioritizes the inherent orderliness of the natural word over theoretically calculated statistical chance.[544] In the current project, we chose a moderate position and adjusted the significance level to $p < 0.01$.

5. Discussion

G-CSF responsiveness is probably multifactorial

In Paper I, we made detailed characterizations of 27 donor lymphocyte subsets during HSC mobilization and collection. Due to small sample size, our study should be considered explorative and hypothesis generating, and the lack of methodological standardization of phenotype definitions (see Chapter 4.3) complicates comparison of single immune cell subsets between studies. Nevertheless, our results strongly suggest a dichotomous G-CSF immune cell mobilizing response in healthy individuals, involving 24 of 27 investigated subsets, irrespective of donor age, G-CSF dose, immune cell concentrations in peripheral blood prior to mobilization and in the stem cell graft (Paper I, Figure 4).

Variable G-CSF HSC mobilizing response due to BM-toxic effects of chemotherapeutics is well-known in autologous stem cell mobilization. Furthermore, autologous stem cell donor response to a single dose of G-CSF, in terms of neutrophil peak the following day, predicts neutrophil and especially platelet engraftment and defense to infection,[545] and low G-CSF responsiveness with regard to HSC mobilization is associated with prolonged neutropenia.[546] Less is known about the mechanisms for the variable stem cell mobilization that is observed even in healthy donors (see Chapter 1.1) and the possible impact on recipient engraftment and clinical outcome. Age was identified as the most important predictive factor for HSC mobilization in healthy individuals (Table 1). Even though age had little impact on the proportion of BM HPCs, the *in vitro* proliferative response of BM HPCs was lower in elderly individuals.[547]

Several potential mechanisms may modulate G-CSF immune cell mobilizing response and lead to variable immune cell composition of the graft. G-CSF acts through both direct G-CSFR dependent effects and indirect cytokine-mediated mechanisms, and HSC G-CSFR expression is not required for mobilization.[70] On the other hand, G-CSFR is widely expressed on myeloid cells and has recently been identified on most lymphoid subsets (see Chapter 1.1), indicating possible involvement in immune cell mobilization.

Heterogeneous immune cell mobilization may be partially due to variable regulation of G-CSFR expression and structural variation in the G-CSFR. Pathogenic mutations in G-CSFR are found in several myeloid disorders and are potential leukemogenic factors[60,548]. Targeted G-CSFR mutations in murine models showed normal resting granulopoiesis but hyperproliferation of neutrophils in response to G-CSF.[549,550] During HSC mobilization of myeloma patients, the CSFR3 variant rs3917924 showed association to HSC mobilization potential and neutrophil engraftment.[551] In a study of 303 autologous and allogeneic HSC donors, 16 missense G-CSFR SNPs were screened, and three of those (rs3918001, rs 3918018 and rs3918019) had potential impact on peripheral blood $CD34^+$ cell enrichment.[552] The possible impact of G-CSFR genetic polymorphisms for immune cell mobilization remains to be elucidated.

Direct G-CSFR–mediated effects may also lead to functional alterations of a broad spectrum of immune cell subsets with potential implications for recipient outcome. For example, by use of artificial antigen-presenting cells, direct antigen-dependent and –independent effects of G-CSF on isolated $CD8^+$ T cells have been demonstrated.[553] The effects were synergistic to the previous known indirect effects, were detectable both at the RNA and protein levels and involved several central elements in T cell activation: surface activation markers, miR-155 expression and ERK1/2 and CD3ζ/Lymphocyte-specific protein tyrosine kinase (Lck) signaling pathways.[553]

G-CSF is part of an extensive immunoregulatory network, and the variability of multiple extracellular and intracellular receptors and signaling molecules potentially influences the G-CSF response. For example, SOCS3 is a negative regulator of G-CSF signaling, and modification of its protein structure leads to hyper-responsiveness to G-CSF with regard to granulopoiesis and inflammatory response in murine models.[554] SOCS3-deficient mice have prolonged STAT3 activation and enhanced cloning frequency, proliferative capacity and survival.[555]

CXCL12 is a key mediator of HSC mobilization (Chapter 1.1), and the experience from autologous stem cell mobilization has revealed associations between CXCL12 genetic polymorphisms and HSC mobilization capacity and neutrophil

engraftment.[556] As CXCR4, the receptor of CXCL12, is broadly expressed on leukocytes,[336] CXCL12 SNPs may have implications also for immune cell mobilization. Immune cell migratory capacity is possibly a prerequisite for G-CSF responsiveness and is potentially influenced by the expression of multiple other chemokines, chemokine receptors and adhesion molecules. To mention a few examples, G-CSF leads to STAT3-dependent CXCR2-mediated neutrophil chemotaxis [557] and the general neutrophil chemotactic activity and phagocytic functionality is reduced by G-CSF, not only in healthy donors, but also in their HSC graft recipients up to 4 weeks post transplant.[558] G-CSF mobilization of healthy donors also alters the conformation of the integrin leukocyte function-associated antigen-1 (LFA-1) and thereby inhibits interaction with its receptor ICAM-1, potentially reducing activation, proliferation and migration of $CD4^+$ T cells.[559] *In vitro* studies indicate that this influence of G-CSF on LFA-1 directly suppresses $CD4^+$ migration, adhesiveness and release of inflammatory cytokines by downregulation of Lck and Zeta-chain–associated protein kinase 70 (ZAP-70).[560]

In addition to the adhesion molecules investigated in Paper II, CD44 was included in the antibody panel of Paper III. CD44 is not only a receptor for OPN, but has multiple functions including serving as an adhesion molecule.[325] CD44 is a major E-selectin ligand controlling $CD4^+$ and $CD8^+$ T cell migration and adhesion to and extravasation from inflamed endothelium.[561-564] CD44 shows highlevels of expression in T memory cells and is thought to promote effector cell survival by limitation of Fas-mediated death and activation of the PI3K/Akt signaling pathway.[565]

During G-CSF therapy, we observed significantly reduced CD44 expression in $CD19^+$ B cells. A functional role of CD44 in murine B cell activation and chemotaxis has been described.[566] High levels of expression of CD44 in autoantibody-secreting B cells has been reported in murine cGVHD,[567] and a reduced level of CD44 in G-CSF–mobilized B cells may contribute to attenuate GVHD.

Finally, the migration of immune cells is also linked to sphingolipid metabolism.[568] G-CSFR signaling leading to STAT3 phosphorylation and increased neutrophil CXCR2 expression, and migratory capacity depends on translocation of G-CSFR regulated by the ceramide synthetase CerS2.[568] CerS2 gene deletion leads to anti-

inflammatory effects through reduced G-CSF–induced neutrophil migration in murine models[568].

Apheresis device dependent and immune subset specific effects on the profile of soluble mediators need clarification

In our second paper, we found significant differences in the graft and peripheral blood mediator profiles between donors harvested with Cobe Spectra and Spectra Optia. The pre-apheresis peripheral blood concentrations of neutrophils, monocytes, lymphocytes and platelets did not differ significantly between the two groups, but they were significantly higher in grafts harvested with Cobe Spectra compared to Spectra Optia. We considered the clinical significance of this observation to be uncertain. In a recent study, a similar difference in graft leukocyte and platelet concentrations between samples taken using Cobe Spectra and Spectra Optia was reported, as well as a lower incidence of aGVHD in recipients of grafts harvested with Spectra Optia.[144] The study was small and reported contradictory increased cGVHD associated with Spectra Optia. The results of other comparisons of the two devices with respect to graft content are also contradictory.[142,143,569,570]

From autologous stem cell collection, it is known that not only the apheresis device used but also the apheresis settings influence graft composition.[571,572] Even though apheresis procedures are supposed to be standardized and automated, the procedure settings still, to a large extent, need to be individualized depending on donor and patient characteristics (e.g. age, weight, mobilization efficiency, disease status, comorbidity) and events occurring during the apheresis procedure (e.g. access problems, platelet aggregation, electrolyte and fluid balance disturbances). Thus, great variability in processed blood volume, processing time, ACD-A/whole blood ratio, collection efficiency and graft composition can be expected not only between different apheresis devices and different protocols used in the same device but also between different donors harvested with the same protocol/device. Due to multiple patient, donor and apheresis procedure confounding factors, very large studies will be needed to confirm device-dependent differences in post-transplant clinical outcome.

Donor G-CSF treatment increased the systemic level of most investigated mediators, but generally the increase was modest, with the exception of a more than 50-fold rise

in MMP-8 concentration. MMP-8 is thought to be a marker of inflammation severity and an important mediator in chronic inflammation [573] and has recently shown anti-tumor and complex immunoregulatory functions in gastrointestinal cancer.[574] The G-CSF–induced systemic increase of several pro-inflammatory cytokines including TNFα and IL-6 is not an unexpected response as G-CSF is considered a component of the pro-inflammatory program normally triggered by infection. In a related project, we found increased C-reactive protein (CRP) during G-CSF treatment in the same donor cohort.[575] These results align with several other published studies reporting an acute phase-like response to G-CSF injections with increased IL-6 and CRP levels.[576,577] Both G-CSF treatment and apheresis contribute to the inflammatory response in healthy donors, accompanied by increased serum ferritin and iron levels.[577] Apheresis in healthy donors also leads to increased protein-limited oxidative stress that is counteracted by increased antioxidant capacity thought to limit tissue damage.[577]

In contrast, a recent study demonstrated reduced *in vitro* secretion of inflammatory cytokines in isolated $CD4^+$ cells from G-CSF–treated healthy donors.[560] $CD4^+$ cells were stimulated with ICAM-1 and anti-$CD3^+$, and downregulated Lck and ZAP-70 expression was proposed as the mechanism for the observed reduced LFA-1-ICAM mediated $CD4^+$ migration *in vitro*.[560] The significant peripheral blood concentration increase for virtually all immune cell subsets during G-CSF administration (see Paper I, Figure 2) may explain these apparent contradictory results with respect to G-CSF induction of pro-inflammatory cytokines. The peripheral blood increase in immune cell numbers by far exceeded the increase in mediator levels during G-CSF therapy (see Paper II, Table I), and the G-CSF effect on cytokine secretion probably differs between various immune cell subpopulations. Thus a total increase of systemic cytokines may be possible, despite reduced secretion in certain immune cell subsets.

Donor heterogeneity–an undervalued outcome-predictive factor?

The high degree of variability characterizing the entire multi-step process of allogeneic HSCT has been emphasized throughout this thesis (summarized in Figure 1 and Table 7). As discussed in a recent review, the current project confirms and

augments the impression of extensive untreated healthy donor heterogeneity encompassing both innate and adaptive immunity, and the variability is further enhanced during G-CSF treatment.[578] There are great controversies around the effects of G-CSF on graft composition, as well as about the importance of graft composition on clinical outcome. These disagreements may be explained both by the already recognized variability and by the still unexplored heterogeneity of donor characteristics, allogeneic HSC mobilization and collection procedures. Outcomes after HSCT using MUDs or MRDs are now comparable (see p. 3). Improved immunosuppression potentially reduces the importance of immunological donor-host incompatibility and increases the relative impact of donor traits beyond the already recognized factors of age and HLA and ABO types.[24] The impacts of a wider range of donor factors on graft quality and recipient outcome need to be explored in studies controlled for confounding patient factors.

There is also a need for more precise HSC graft specification with regard to infused cell types and doses. HSC graft specifications are, to a limited degree, standardized. The required HSC dose for allotransplantation is usually requested as a minimum dose, and the final infused dose is influenced by individual and local factors like the donor mobilization response, the donor/recipient weight ratio and the capacity and routines at the collection center (Figure 1). For other immune cell subsets, the requested and infused doses are even less clearly defined.

Furthermore, based on genetic differences, the distribution of several donor traits may vary between regions. Confounding factors due to heterogeneity in donor origin may be enhanced by the small sample size study design typically found in this field. These factors add to the extensive variability in transplant courses due to recipient diversity, encompassing disease, comorbidity, conditioning therapy and immunosuppression.

Recently, a risk stratification model for acute and chronic GVHD was developed based on retrospective multivariate analyses of clinical and genomic factors.[579] The failure of this algorithm to stratify risk when repeated on another patient population may illustrate the effects of the extensive heterogeneity of the transplant process.[580] The risk factors are probably largely individual and dependent on multiple clinical

and immunological factors; hence a general risk prediction scheme may be hard to construct for inhomogeneous populations. The degree of complexity included in both recipient and donor multivariable heterogeneity may also explain the failure of two recent studies to identify clinically important effects of donor heterogeneity.[26,581] To handle the full range of patient and donor heterogeneity, powerful biostatistical tools may be valuable, to inform individualized decision making throughout the transplant course. In principle, standardization of graft characteristics and cell doses is desirable, but is hard to achieve in practice due to the extreme variability. To improve the treatment of the heterogeneous patient population with individual donors, taking a more personalized approach may be more advantageous than efforts at standardization.

The principles of personalized medicine already apply to allogeneic HSCT, with adaption of conditioning therapy and immunosuppression depending on age, diagnosis, disease stage, remission status and comorbidity. Based on increased knowledge of the importance of donor heterogeneity, the personalization of therapy could be extended beyond choice of donor type and source, in terms of HLA compatibility, HSC and T cell doses. Donor selection may be refined by inclusion of updated criteria based on total assessment of patient and donor traits and individual risk. However, the availability of donors is usually a limiting factor, and individualized treatment of the donor and graft are consequently more relevant options. Predictions of donor HSC and immune cell mobilization capacity compared with patient characteristics may be the decisive factor in the choice of alternative mobilizing agents or combinations of two or more agents, as well as the dosage and duration of mobilization treatment. Similarly, the collected stem cell graft may be adapted to the individual recipient regarding doses and features of both progenitor and mature immune cells using existing *ex vivo* depletion and enrichment techniques.

Intervention with the graft components further increases the possibilities for individualization. Prediction of risk based on donor and graft specifications may influence post-transplant immunosuppression and/or adoptive immunotherapy regimen for the recipient. Pharmacological donor intervention, in addition to mobilization therapy, may even be considered. For example, 3-hydroxy-3-

methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (statins) not only lowers serum cholesterol but also has multiple immunoregulatory effects, and statin treatment of the sibling donor but not the recipient has been shown to reduce the frequency of aGVHD.[582]

Small sample size and heterogeneity–can we trust the results?

The major limitation of the present study is the small sample size. However, the study population is a consecutive group of patients and donors. Based on the national organization of allotransplantation activity in Norway, they represent all patients from a defined geographical area (western, middle and northern regions) transplanted during a defined time period with family donors. It is thus a relatively homogeneous group with regard to type of donor and also with regard to conditioning regimen and GVHD prophylaxis. However, additional larger studies are needed to confirm our results and clarify whether our observations are relevant also for other groups of allotransplant recipients, such as patients receiving grafts from MUDs.

Our four papers focus on different parts of the donor and recipient immunoregulatory systems. The donors and recipients included in the different papers overlapped to a great extent, which opened an opportunity to compare the results on an individual level in order to reduce the uncertainty associated with the small sample size and to assess different parts of the immunoregulatory networks in a more complete manner. In general, the results from all papers were characterized by a high degree of donor heterogeneity both prior to and during stem cell mobilization. The heterogeneity with respect to G-Rsp was most distinct, presumably with the greatest potential for clinical application, and was compared to the results from the three other papers to reveal common trends or contradictions.

First, a possible association between donor G-Rsp and recipient post-transplant mediator profile was investigated. The unexpected observation of an association between the early post-transplant mediator profile and the overall survival of the recipients could not be explained by patient factors such as comorbidity or remission status (Paper II). Donor G-Rsp was available for 8 of the 16 recipients and identical hierarchical cluster analyses of recipient post-transplant mediator profiles and donor

G-Rsp were repeated on these subsets and resulted in identical distribution of patients and donors compared to the original clustering analyses (Paper I, Figure 4 and Paper II, Figure 4B). Kaplan Meier survival analysis repeated on this smaller recipient subset showed consistent results compared to the original analysis, with inferior mean overall survival of 266 days for the three patients in the lower cluster compared to 1429 for the five recipients in the upper cluster (p = 0.022).

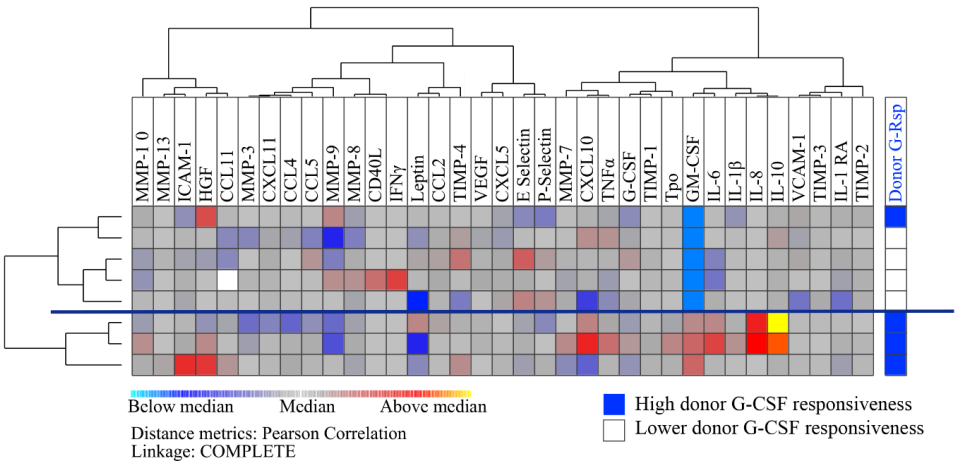

Figure 4. The recipient post-transplant mediator profile compared to donor G-Rsp. Unsupervised hierarchical clustering analysis based on the first day post-transplant mediator profile of a subset of 8 HSCT recipients. The column to the right shows the corresponding donor G-Rsp.

When comparing the clustering analyses of the plasma mediator profiles of the patients with the G-Rsp of the respective donors, the clustering was identical for 7 out of 8 donor/recipient pairs (Figure 4, p = 0.028, Pearson Chi-Square test). Three of four recipients of stem cell products from donors with high G-Rsp were localized in the lower cluster with inferior overall survival, whereas the fourth recipient (Figure 4) was diagnosed with aGVHD grade II and was still alive at termination of the study. Certain conclusions cannot be drawn based on this limited data set, but results in Paper I and II do not seem contradictory and suggest a possible association between donor-related early post-transplant events and transplant outcome.

Second, if it is confirmed that G-Rsp is important for the clinical outcome of the patient, new strategies to improve product quality and individualize the treatment based on both donor and patient features could be beneficial. It would then be

advantageous to be able to predict donor G-Rsp to optimize donor mobilization, harvest and graft processing. As mentioned above, adhesion molecules are important for G-CSF–mediated stem cell mobilization and T cell immunomodulation. CD44 is an adhesion molecule and also a functional osteopontin-receptor, which was evaluated in Paper III. We used the Mann-Whitney U test to compare pre-treatment T cell CD44 levels available from Paper III for 12 of the donors in Paper I with the G-Rsps of the same donors. We found a significant association between the $CD3^+$ cells' level of CD44 expressed as CD44-Ax 488 MFI and lower or higher G-Rsp defined as G-CSF immune cell mobilizing response ($p = 0.030$).

In the autologous setting, CD44 polymorphisms influenced G-CSF–induced stem cell mobilization,[583] and BM $CD34^+$ HSC expression of several adhesion molecules including CD44 correlates to stem cell yield in both autologous and allogeneic PBSC collections.[325] High levels of adhesion molecules might reflect generally increased migratory capacity. CD44 also facilitates T cell proliferation,[584] and one may speculate whether this mechanism contributed to the significant increases in leukocytes concentrations observed in donors with high levels of expression of CD44 during G-CSF treatment. CD44 is required for adhesion of lymphocytes to high endothelial venules [584] and extravasation of activated T cells into inflammatory sites.[585] High T cell CD44 expression was also recently shown to enhance the strength of T cell receptor signaling and promote induction of the Th17 subset.[586] CD44 mediates lymphocyte binding to dermal endothelium in acute cutaneous GVHD,[587] and $CD44^{hi}$ effector memory $CD8^+$ T cells have been identified as the dominant population in murine aGVHD.[588] Antibody blockade of CD44 attenuates disease activity in animal models of various autoimmune diseases and is considered especially valuable in inflammatory liver disease due to unique leukocyte adhesion without endothelial rolling in the liver vasculature.[589] Based on these observations, CD44 blockade may help prevent aGVHD.

We hypothesize that analysis of T cell CD44 expression represents a potential means of predicting G-Rsp and individualizing stem cell mobilization and harvest of the donor, manipulation of the graft and preparation and post-transplant follow up of the recipient.

Third, we looked for associations between metabolomics profiles and G-Rsp. G-Rsp was available from Paper I for 13 of the 15 participants in Paper IV. We performed bioinformatics analyses with two unsupervised hierarchical cluster analyses as described in Papers I and II based on the donor serum levels of 641 metabolites prior to and during G-CSF treatment (Figure 5). Based on their total metabolomic profiles, the donors could be separated into two different subsets with 10 and 5 donors, respectively, both prior to and during G-CSF administration. With the exception of two donors, the distribution of individuals into two subsets was identical before and after G-CSF treatment.

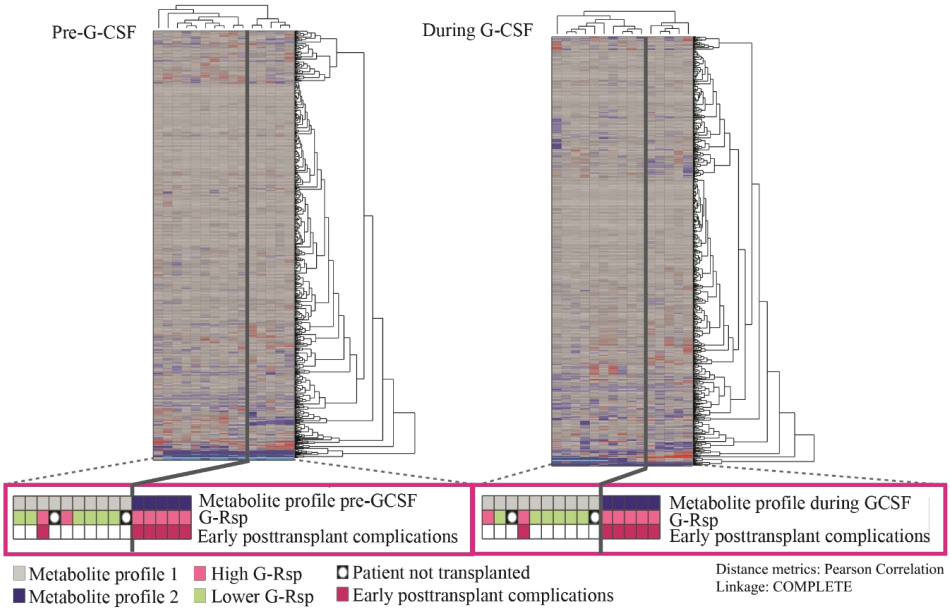

Figure 5. The donor pre-G-CSF metabolomics profile compared to G-Rsp and early post-transplant complications. Unsupervised hierarchical clustering analysis based on donor pre-transplant levels of 641 identified metabolites. Two donors clusters called Metabolite profile 1 (10 donors) and Metabolite profile 2 (5 donors) were identified both prior to and during G-CSF administration. Below the heatmap the Metabolite profiles 1 and 2 prior to and during G-CSF are compared to the responsiveness to G-CSF in the same donors and to the incidence of early post-transplant complications in the corresponding recipients. Evaluated by Pearson Chi-Square test, the donor metabolite profiles both prior to and during G-CSF were associated to G-CSF responsiveness ($p = 0.008$) and to early post-transplant complications in the recipient ($p = 0.001$).

Comparing these metabolomics bioinformatics results to G-Rsp, 5 of the 7 donors with high G-Rsp belonged to the right donor clusters both prior to and during G-CSF, whereas all six donors with lower G-Rsp were included in the left clusters (Figure 5).

Thus, the G-Rsp differed significantly based on serum metabolite profile both prior to and during G-CSF treatment (p = 0.008, Pearson Chi-Square test).

Paracetamol is used as an analgesic for skeletal pain during HSC mobilization. Prior to G-CSF treatment, paracetamol metabolites were detected at low levels in a minority of the donors (Figure 6). During G-CSF administration, the levels rose substantially and were significantly higher in the donors classified as highly G-CSF responsive (p = 0.036, Pearson Chi-Square test), indicating that donors with the strongest immune cell mobilizing effect from G-CSF had a stronger need for analgesics (i. e. had more toxicity) during mobilization.

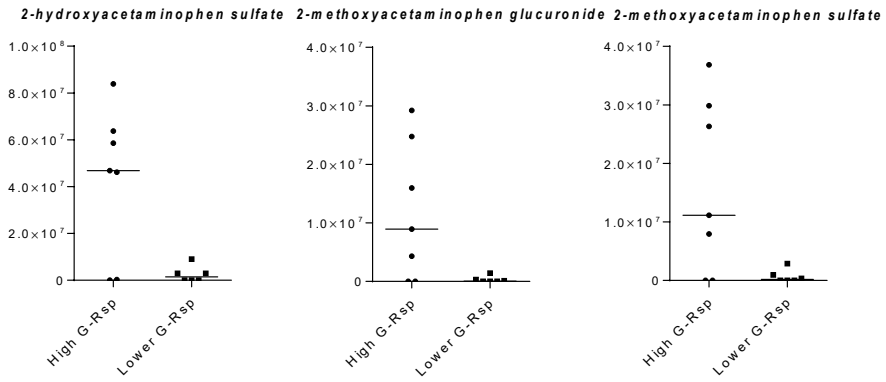

Figure 6. The level of paracetamol metabolites during G-CSF administration in donors with high and lower G-Rsp. In addition to the three representative metabolites shown, 3-(cystein-S-yl) and 3-(N-acetyl-L-cysteine-S-yl) acetaminophen, 4-acetamidophenol, 4-acetamidophenylglucoronide and 4-acetaminophen sulfate showed similar patterns.

Furthermore, out of the six recipients diagnosed with early transplant-related complications (five with aGVHD and one with multi-organ failure), five received grafts from donors included in the right cluster both prior to and during G-CSF, while only one stem cell graft originated from a donor included in left cluster (Figure 5). Thus, both the untreated and G-CSF-treated donor serum metabolomic profiles seem to predict the risk of early post-transplant complications (p = 0.001, Pearson Chi-Square test).

This possible association between donor metabolic status and recipient outcome aligns with a murine model in which obese donors were studied.[590] Obesity was reported to induce expansion of the BM HSC and myeloid progenitor compartment, and the HSC inclination to differentiate into pro-inflammatory macrophage

phenotypes was transferrable to the recipient.[590] These observations suggest that donor nutritional status, diet and total metabolomic profiles may be important to HSCT outcome. Moreover, targeting of metabolic pathways in order to prevent GVHD while pre-serving the GVL effect (see p. 41) may be useful not only post transplant, but also for optimizing stem cell grafts. In case of pre-transplant *in vitro* expansion and modulation of cellular graft components, the possible effects of the availability of different metabolites during cultivation should gain greater attention.[481]

To summarize, our observation of heterogeneity in donor G-Rsp with possible clinical implications for recipient outcome in Paper I is not contradicted by the other papers. On the contrary, through identification of possible associations between G-Rsp identified in Paper I and central aspects of Papers II, III and IV, we have identified new potential associations between donor features and recipient outcomes. Thus, even though the small numbers of investigated donors necessitates great caution in interpretating our results, there seems to be good agreement between the suggested importance of donor G-Rsp and recipient early post-transplant mediator profile for clinical outcome. Furthermore, both donor T cell expression of the adhesion molecule CD44 and the total metabolomic profile of the donor prior to G-CSF treatment seem to predict G-Rsp. Thus, the donor trait G-Rsp does not seem to be limited to the concentration change of various immunomodulatory cells, but rather seems to be associated to a wider range of immune system characteristics and to systemic metabolism. These observations further strengthen our hypothesis that heterogeneity of the stem cell donors and hitherto unknown donor factors represent potential therapeutic aspects of allogeneic stem cell transplantation.

6. Conclusions and future perspectives

Our present studies demonstrate extensive heterogeneous effects of allogeneic HSC mobilization and collection on the donor profile of immune cells, soluble mediators and metabolites that is also reflected in the stem cell graft. In recent years, it has become increasingly evident that the interplay of multiple clinical and immunological factors influences the course of recovery after allogeneic HSCT. Donor heterogeneity may represent a hitherto underestimated factor contributing to the risk of complications after allogeneic stem cell transplantation.

The explorative approach of the current project generated new hypotheses:

1. The donor G-CSF immune cell mobilizing response (G-CSF responsiveness), and not only graft composition, is important for recipient clinical outcome.
2. The pre-mobilization, pre-harvesting immunological and metabolic status of the donor predicts the response to G-CSF and the composition of the stem cell graft.
3. Individualized adaption of HSC mobilization, modification of the graft and adjustment of recipient immunosuppression can therefore potentially improve HSCT therapy.

Larger studies are needed to establish a more detailed and comprehensive characterization of the donor immune system and metabolomics profile. Advanced, multiplexed technology and bioinformatics tools will be necessary to overcome the challenges associated with pervasive immunological and metabolic heterogeneity and interconnectedness, thereby enabling elucidation of underlying mechanisms and potential targets for modification.

There has been little improvement in survival in acute high-risk leukemia over the last three decades. Allogeneic HSCT is, to date, the best curative treatment option for many patients. Despite numerous advances in HSCT over the past decades with improved post-transplant outcomes, this treatment is still encumbered with serious complications and there is continued need for novel therapeutic strategies.

7. References

1. Thomas ED, Storb R, Clift RA, Fefer A, Johnson L, Neiman PE, Lerner KG, Glucksberg H, Buckner CD. Bone-marrow transplantation (second of two parts). N Engl J Med **1975**;292: 895-902.
2. Gratwohl A, Pasquini MC, Aljurf M, Atsuta Y, Baldomero H, Foeken L, Gratwohl M, Bouzas LF, Confer D, Frauendorfer K, Gluckman E, Greinix H, Horowitz M, Iida M, Lipton J, Madrigal A, Mohty M, Noel L, Novitzky N, Nunez J, Oudshoorn M, Passweg J, van Rood J, Szer J, Blume K, Appelbaum FR, Kodera Y, Niederwieser D, Worldwide Network for B, Marrow T. One million haemopoietic stem-cell transplants: a retrospective observational study. Lancet Haematol **2015**;2: e91-100.
3. Duhrsen U, Villeval JL, Boyd J, Kannourakis G, Morstyn G, Metcalf D. Effects of recombinant human granulocyte colony-stimulating factor on hematopoietic progenitor cells in cancer patients. Blood **1988**;72: 2074-81.
4. Baumann I, Testa NG, Lange C, de Wynter E, Luft T, Dexter TM, van Hoef ME, Howell A. Haemopoietic cells mobilised into the circulation by lenograstim as alternative to bone marrow for allogeneic transplants. Lancet **1993**;341: 369.
5. Korbling M, Przepiorka D, Huh YO, Engel H, van Besien K, Giralt S, Andersson B, Kleine HD, Seong D, Deisseroth AB, et al. Allogeneic blood stem cell transplantation for refractory leukemia and lymphoma: potential advantage of blood over marrow allografts. Blood **1995**;85: 1659-65.
6. Switzer GE, Goycoolea JM, Dew MA, Graeff EC, Hegland J. Donating stimulated peripheral blood stem cells vs bone marrow: do donors experience the procedures differently? Bone Marrow Transplant **2001**;27: 917-23.
7. Passweg JR, Baldomero H, Basak GW, Chabannon C, Corbacioglu S, Duarte R, Kuball J, Lankester A, Montoto S, de Latour RP, Snowden JA, Styczynski J, Yakoub-Agha I, Arat M, Mohty M, Kroger N. The EBMT activity survey report 2017: a focus on allogeneic HCT for nonmalignant indications and on the use of non-HCT cell therapies. Bone Marrow Transplant **2019**.
8. Armand P, Kim HT, Logan BR, Wang Z, Alyea EP, Kalaycio ME, Maziarz RT, Antin JH, Soiffer RJ, Weisdorf DJ, Rizzo JD, Horowitz MM, Saber W. Validation and refinement of the Disease Risk Index for allogeneic stem cell transplantation. Blood **2014**;123: 3664-71.
9. Sorror ML, Maris MB, Storb R, Baron F, Sandmaier BM, Maloney DG, Storer B. Hematopoietic cell transplantation (HCT)-specific comorbidity index: a new tool for risk assessment before allogeneic HCT. Blood **2005**;106: 2912-9.
10. Sorror ML, Storb RF, Sandmaier BM, Maziarz RT, Pulsipher MA, Maris MB, Bhatia S, Ostronoff F, Deeg HJ, Syrjala KL, Estey E, Maloney DG, Appelbaum FR, Martin PJ, Storer BE. Comorbidity-age index: a clinical measure of biologic age before allogeneic hematopoietic cell transplantation. J Clin Oncol **2014**;32: 3249-56.
11. Gratwohl A, Stern M, Brand R, Apperley J, Baldomero H, de Witte T, Dini G, Rocha V, Passweg J, Sureda A, Tichelli A, Niederwieser D, European Group for B, Marrow T, the European Leukemia N. Risk score for outcome after allogeneic hematopoietic stem cell transplantation: a retrospective analysis. Cancer **2009**;115: 4715-26.
12. Bacigalupo A, Ballen K, Rizzo D, Giralt S, Lazarus H, Ho V, Apperley J, Slavin S, Pasquini M, Sandmaier BM, Barrett J, Blaise D, Lowski R, Horowitz M. Defining the intensity of conditioning regimens: working definitions. Biol Blood Marrow Transplant **2009**;15: 1628-33.
13. Gyurkocza B, Sandmaier BM. Conditioning regimens for hematopoietic cell transplantation: one size does not fit all. Blood **2014**;124: 344-53.
14. Zeiser R, Blazar BR. Acute Graft-versus-Host Disease - Biologic Process, Prevention, and Therapy. N Engl J Med **2017**;377: 2167-79.
15. Lee SJ, Klein J, Haagenson M, Baxter-Lowe LA, Confer DL, Eapen M, Fernandez-Vina M, Flomenberg N, Horowitz M, Hurley CK, Noreen H, Oudshoorn M, Petersdorf E, Setterholm M, Spellman S, Weisdorf D, Williams TM, Anasetti C. High-resolution donor-recipient HLA matching contributes to the success of unrelated donor marrow transplantation. Blood **2007**;110: 4576-83.
16. Petersdorf EW. The major histocompatibility complex: a model for understanding graft-versus-host disease. Blood **2013**;122: 1863-72.
17. Sanchez-Mazas A, Vidan-Jeras B, Nunes JM, Fischer G, Little A-M, Bekmane U, Buhler S, Buus S, Claas FHJ, Dormoy A, Dubois V, Eglite E, Eliaou JF, Gonzalez-Galarza F, Grubic Z, Ivanova M, Lie B, Ligeiro D, Lokki ML, da Silva BM, Martorell J, Mendonça D, Middleton D, Voniatis DP, Papasteriades C, Poli F, Riccio ME, Vlachou MS, Sulcebe G, Tonks S, Nevessignsky MT, Vangenot C, van Walraven A-M, Tiercy J-M. Strategies to work with HLA data in human populations for

histocompatibility, clinical transplantation, epidemiology and population genetics: HLA-NET methodological recommendations **2012**;39: 459-76.
18. Tiercy JM. How to select the best available related or unrelated donor of hematopoietic stem cells? Haematologica **2016**;101: 680-7.
19. Barker JN, Boughan K, Dahi PB, Devlin SM, Maloy MA, Naputo K, Mazis CM, Davis E, Nhaissi M, Wells D, Cooper C, Ponce DM, Kernan N, Scaradavou A, Giralt SA, Papadopoulos EB, Politikos I. Racial disparities in access to HLA-matched unrelated donor transplants: a prospective 1312-patient analysis. Blood Adv **2019**;3: 939-44.
20. Saber W, Opie S, Rizzo JD, Zhang MJ, Horowitz MM, Schriber J. Outcomes after matched unrelated donor versus identical sibling hematopoietic cell transplantation in adults with acute myelogenous leukemia. Blood **2012**;119: 3908-16.
21. Wang Y, Liu QF, Xu LP, Liu KY, Zhang XH, Ma X, Fan ZP, Wu DP, Huang XJ. Haploidentical vs identical-sibling transplant for AML in remission: a multicenter, prospective study. Blood **2015**;125: 3956-62.
22. Fuchs EJ. Related haploidentical donors are a better choice than matched unrelated donors: Point. Blood Adv **2017**;1: 397-400.
23. Shaw BE. Related haploidentical donors are a better choice than matched unrelated donors: Counterpoint. Blood Adv **2017**;1: 401-6.
24. Kollman C, Spellman SR, Zhang MJ, Hassebroek A, Anasetti C, Antin JH, Champlin RE, Confer DL, DiPersio JF, Fernandez-Vina M, Hartzman RJ, Horowitz MM, Hurley CK, Karanes C, Maiers M, Mueller CR, Perales MA, Setterholm M, Woolfrey AE, Yu N, Eapen M. The effect of donor characteristics on survival after unrelated donor transplantation for hematologic malignancy. Blood **2016**;127: 260-7.
25. Wang Y, Wu DP, Liu QF, Xu LP, Liu KY, Zhang XH, Xu Y, Huang F, Huang XJ. Donor and recipient age, gender and ABO incompatibility regardless of donor source: validated criteria for donor selection for haematopoietic transplants. Leukemia **2018**;32: 492-8.
26. Shaw BE, Logan BR, Spellman SR, Marsh SGE, Robinson J, Pidala J, Hurley C, Barker J, Maiers M, Dehn J, Wang H, Haagenson M, Porter D, Petersdorf EW, Woolfrey A, Horowitz MM, Verneris M, Hsu KC, Fleischhauer K, Lee SJ. Development of an Unrelated Donor Selection Score Predictive of Survival after HCT: Donor Age Matters Most. Biol Blood Marrow Transplant **2018**;24: 1049-56.
27. Kroger N, Zabelina T, de Wreede L, Berger J, Alchalby H, van Biezen A, Milpied N, Volin L, Mohty M, Leblond V, Blaise D, Finke J, Schaap N, Robin M, de Witte T, Blood MDSsotCLWPotEGf, Marrow T. Allogeneic stem cell transplantation for older advanced MDS patients: improved survival with young unrelated donor in comparison with HLA-identical siblings. Leukemia **2013**;27: 604-9.
28. Anderlini P. Sixty as the new forty: considerations on older related stem cell donors. Bone Marrow Transplant **2017**;52: 15-9.
29. Watz E, Remberger M, Ringden O, Lundahl J, Ljungman P, Mattsson J, Wikman A, Uhlin M. Analysis of donor and recipient ABO incompatibility and antibody-associated complications after allogeneic stem cell transplantation with reduced-intensity conditioning. Biol Blood Marrow Transplant **2014**;20: 264-71.
30. Booth GS, Gehrie EA, Bolan CD, Savani BN. Clinical guide to ABO-incompatible allogeneic stem cell transplantation. Biol Blood Marrow Transplant **2013**;19: 1152-8.
31. Fonseca M, Jakubowski AA, Devlin SM, Young JW, Fatmi SA, Maloy M, Giralt S, Ponce D, Markova A. HLA-a*0101 Expression Correlates with Increased Risk of Severe Cutaneous Acute Graft-Versus-Host Disease after Allogeneic Hematopoietic Stem Cell Transplantation. Blood **2018**;132.
32. Holtick U, Albrecht M, Chemnitz JM, Theurich S, Skoetz N, Scheid C, von Bergwelt-Baildon M. Bone marrow versus peripheral blood allogeneic haematopoietic stem cell transplantation for haematological malignancies in adults. Cochrane Database Syst Rev **2014**: CD010189.
33. Pulsipher MA, Logan BR, Kiefer DM, Chitphakdithai P, Riches ML, Rizzo JD, Anderlini P, Leitman OF, Kobusingye H, Besser RM, Miller JP, Drexler RJ, Abdel-Mageed A, Ahmed IA, Akard LP, Artz AS, Ball ED, Bayer RL, Bigelow C, Bolwell BJ, Broun ER, Delgado DC, Duckworth K, Dvorak CC, Hahn TE, Haight AE, Hari PN, Hayes-Lattin BM, Jacobsohn DA, Jakubowski AA, Kasow KA, Lazarus HM, Liesveld JL, Linenberger M, Litzow MR, Longo W, Magalhaes-Silverman M, McCarty JM, McGuirk JP, Mori S, Parameswaran V, Prasad VK, Rowley SD, Rybka WB, Sahdev I, Schriber JR, Selby GB, Shaughnessy PJ, Shenoy S, Spitzer T, Tse WT, Uberti JP, Vusirikala M, Waller EK, Weisdorf DJ, Yanik GA, Navarro WH, Horowitz MM, Switzer GE, Confer DL, Shaw BE. Related peripheral blood stem cell donors experience more severe symptoms and less complete recovery at one year compared to unrelated donors. Haematologica **2019**;104: 844-54.

34. Anderlini P, Przepiorka D, Seong C, Smith TL, Huh YO, Lauppe J, Champlin R, Korbling M. Factors affecting mobilization of CD34+ cells in normal donors treated with filgrastim. Transfusion **1997**;37: 507-12.
35. Vasu S, Leitman SF, Tisdale JF, Hsieh MM, Childs RW, Barrett AJ, Fowler DH, Bishop MR, Kang EM, Malech HL, Dunbar CE, Khuu HM, Wesley R, Yau YY, Bolan CD. Donor demographic and laboratory predictors of allogeneic peripheral blood stem cell mobilization in an ethnically diverse population. Blood **2008**;112: 2092-100.
36. Zhang C, Chen XH, Zhang X, Gao L, Gao L, Kong PY, Peng XG, Sun AH, Gong Y, Zeng DF, Wang QY. Stem cell collection in unmanipulated HLA-haploidentical/mismatched related transplantation with combined granulocyte-colony stimulating factor-mobilised blood and bone marrow for patients with haematologic malignancies: the impact of donor characteristics and procedural settings. Transfus Med **2010**;20: 169-77.
37. Bertani G, Santoleri L, Martino M, Fedele R, Moscato T, Marenco P, Grillo G, Zucchetti E, Lotesoriere I, Lando G, Cesana C, Cairoli R, Rossini S. Identification of hematopoietic progenitor cell donor characteristics predicting successful mobilization: results of an Italian multicenter study. Transfusion **2014**;54: 2028-33.
38. Al-Ali HK, Bourgeois M, Krahl R, Edel E, Leiblein S, Poenisch W, Basara N, Lange T, Niederwieser D. The impact of the age of HLA-identical siblings on mobilization and collection of PBSCs for allogeneic hematopoietic cell transplantation. Bone Marrow Transplant **2011**;46: 1296-302.
39. Switzer GE, Bruce J, Kiefer DM, Kobusingye H, Drexler R, Besser RM, Confer DL, Horowitz MM, King RJ, Shaw BE, Riches M, Hayes-Lattin B, Linenberger M, Bolwell B, Rowley SD, Litzow MR, Pulsipher MA. Health-Related Quality of Life among Older Related Hematopoietic Stem Cell Donors (>60 Years) Is Equivalent to That of Younger Related Donors (18 to 60 Years): A Related Donor Safety Study. Biol Blood Marrow Transplant **2017**;23: 165-71.
40. Artz AS. Older patients/older donors: choosing wisely. Hematology Am Soc Hematol Educ Program **2013**;2013: 70-5.
41. Wang YT, Zhao XY, Zhao XS, Xu LP, Zhang XH, Wang Y, Liu KY, Chang YJ, Huang XJ. The impact of donor characteristics on the immune cell composition of mixture allografts of granulocyte-colony-stimulating factor-mobilized marrow harvests and peripheral blood harvests. Transfusion **2015**;55: 2874-81.
42. Canaani J, Savani BN, Labopin M, Huang XJ, Ciceri F, Arcese W, Tischer J, Koc Y, Bruno B, Gulbas Z, Blaise D, Maertens J, Ehninger G, Mohty M, Nagler A. Impact of ABO incompatibility on patients' outcome after haploidentical hematopoietic stem cell transplantation for acute myeloid leukemia - a report from the Acute Leukemia Working Party of the EBMT. Haematologica **2017**;102: 1066-74.
43. Ljungman P, Brand R, Hoek J, de la Camara R, Cordonnier C, Einsele H, Styczynski J, Ward KN, Cesaro S, Infectious Diseases Working Party of the European Group for B, Marrow T. Donor cytomegalovirus status influences the outcome of allogeneic stem cell transplant: a study by the European group for blood and marrow transplantation. Clin Infect Dis **2014**;59: 473-81.
44. Holig K, Kramer M, Kroschinsky F, Bornhauser M, Mengling T, Schmidt AH, Rutt C, Ehninger G. Safety and efficacy of hematopoietic stem cell collection from mobilized peripheral blood in unrelated volunteers: 12 years of single-center experience in 3928 donors. Blood **2009**;114: 3757-63.
45. Wang W, Huang H, Halagan M, Vierra-Green C, Heuer M, Brelsford JE, Haagenson M, Scheuermann RH, Telenti A, Biggs W, Pearson NM, Udell J, Spellman S, Maiers M, Kennedy CJ. Chromosome Y-encoded antigens associate with acute graft-versus-host disease in sex-mismatched stem cell transplant. Blood Adv **2018**;2: 2419-29.
46. Spierings E. Minor histocompatibility antigens: past, present, and future. Tissue Antigens **2014**;84: 374-60.
47. Martin PJ, Levine DM, Storer BE, Warren EH, Zheng X, Nelson SC, Smith AG, Mortensen BK, Hansen JA. Genome-wide minor histocompatibility matching as related to the risk of graft-versus-host disease. Blood **2017**;129: 791-8.
48. Faridi RM, Kemp TJ, Dharmani-Khan P, Lewis V, Tripathi G, Rajalingam R, Daly A, Berka N, Storek J, Masood Khan F. Donor-Recipient Matching for KIR Genotypes Reduces Chronic GVHD and Missing Inhibitory KIR Ligands Protect against Relapse after Myeloablative, HLA Matched Hematopoietic Cell Transplantation. PLoS One **2016**;11: e0158242.
49. Heidenreich S, Kroger N. Reduction of Relapse after Unrelated Donor Stem Cell Transplantation by KIR-Based Graft Selection. Front Immunol **2017**;8: 41.
50. Sieff CA, Niemeyer CM, Mentzer SJ, Faller DV. Interleukin-1, tumor necrosis factor, and the production of colony-stimulating factors by cultured mesenchymal cells. Blood **1988**;72: 1316-23.
51. Bazan JF. Haemopoietic receptors and helical cytokines. Immunol Today **1990**;11: 350-4.

52. Hill CP, Osslund TD, Eisenberg D. The structure of granulocyte-colony-stimulating factor and its relationship to other growth factors. Proc Natl Acad Sci U S A **1993**;90: 5167-71.
53. Metcalf D. The molecular control of cell division, differentiation commitment and maturation in haemopoietic cells. Nature **1989**;339: 27-30.
54. Demetri GD, Griffin JD. Granulocyte colony-stimulating factor and its receptor. Blood **1991**;78: 2791-808.
55. Avalos BR. Molecular analysis of the granulocyte colony-stimulating factor receptor. Blood **1996**;88: 761-77.
56. Layton JE, Hall NE. The interaction of G-CSF with its receptor. Front Biosci **2006**;11: 3181-9.
57. Palande K, Meenhuis A, Jevdjovic T, Touw IP. Scratching the surface: signaling and routing dynamics of the CSF3 receptor. Front Biosci (Landmark Ed) **2013**;18: 91-105.
58. Touw IP, van de Geijn GJ. Granulocyte colony-stimulating factor and its receptor in normal myeloid cell development, leukemia and related blood cell disorders. Front Biosci **2007**;12: 800-15.
59. Dwivedi P, Greis KD. Granulocyte colony-stimulating factor receptor signaling in severe congenital neutropenia, chronic neutrophilic leukemia, and related malignancies. Exp Hematol **2017**;46: 9-20.
60. Mehta HM, Futami M, Glaubach T, Lee DW, Andolina JR, Yang Q, Whichard Z, Quinn M, Lu HF, Kao WM, Przychodzen B, Sarkar CA, Minella A, Maciejewski JP, Corey SJ. Alternatively spliced, truncated GCSF receptor promotes leukemogenic properties and sensitivity to JAK inhibition. Leukemia **2014**;28: 1041-51.
61. Mielcarek M, Graf L, Johnson G, Torok-Storb B. Production of interleukin-10 by granulocyte colony-stimulating factor-mobilized blood products: A mechanism for monocyte-mediated suppression of T-cell proliferation. Blood **1998**;92: 215-22.
62. Sloand EM, Kim S, Maciejewski JP, Van Rhee F, Chaudhuri A, Barrett J, Young NS. Pharmacologic doses of granulocyte colony-stimulating factor affect cytokine production by lymphocytes in vitro and in vivo. Blood **2000**;95: 2269-74.
63. Morikawa K, Morikawa S, Nakamura M, Miyawaki T. Characterization of granulocyte colony-stimulating factor receptor expressed on human lymphocytes. Br J Haematol **2002**;118: 296-304.
64. Franzke A, Piao W, Lauber J, Gatzlaff P, Konecke C, Hansen W, Schmitt-Thomsen A, Hertenstein B, Buer J, Ganser A. G-CSF as immune regulator in T cells expressing the G-CSF receptor: implications for transplantation and autoimmune diseases. Blood **2003**;102: 734-9.
65. Friedenstein AJ, Chailakhyan RK, Latsinik NV, Panasyuk AF, Keiliss-Borok IV. Stromal cells responsible for transferring the microenvironment of the hemopoietic tissues. Cloning in vitro and retransplantation in vivo. Transplantation **1974**;17: 331-40.
66. Mendez-Ferrer S, Lucas D, Battista M, Frenette PS. Haematopoietic stem cell release is regulated by circadian oscillations. Nature **2008**;452: 442-7.
67. Massberg S, Schaerli P, Knezevic-Maramica I, Kollnberger M, Tubo N, Moseman EA, Huff IV, Junt T, Wagers AJ, Mazo IB, von Andrian UH. Immunosurveillance by hematopoietic progenitor cells trafficking through blood, lymph, and peripheral tissues. Cell **2007**;131: 994-1008.
68. Morici G, Zangla D, Santoro A, Pelosi E, Petrucci E, Gioia M, Bonanno A, Profita M, Bellia V, Testa U, Bonsignore MR. Supramaximal exercise mobilizes hematopoietic progenitors and reticulocytes in athletes. Am J Physiol Regul Integr Comp Physiol **2005**;289: R1496-503.
69. Kucia M, Zhang YP, Reca R, Wysoczynski M, Machalinski B, Majka M, Ildstad ST, Ratajczak J, Shields CB, Ratajczak MZ. Cells enriched in markers of neural tissue-committed stem cells reside in the bone marrow and are mobilized into the peripheral blood following stroke. Leukemia **2006**;20: 18-28.
70. Liu F, Poursine-Laurent J, Link DC. Expression of the G-CSF receptor on hematopoietic progenitor cells is not required for their mobilization by G-CSF. Blood **2000**;95: 3025-31.
71. Kollet O, Dar A, Shivtiel S, Kalinkovich A, Lapid K, Sztainberg Y, Tesio M, Samstein RM, Goichberg P, Spiegel A, Elson A, Lapidot T. Osteoclasts degrade endosteal components and promote mobilization of hematopoietic progenitor cells. Nat Med **2006**;12: 657-64.
72. Li S, Li T, Chen Y, Nie Y, Li C, Liu L, Li Q, Qiu L. Granulocyte Colony-Stimulating Factor Induces Osteoblast Inhibition by B Lymphocytes and Osteoclast Activation by T Lymphocytes during Hematopoietic Stem/Progenitor Cell Mobilization. Biol Blood Marrow Transplant **2015**;21: 1384-91.
73. Cashman J, Clark-Lewis I, Eaves A, Eaves C. Stromal-derived factor 1 inhibits the cycling of very primitive human hematopoietic cells in vitro and in NOD/SCID mice. Blood **2002**;99: 792-9.
74. Semerad CL, Christopher MJ, Liu F, Short B, Simmons PJ, Winkler I, Levesque JP, Chappel J, Ross FP, Link DC. G-CSF potently inhibits osteoblast activity and CXCL12 mRNA expression in the bone marrow. Blood **2005**;106: 3020-7.
75. Christopher MJ, Link DC. Granulocyte colony-stimulating factor induces osteoblast apoptosis and inhibits osteoblast differentiation. J Bone Miner Res **2008**;23: 1765-74.

76. Levesque JP, Hendy J, Takamatsu Y, Simmons PJ, Bendall LJ. Disruption of the CXCR4/CXCL12 chemotactic interaction during hematopoietic stem cell mobilization induced by GCSF or cyclophosphamide. J Clin Invest **2003**;111: 187-96.
77. Marquez-Curtis L, Jalili A, Deiteren K, Shirvaikar N, Lambeir AM, Janowska-Wieczorek A. Carboxypeptidase M expressed by human bone marrow cells cleaves the C-terminal lysine of stromal cell-derived factor-1alpha: another player in hematopoietic stem/progenitor cell mobilization? Stem Cells **2008**;26: 1211-20.
78. Christopherson KW, Cooper S, Hangoc G, Broxmeyer HE. CD26 is essential for normal G-CSF-induced progenitor cell mobilization as determined by CD26-/- mice. Exp Hematol **2003**;31: 1126-34.
79. Ross EA, Freeman S, Zhao Y, Dhanjal TS, Ross EJ, Lax S, Ahmed Z, Hou TZ, Kalia N, Egginton S, Nash G, Watson SP, Frampton J, Buckley CD. A novel role for PECAM-1 (CD31) in regulating haematopoietic progenitor cell compartmentalization between the peripheral blood and bone marrow. PLoS One **2008**;3: e2338.
80. Tesio M, Golan K, Corso S, Giordano S, Schajnovitz A, Vagima Y, Shivtiel S, Kalinkovich A, Caione L, Gammaitoni L, Laurenti E, Buss EC, Shezen E, Itkin T, Kollet O, Petit I, Trumpp A, Christensen J, Aglietta M, Piacibello W, Lapidot T. Enhanced c-Met activity promotes G-CSF-induced mobilization of hematopoietic progenitor cells via ROS signaling. Blood **2011**;117: 419-28.
81. Katayama Y, Battista M, Kao WM, Hidalgo A, Peired AJ, Thomas SA, Frenette PS. Signals from the sympathetic nervous system regulate hematopoietic stem cell egress from bone marrow. Cell **2006**;124: 407-21.
82. Levesque JP, Hendy J, Takamatsu Y, Williams B, Winkler IG, Simmons PJ. Mobilization by either cyclophosphamide or granulocyte colony-stimulating factor transforms the bone marrow into a highly proteolytic environment. Exp Hematol **2002**;30: 440-9.
83. Petit I, Szyper-Kravitz M, Nagler A, Lahav M, Peled A, Habler L, Ponomaryov T, Taichman RS, Arenzana-Seisdedos F, Fujii N, Sandbank J, Zipori D, Lapidot T. G-CSF induces stem cell mobilization by decreasing bone marrow SDF-1 and up-regulating CXCR4. Nat Immunol **2002**;3: 687-94.
84. Levesque JP, Hendy J, Winkler IG, Takamatsu Y, Simmons PJ. Granulocyte colony-stimulating factor induces the release in the bone marrow of proteases that cleave c-KIT receptor (CD117) from the surface of hematopoietic progenitor cells. Exp Hematol **2003**;31: 109-17.
85. Adamiak M, Abdelbaset-Ismail A, Suszynska M, Abdel-Latif A, Ratajczak J, Ratajczak MZ. Novel evidence that the mannan-binding lectin pathway of complement activation plays a pivotal role in triggering mobilization of hematopoietic stem/progenitor cells by activation of both the complement and coagulation cascades. Leukemia **2017**;31: 262-5.
86. Ratajczak MZ, Adamiak M, Plonka M, Abdel-Latif A, Ratajczak J. Mobilization of hematopoietic stem cells as a result of innate immunity-mediated sterile inflammation in the bone marrow microenvironment-the involvement of extracellular nucleotides and purinergic signaling. Leukemia **2018**;32: 1116-23.
87. Ratajczak MZ, Reca R, Wysoczynski M, Kucia M, Baran JT, Allendorf DJ, Ratajczak J, Ross GD. Transplantation studies in C3-deficient animals reveal a novel role of the third complement component (C3) in engraftment of bone marrow cells. Leukemia **2004**;18: 1482-90.
88. Jalili A, Shirvaikar N, Marquez-Curtis L, Qiu Y, Korol C, Lee H, Turner AR, Ratajczak MZ, Janowska-Wieczorek A. Fifth complement cascade protein (C5) cleavage fragments disrupt the SDF-1/CXCR4 axis: further evidence that innate immunity orchestrates the mobilization of hematopoietic stem/progenitor cells. Exp Hematol **2010**;38: 321-32.
89. Ratajczak MZ, Lee H, Wysoczynski M, Wan W, Marlicz W, Laughlin MJ, Kucia M, Janowska-Wieczorek A, Ratajczak J. Novel insight into stem cell mobilization-plasma sphingosine-1-phosphate is a major chemoattractant that directs the egress of hematopoietic stem progenitor cells from the bone marrow and its level in peripheral blood increases during mobilization due to activation of complement cascade/membrane attack complex. Leukemia **2010**;24: 976-85.
90. Golan K, Vagima Y, Ludin A, Itkin T, Cohen-Gur S, Kalinkovich A, Kollet O, Kim C, Schajnovitz A, Ovadya Y, Lapid K, Shivtiel S, Morris AJ, Ratajczak MZ, Lapidot T. S1P promotes murine progenitor cell egress and mobilization via S1P1-mediated ROS signaling and SDF-1 release. Blood **2012**;119: 2478-88.
91. Korbling M, Huh YO, Durett A, Mirza N, Miller P, Engel H, Anderlini P, van Besien K, Andreeff M, Przepiorka D, Deisseroth AB, Champlin RE. Allogeneic blood stem cell transplantation: peripheralization and yield of donor-derived primitive hematopoietic progenitor cells (CD34+ Thy-1dim) and lymphoid subsets, and possible predictors of engraftment and graft-versus-host disease. Blood **1995**;86: 2842-8.

92. Pulsipher MA, Chitphakdithai P, Logan BR, Shaw BE, Wingard JR, Lazarus HM, Waller EK, Seftel M, Stroncek DF, Lopez AM, Maharaj D, Hematti P, O'Donnell PV, Loren AW, Leitman SF, Anderlini P, Goldstein SC, Levine JE, Navarro WH, Miller JP, Confer DL. Acute toxicities of unrelated bone marrow versus peripheral blood stem cell donation: results of a prospective trial from the National Marrow Donor Program. Blood **2013**;121: 197-206.
93. Rhodes B, Anderlini P. Allogeneic peripheral blood stem cell collection as of 2008. Transfus Apher Sci **2008**;38: 219-27.
94. Anderlini P, Donato M, Chan KW, Huh YO, Gee AP, Lauppe MJ, Champlin RE, Korbling M. Allogeneic blood progenitor cell collection in normal donors after mobilization with filgrastim: the M.D. Anderson Cancer Center experience. Transfusion **1999**;39: 555-60.
95. Holig K. G-CSF in Healthy Allogeneic Stem Cell Donors. Transfus Med Hemother **2013**;40: 225-35.
96. Tayebi H, Kuttler F, Saas P, Lienard A, Petracca B, Lapierre V, Ferrand C, Fest T, Cahn J, Blaise D, Kuentz M, Herve P, Tiberghien P, Robinet E. Effect of granulocyte colony-stimulating factor mobilization on phenotypical and functional properties of immune cells. Exp Hematol **2001**;29: 458-70.
97. Theilgaard-Monch K, Raaschou-Jensen K, Palm H, Schjodt K, Heilmann C, Vindelov L, Jacobsen N, Dickmeiss E. Flow cytometric assessment of lymphocyte subsets, lymphoid progenitors, and hematopoietic stem cells in allogeneic stem cell grafts. Bone Marrow Transplant **2001**;28: 1073-82.
98. Sapoznikov A, Pewzner-Jung Y, Kalchenko V, Krauthgamer R, Shachar I, Jung S. Perivascular clusters of dendritic cells provide critical survival signals to B cells in bone marrow niches. Nat Immunol **2008**;9: 388-95.
99. Libregts SF, Nolte MA. Parallels between immune driven-hematopoiesis and T cell activation: 3 signals that relay inflammatory stress to the bone marrow. Exp Cell Res **2014**;329: 239-47.
100. Martins A, Han J, Kim SO. The multifaceted effects of granulocyte colony-stimulating factor in immunomodulation and potential roles in intestinal immune homeostasis. IUBMB Life **2010**;62: 611-7.
101. Dunn SM, Coles LS, Lang RK, Gerondakis S, Vadas MA, Shannon MF. Requirement for nuclear factor (NF)-kappa B p65 and NF-interleukin-6 binding elements in the tumor necrosis factor response region of the granulocyte colony-stimulating factor promoter. Blood **1994**;83: 2469-79.
102. Cai XY, Gommoll CP, Jr., Justice L, Narula SK, Fine JS. Regulation of granulocyte colony-stimulating factor gene expression by interleukin-17. Immunol Lett **1998**;62: 51-8.
103. Schwarzenberger P, Huang W, Ye P, Oliver P, Manuel M, Zhang Z, Bagby G, Nelson S, Kolls JK. Requirement of endogenous stem cell factor and granulocyte-colony-stimulating factor for IL-17-mediated granulopoiesis. J Immunol **2000**;164: 4783-9.
104. Bajrami B, Zhu H, Kwak HJ, Mondal S, Hou Q, Geng G, Karatepe K, Zhang YC, Nombela-Arrieta C, Park SY, Loison F, Sakai J, Xu Y, Silberstein LE, Luo HR. G-CSF maintains controlled neutrophil mobilization during acute inflammation by negatively regulating CXCR2 signaling. J Exp Med **2016**;213: 1999-2018.
105. Kudo T, Matsumoto T, Nakamichi I, Yada S, Esaki M, Jo Y, Ohji Y, Yao T, Iida M. Recombinant human granulocyte colony-stimulating factor reduces colonic epithelial cell apoptosis and ameliorates murine dextran sulfate sodium-induced colitis. Scand J Gastroenterol **2008**;43: 689-97.
106. Valente JF, Alexander JW, Li BG, Noel JG, Custer DA, Ogle JD, Ogle CK. Effect of in vivo infusion of granulocyte colony-stimulating factor on immune function. Shock **2002**;17: 23-9.
107. Hebert JC, O'Reilly M, Yuenger K, Shatney L, Yoder DW, Barry B. Augmentation of alveolar macrophage phagocytic activity by granulocyte colony stimulating factor and interleukin-1: influence of splenectomy. J Trauma **1994**;37: 909-12.
108. Miller JS, Prosper F, McCullar V. Natural killer (NK) cells are functionally abnormal and NK cell progenitors are diminished in granulocyte colony-stimulating factor-mobilized peripheral blood progenitor cell collections. Blood **1997**;90: 3098-105.
109. Reca R, Cramer D, Yan J, Laughlin MJ, Janowska-Wieczorek A, Ratajczak J, Ratajczak MZ. A novel role of complement in mobilization: immunodeficient mice are poor granulocyte-colony stimulating factor mobilizers because they lack complement-activating immunoglobulins. Stem Cells **2007**;25: 3093-100.
110. Wen Q, Kong Y, Zhao HY, Zhang YY, Han TT, Wang Y, Xu LP, Zhang XH, Huang XJ. G-CSF-induced macrophage polarization and mobilization may prevent acute graft-versus-host disease after allogeneic hematopoietic stem cell transplantation. Bone Marrow Transplant **2019**.
111. Arpinati M, Green CL, Heimfeld S, Heuser JE, Anasetti C. Granulocyte-colony stimulating factor mobilizes T helper 2-inducing dendritic cells. Blood **2000**;95: 2484-90.
112. Vuckovic S, Kim M, Khalil D, Turtle CJ, Crosbie GV, Williams N, Brown L, Williams K, Kelly C, Stravos P, Rodwell R, Hill GR, Wright S, Taylor K, Gill D, Marlton P, Bradstock K, Hart DN.

Granulocyte-colony stimulating factor increases CD123hi blood dendritic cells with altered CD62L and CCR7 expression. Blood **2003**;101: 2314-7.
113. Tayebi H, Lapierre V, Saas P, Lienard A, Sutton L, Milpied N, Attal M, Cahn JY, Kuentz M, Blaise D, Herve P, Tiberghien P, Robinet E, Societe Francaise de Greffe de Moelle et de Therapie C. Enhanced activation of B cells in a granulocyte colony-stimulating factor-mobilized peripheral blood stem cell graft. Br J Haematol **2001**;114: 698-700.
114. Rutella S, Pierelli L, Bonanno G, Sica S, Ameglio F, Capoluongo E, Mariotti A, Scambia G, d'Onofrio G, Leone G. Role for granulocyte colony-stimulating factor in the generation of human T regulatory type 1 cells. Blood **2002**;100: 2562-71.
115. Sun LX, Ren HY, Shi YJ, Wang LH, Qiu ZX. Recombinant human granulocyte colony-stimulating factor significantly decreases the expression of CXCR3 and CCR6 on T cells and preferentially induces T helper cells to a T helper 17 phenotype in peripheral blood harvests. Biol Blood Marrow Transplant **2009**;15: 835-43.
116. Toh HC, Sun L, Soe Y, Wu Y, Phoon YP, Chia WK, Wu J, Wong KY, Tan P. G-CSF induces a potentially tolerant gene and immunophenotype profile in T cells in vivo. Clin Immunol **2009**;132: 83-92.
117. Tanaka J, Tutumi Y, Li Z, Katho N, Sugita J, Mori A, Toyoshima N, Ota S, Kobayashi M, Kasai M, Asaka M, Imamura M. Induction of CD94/NKG2A expression on T cells in mixed lymphocyte culture by CD14+ cells from granulocyte colony-stimulating factor-mobilized peripheral blood mononuclear cells. Br J Haematol **2002**;117: 751-4.
118. Blache C, Chauvin JM, Marie-Cardine A, Contentin N, Pommier P, Dedreux I, Francois S, Jacquot S, Bastit D, Boyer O. Reduced frequency of regulatory T cells in peripheral blood stem cell compared to bone marrow transplantations. Biol Blood Marrow Transplant **2010**;16: 430-4.
119. Vela-Ojeda J, Montiel-Cervantes L, Granados-Lara P, Reyes-Maldonado E, Garcia-Latorre E, Garcia-Chavez J, Majluf-Cruz A, Mayani H, Borbolla-Escoboza JR, Esparza MG. Role of CD4+CD25+highFoxp3+CD62L+ regulatory T cells and invariant NKT cells in human allogeneic hematopoietic stem cell transplantation. Stem Cells Dev **2010**;19: 333-40.
120. Ding L, Zhu H, Yang Y, Yan HM, Zhang HH, Han DM, Wang ZD, Zheng XL, Liu J, Zhu L, Mei X, Guo ZK, Wang HX. The absolute number of regulatory T cells in unmanipulated peripheral blood grafts predicts the occurrence of acute graft-versus-host disease post haplo-identical hematopoietic stem cell transplantation. Leuk Res **2017**;56: 13-20.
121. Khalili JS, Karandish S, Bryan S, Molldrem J, McMannis J, Komanduri KV. 355: GCSF Decreases CD4+CD25+CD127lo Regulatory T Cell Proliferation Index in Stem Cell DonorsBiology of Blood and Marrow Transplantation **2008**;14: 130.
122. Xuan L, Wu X, Qiu D, Gao L, Liu H, Fan Z, Huang F, Jin Z, Sun J, Li Y, Liu Q. Regulatory gammadelta T cells induced by G-CSF participate in acute graft-versus-host disease regulation in G-CSF-mobilized allogeneic peripheral blood stem cell transplantation. J Transl Med **2018**;16: 144.
123. Vendramin A, Gimondi S, Bermema A, Longoni P, Rizzitano S, Corradini P, Carniti C. Graft monocytic myeloid-derived suppressor cell content predicts the risk of acute graft-versus-host disease after allogeneic transplantation of granulocyte colony-stimulating factor-mobilized peripheral blood stem cells. Biol Blood Marrow Transplant **2014**;20: 2049-55.
124. Lv M, Zhao XS, Hu Y, Chang YJ, Zhao XY, Kong Y, Zhang XH, Xu LP, Liu KY, Huang XJ. Monocytic and promyelocytic myeloid-derived suppressor cells may contribute to G-CSF-induced immune tolerance in haplo-identical allogeneic hematopoietic stem cell transplantation. Am J Hematol **2015**;90: E9-E16.
125. D'Aveni M, Rossignol J, Coman T, Sivakumaran S, Henderson S, Manzo T, Santos e Sousa P, Bruneau J, Fouquet G, Zavala F, Alegria-Prevot O, Garfa-Traore M, Suarez F, Trebeden-Negre H, Mohty M, Bennett CL, Chakraverty R, Hermine O, Rubio MT. G-CSF mobilizes CD34+ regulatory monocytes that inhibit graft-versus-host disease. Sci Transl Med **2015**;7: 281ra42.
126. Collin M, Bigley V. Human dendritic cell subsets: an update. Immunology **2018**;154: 3-20.
127. Dreger P, Haferlach T, Eckstein V, Jacobs S, Suttorp M, Loffler H, Muller-Ruchholtz W, Schmitz N. G-CSF-mobilized peripheral blood progenitor cells for allogeneic transplantation: safety, kinetics of mobilization, and composition of the graft. Br J Haematol **1994**;87: 609-13.
128. Kroschinsky F, Holig K, Poppe-Thiede K, Zimmer K, Ordemann R, Blechschmidt M, Oelschlaegel U, Bornhauser M, Rall G, Rutt C, Ehninger G. Single-dose pegfilgrastim for the mobilization of allogeneic CD34+ peripheral blood progenitor cells in healthy family and unrelated donors. Haematologica **2005**;90: 1665-71.
129. Holzmann J, Balser S, Windisch J. Totality of the evidence at work: The first U.S. biosimilar. Expert Opin Biol Ther **2016**;16: 137-42.

130. Bonig H, Becker PS, Schwebig A, Turner M. Biosimilar granulocyte-colony-stimulating factor for healthy donor stem cell mobilization: need we be afraid? Transfusion **2015**;55: 430-9.
131. Schmitt M, Hoffmann JM, Lorenz K, Publicover A, Schmitt A, Nagler A. Mobilization of autologous and allogeneic peripheral blood stem cells for transplantation in haematological malignancies using biosimilar G-CSF. Vox Sang **2016**;111: 178-86.
132. Pahnke S, Egeland T, Halter J, Hagglund H, Shaw BE, Woolfrey AE, Szer J. Current use of biosimilar G-CSF for haematopoietic stem cell mobilisation. Bone Marrow Transplant **2018**.
133. Patel MM, Shah PJ, Patel BM. Insights of biosimilars through SWOT analysis. Expert Opin Biol Ther **2014**;14: 139-44.
134. Stroncek DF, Clay ME, Jaszcz W, Lennon S, Smith J, McCullough J. Collection of two peripheral blood stem cell concentrates from healthy donors. Transfus Med **1999**;9: 37-50.
135. Duong HK, Savani BN, Copelan E, Devine S, Costa LJ, Wingard JR, Shaughnessy P, Majhail N, Perales MA, Cutler CS, Bensinger W, Litzow MR, Mohty M, Champlin RE, Leather H, Giralt S, Carpenter PA. Peripheral blood progenitor cell mobilization for autologous and allogeneic hematopoietic cell transplantation: guidelines from the American Society for Blood and Marrow Transplantation. Biol Blood Marrow Transplant **2014**;20: 1262-73.
136. Hoglund M, Smedmyr B, Simonsson B, Totterman T, Bengtsson M. Dose-dependent mobilisation of haematopoietic progenitor cells in healthy volunteers receiving glycosylated rHuG-CSF. Bone Marrow Transplant **1996**;18: 19-27.
137. Engelhardt M, Bertz H, Afting M, Waller CF, Finke J. High-versus standard-dose filgrastim (rhG-CSF) for mobilization of peripheral-blood progenitor cells from allogeneic donors and CD34(+) immunoselection. J Clin Oncol **1999**;17: 2160-72.
138. Kroger N, Renges H, Sonnenberg S, Kruger W, Gutensohn K, Dielschneider T, Cortes-Dericks L, Zander AR. Stem cell mobilisation with 16 microg/kg vs 10 microg/kg of G-CSF for allogeneic transplantation in healthy donors. Bone Marrow Transplant **2002**;29: 727-30.
139. Arbona C, Prosper F, Benet I, Mena F, Solano C, Garcia-Conde J. Comparison between once a day vs twice a day G-CSF for mobilization of peripheral blood progenitor cells (PBPC) in normal donors for allogeneic PBPC transplantation. Bone Marrow Transplant **1998**;22: 39-45.
140. Molineux G. The design and development of pegfilgrastim (PEG-rmetHuG-CSF, Neulasta). Curr Pharm Des **2004**;10: 1235-44.
141. Moog R. Apheresis techniques for collection of peripheral blood progenitor cells. Transfus Apher Sci **2004**;31: 207-20.
142. Brauninger S, Bialleck H, Thorausch K, Felt T, Seifried E, Bonig H. Allogeneic donor peripheral blood "stem cell" apheresis: prospective comparison of two apheresis systems. Transfusion **2012**;52: 1137-45.
143. Ikeda K, Minakawa K, Muroi K, Fujiwara SI, Yamada-Fujiwara M, Fujimori Y, Tanosaki R, Ohto H, Study Group for Peripheral Blood Stem Cell Collection CTCTJSoTM, Cell T. Prospective randomized and crossover comparison of two apheresis machines for peripheral blood stem cell collection: a multicenter study. Transfusion **2016**;56: 2839-47.
144. Wang T, Remberger M, Axdorph Nygell U, Sundin M, Bjorklund A, Mattsson J, Uhlin M, Watz E. Change of apheresis device decreased the incidence of severe acute graft-versus-host disease among patients after allogeneic stem cell transplantation with sibling donors. Transfusion **2018**;58: 1442-51.
145. Wu FY, Heng KK, Salleh RB, Soh TG, Lee JJ, Mah J, Linn YC, Loh Y, Hwang W, Tan LK, Law P, Goh YT. Comparing peripheral blood stem cell collection using the COBE Spectra, Haemonetics MCS+, and Baxter Amicus. Transfus Apher Sci **2012**;47: 345-50.
146. Reddy RL. Mobilization and collection of peripheral blood progenitor cells for transplantation. Transfus Apher Sci **2005**;32: 63-72.
147. Keeney M, Chin-Yee I, Weir K, Popma J, Nayar R, Sutherland DR. Single platform flow cytometric absolute CD34+ cell counts based on the ISHAGE guidelines. International Society of Hematotherapy and Graft Engineering. Cytometry **1998**;34: 61-70.
148. Machaczka M, Hagglund H, Staver E, Joks M, Hassan M, Wahlin BE, Axdorph Nygell U. G-CSF mobilized peripheral blood stem cell collection for allogeneic transplantation in healthy donors: Analysis of factors affecting yield. J Clin Apher **2017**;32: 384-91.
149. Gasova Z, Marinov I, Vodvarkova S, Bohmova M, Bhuyian-Ludvikova Z. PBPC collection techniques: standard versus large volume leukapheresis (LVL) in donors and in patients. Transfus Apher Sci **2005**;32: 167-76.
150. Dzieczkowski JS, McGonigal M, Cook J, Sugrue M, Andersen J, Anderson KC. A comparison of peripheral blood stem cell apheresis using the Fenwal CS3000 Plus and COBE Spectra. Transfus Sci **1995**;16: 71-7.

151. Brauninger S, Bialleck H, Thorausch K, Seifried E, Bonig H. Mobilized allogeneic peripheral stem/progenitor cell apheresis with Spectra Optia v.5.0, a novel, automatic interface-controlled apheresis system: results from the first feasibility trial. Vox Sang **2011**;101: 237-46.
152. Switzer GE, Bruce JG, Harrington D, Haagenson M, Drexler R, Foley A, Confer D, Bishop M, Anderlini P, Rowley S, Leitman SF, Anasetti C, Wingard JR. Health-related quality of life of bone marrow versus peripheral blood stem cell donors: a prespecified subgroup analysis from a phase III RCT-BMTCTN protocol 0201. Biol Blood Marrow Transplant **2014**;20: 118-27.
153. Neyrinck MM, Vrielink H, Joint Task Force for E, Certification. Calculations in apheresis. J Clin Apher **2015**;30: 38-42.
154. Moog R, Muller N. Technical aspects and performance in collecting peripheral blood progenitor cells. Ann Hematol **1998**;77: 143-7.
155. Pulsipher MA, Chitphakdithai P, Miller JP, Logan BR, King RJ, Rizzo JD, Leitman SF, Anderlini P, Haagenson MD, Kurian S, Klein JP, Horowitz MM, Confer DL. Adverse events among 2408 unrelated donors of peripheral blood stem cells: results of a prospective trial from the National Marrow Donor Program. Blood **2009**;113: 3604-11.
156. Holig K, Blechschmidt M, Kramer M, Zimmer K, Kroschinsky F, Poppe-Thiede K, Bornhauser M, Ehninger G. Peripheral blood stem cell collection in allogeneic donors: impact of venous access. Transfusion **2012**;52: 2600-5.
157. Sadler DJ, Gordon AC, Klassen J, Saliken JC, So CB, Gray RR. Image-guided central venous catheters for apheresis. Bone Marrow Transplant **1999**;23: 179-82.
158. Lee G, Arepally GM. Anticoagulation techniques in apheresis: from heparin to citrate and beyond. J Clin Apher **2012**;27: 117-25.
159. Monroe DM, Hoffman M. What does it take to make the perfect clot? Arterioscler Thromb Vasc Biol **2006**;26: 41-8.
160. Rosenbaum ER, O'Connell B, Cottler-Fox M. Validation of a formula for predicting daily CD34(+) cell collection by leukapheresis. Cytotherapy **2012**;14: 461-6.
161. Deneys V, Fabry A, Van Hooydonk M, Sonet A, Andre M, Bourgeois M, Botson F. Efficiency of autologous stem cell collection: Comparison of three different cell separators. Transfus Apher Sci **2017**;56: 35-8.
162. Martin-Antonio B, Carmona M, Falantes J, Gil E, Baez A, Suarez M, Marin P, Espigado I, Urbano-Ispizua A. Impact of constitutional polymorphisms in VCAM1 and CD44 on CD34+ cell collection yield after administration of granulocyte colony-stimulating factor to healthy donors. Haematologica **2011**;96: 102-9.
163. Maie K, Fuji S, Tajima K, Tatsuno M, Yamagata S, Takahashi N, Ueda R, Hashimoto H, Takano K, Inoue Y, Ito A, Hayashi Y, Okinaka K, Kurosawa S, Kim SW, Tanosaki R, Heike Y, Yamashita T, Fukuda T. A higher number of infused CD34(+) cells has a positive impact on the clinical outcome after related PBSC transplantation. Bone Marrow Transplant **2014**;49: 1113-5.
164. Pulsipher MA, Chitphakdithai P, Logan BR, Leitman SF, Anderlini P, Klein JP, Horowitz MM, Miller JP, King RJ, Confer DL. Donor, recipient, and transplant characteristics as risk factors after unrelated donor PBSC transplantation: beneficial effects of higher CD34+ cell dose. Blood **2009**;114: 2606-16.
165. Suzuya H, Watanabe T, Nakagawa R, Watanabe H, Okamoto Y, Onishi T, Abe T, Kawano Y, Kagami S, Takaue Y. Factors associated with granulocyte colony-stimulating factor-induced peripheral blood stem cell yield in healthy donors. Vox Sang **2005**;89: 229-35.
166. Liles WC, Rodger E, Broxmeyer HE, Dehner C, Badel K, Calandra G, Christensen J, Wood B, Price TH, Dale DC. Augmented mobilization and collection of CD34+ hematopoietic cells from normal human volunteers stimulated with granulocyte-colony-stimulating factor by single-dose administration of AMD3100, a CXCR4 antagonist. Transfusion **2005**;45: 295-300.
167. Lemery SJ, Hsieh MM, Smith A, Rao S, Khuu HM, Theresa D, Viano JM, Cook L, Goodwin R, Boss C, Calandra G, Geller N, Tisdale J, Childs R. A pilot study evaluating the safety and CD34+ cell mobilizing activity of escalating doses of plerixafor in healthy volunteers. Br J Haematol **2011**;153: 66-75.
168. Nadeau M, George L, Yeager AM, Anwer F, McBride A. Plerixafor as a salvage mobilization strategy for haploidentical peripheral blood allogeneic stem cell transplantation. Clin Case Rep **2015**;3: 728-30.
169. Devine SM, Vij R, Rettig M, Todt L, McGlauchlen K, Fisher N, Devine H, Link DC, Calandra G, Bridger G, Westervelt P, Dipersio JF. Rapid mobilization of functional donor hematopoietic cells without G-CSF using AMD3100, an antagonist of the CXCR4/SDF-1 interaction. Blood **2008**;112: 990-8.

170. Pantin J, Purev E, Tian X, Cook L, Donohue-Jerussi T, Cho E, Reger R, Hsieh M, Khuu H, Calandra G, Geller NL, Childs RW. Effect of high-dose plerixafor on CD34(+) cell mobilization in healthy stem cell donors: results of a randomized crossover trial. Haematologica **2017**;102: 600-9.
171. Chen YB, Le-Rademacher J, Brazauskas R, Kiefer DM, Hamadani M, DiPersio JF, Litzow MR, Craig M, Horwitz ME, Artz AS, McClune BL, Fernandez HF, Duong HK, Kobusingye H, Proue M, Drexler RJ, Horowitz MM, Shaw BE, Miller JP, Hosoba S, Waller EK, Devine SM. Plerixafor alone for the mobilization and transplantation of HLA-matched sibling donor hematopoietic stem cells. Blood Adv **2019**;3: 875-83.
172. Schroeder MA, Rettig MP, Lopez S, Christ S, Fiala M, Eades W, Mir FA, Shao J, McFarland K, Trinkaus K, Shannon W, Deych E, Yu J, Vij R, Stockerl-Goldstein K, Cashen AF, Uy GL, Abboud CN, Westervelt P, DiPersio JF. Mobilization of allogeneic peripheral blood stem cell donors with intravenous plerixafor mobilizes a unique graft. Blood **2017**;129: 2680-92.
173. Jeker B, Novak U, Mansouri Taleghani B, Baerlocher GM, Seipel K, Mueller BU, Bigler M, Betticher D, Luethi JM, Farese S, Ruefer A, Pabst T. NSAID treatment with meloxicam enhances peripheral stem cell mobilization in myeloma. Bone Marrow Transplant **2017**.
174. Emmons R, Niemiro GM, De Lisio M. Exercise as an Adjuvant Therapy for Hematopoietic Stem Cell Mobilization. Stem Cells Int **2016**;2016: 7131359.
175. Lucas D, Battista M, Shi PA, Isola L, Frenette PS. Mobilized hematopoietic stem cell yield depends on species-specific circadian timing. Cell Stem Cell **2008**;3: 364-6.
176. Lucas D, Bruns I, Battista M, Mendez-Ferrer S, Magnon C, Kunisaki Y, Frenette PS. Norepinephrine reuptake inhibition promotes mobilization in mice: potential impact to rescue low stem cell yields. Blood **2012**;119: 3962-5.
177. Sugiyama A, Yujiri T, Tanaka M, Tanaka Y, Nakamura Y, Tanizawa Y. Altered expression of circadian clock genes during peripheral blood stem cell mobilization induced by granulocyte colony-stimulating factor. Chronobiol Int **2015**;32: 934-41.
178. de Haas M, Kerst JM, van der Schoot CE, Calafat J, Hack CE, Nuijens JH, Roos D, van Oers RH, von dem Borne AE. Granulocyte colony-stimulating factor administration to healthy volunteers: analysis of the immediate activating effects on circulating neutrophils. Blood **1994**;84: 3885-94.
179. Watari K, Asano S, Shirafuji N, Kodo H, Ozawa K, Takaku F, Kamachi S. Serum granulocyte colony-stimulating factor levels in healthy volunteers and patients with various disorders as estimated by enzyme immunoassay. Blood **1989**;73: 117-22.
180. Pauksen K, Elfman L, Ulfgren AK, Venge P. Serum levels of granulocyte-colony stimulating factor (G-CSF) in bacterial and viral infections, and in atypical pneumonia. Br J Haematol **1994**;88: 256-60.
181. Siddiq S, Pamphilon D, Brunskill S, Doree C, Hyde C, Stanworth S. Bone marrow harvest versus peripheral stem cell collection for haemopoietic stem cell donation in healthy donors. Cochrane Database Syst Rev **2009**: CD006406.
182. Pamphilon D, Siddiq S, Brunskill S, Doree C, Hyde C, Horowitz M, Stanworth S. Stem cell donation--what advice can be given to the donor? Br J Haematol **2009**;147: 71-6.
183. Tassi C, Tazzari PL, Bonifazi F, Giudice V, Nannetti A, Ricci F, Rizzi S, Bandini G, Conte R. Short- and long-term haematological surveillance of healthy donors of allogeneic peripheral haematopoietic progenitors mobilized with G-CSF: a single institution prospective study. Bone Marrow Transplant **2005**;36: 289-94.
184. Storek J, Dawson MA, Maloney DG. Normal T, B, and NK cell counts in healthy donors at 1 year after blood stem cell harvesting. Blood **2000**;95: 2993-4.
185. Anderlini P, Przepiorka D, Seong D, Miller P, Sundberg J, Lichtiger B, Norfleet F, Chan KW, Champlin R, Korbling M. Clinical toxicity and laboratory effects of granulocyte-colony-stimulating factor (filgrastim) mobilization and blood stem cell apheresis from normal donors, and analysis of charges for the procedures. Transfusion **1996**;36: 590-5.
186. Strauss RG. Mechanisms of adverse effects during hemapheresis. J Clin Apher **1996**;11: 160-4.
187. Majolino I, Cavallaro AM, Bacigalupo A, Rambaldi A, Falda M, Locatelli F, Raimondi R, Pucci G, Mordini N, Barbui T, Locatelli F, Rodeghiero F, Irrera G, Indovina A, Valbonesi M, Bellavita P, Tassi V, Perotti C, Soli M, Bresolin G. Mobilization and collection of PBSC in healthy donors: a retrospective analysis of the Italian Bone Marrow Transplantation Group (GITMO). Haematologica **1997**;82: 47-52.
188. Bandarenko N, Brecher ME, Owen H, Wiley J, Shea T, Serody J. Thrombocytopenia in allogeneic peripheral blood stem cell collections. Transfusion **1996**;36: 668-9.
189. de la Rubia J, de Arriba F, Arbona C, Pascual MJ, Zamora C, Insunza A, Martinez D, Paniagua C, Diaz MA, Sanz MA. Follow-up of healthy donors receiving granulocyte colony-stimulating factor for peripheral blood progenitor cell mobilization and collection. Results of the Spanish Donor Registry. Haematologica **2008**;93: 735-40.

190. Lasky LC, Lin A, Kahn RA, McCullough J. Donor platelet response and product quality assurance in plateletpheresis. Transfusion **1981**;21: 247-60.
191. Stroncek DF, Clay ME, Smith J, Ilstrup S, Oldham F, McCullough J. Changes in blood counts after the administration of granulocyte-colony-stimulating factor and the collection of peripheral blood stem cells from healthy donors. Transfusion **1996**;36: 596-600.
192. Avenarius HJ, Freund M, Deinhardt J, Poliwoda H. Effect of recombinant human granulocyte colony-stimulating factor (rhG-CSF) on circulating platelets. Ann Hematol **1992**;65: 6-9.
193. Shimoda K, Okamura S, Inaba S, Okamura T, Ohga S, Ueda K, Niho Y. Granulocyte colony-stimulating factor and platelet aggregation. Lancet **1993**;341: 633.
194. LeBlanc R, Roy J, Demers C, Vu L, Cantin G. A prospective study of G-CSF effects on hemostasis in allogeneic blood stem cell donors. Bone Marrow Transplant **1999**;23: 991-6.
195. Halter J, Kodera Y, Ispizua AU, Greinix HT, Schmitz N, Favre G, Baldomero H, Niederwieser D, Apperley JF, Gratwohl A. Severe events in donors after allogeneic hematopoietic stem cell donation. Haematologica **2009**;94: 94-101.
196. Agishi T. Birth of the concept and the development of extracorporeal immunomodulation. Ther Apher **2002**;6: 312-6.
197. Hagberg IA, Akkok CA, Lyberg T, Kjeldsen-Kragh J. Apheresis-induced platelet activation:comparison of three types of cell separators. Transfusion **2000**;40: 182-92.
198. Macher S, Sipurzynski-Budrass S, Rosskopf K, Rohde E, Griesbacher A, Groselj-Strele A, Lanzer G, Schallmoser K. Function and activation state of platelets in vitro depend on apheresis modality. Vox Sang **2010**;99: 332-40.
199. Weiss R, Eichhorn T, Spittler A, Micusik M, Fischer MB, Weber V. Release and cellular origin of extracellular vesicles during circulation of whole blood over adsorbent polymers for lipid apheresis. J Biomed Mater Res B Appl Biomater **2017**;105: 636-46.
200. Ghio M, Contini P, Ansaldi F, Ubezio G, Setti M, Risso M, Tripodi G. A possible role of soluble HLA-I molecule in the immunomodulatory effects of therapeutic apheresis. Blood Transfus **2014**;12 Suppl 1: s167-9.
201. Ghio M, Contini P, Ansaldi F, Ubezio G, Setti M, Risso M, Tripodi G. Immunomodulation due to plasma or plasma-platelet apheresis donation: Events occurring during donation procedures. J Clin Apher **2015**;30: 204-11.
202. Huang Z, Gao D, Letteri JJ, Clark WR. Blood-membrane interactions during dialysis. Semin Dial **2009**;22: 623-8.
203. Mortzell Henriksson M, Newman E, Witt V, Derfler K, Leitner G, Eloot S, Dhondt A, Deeren D, Rock G, Ptak J, Blaha M, Lanska M, Gasova Z, Hrdlickova R, Ramlow W, Prophet H, Liumbruno G, Mori E, Griskevicius A, Audzijoniene J, Vrielink H, Rombout S, Aandahl A, Sikole A, Tomaz J, Lalic K, Mazic S, Strineholm V, Brink B, Berlin G, Dykes J, Toss F, Axelsson CG, Stegmayr B, Nilsson T, Norda R, Knutson F, Ramsauer B, Wahlstrom A. Adverse events in apheresis: An update of the WAA registry data. Transfus Apher Sci **2016**;54: 2-15.
204. Nagler A, Korenstein-Ilan A, Amiel A, Avivi L. Granulocyte colony-stimulating factor generates epigenetic and genetic alterations in lymphocytes of normal volunteer donors of stem cells. Exp Hematol **2004**;32: 122-30.
205. Makita K, Ohta K, Mugitani A, Hagihara K, Ohta T, Yamane T, Hino M. Acute myelogenous leukemia in a donor after granulocyte colony-stimulating factor-primed peripheral blood stem cell harvest. Bone Marrow Transplant **2004**;33: 661-5.
206. Bennett CL, Evens AM, Andritsos LA, Balasubramanian L, Mai M, Fisher MJ, Kuzel TM, Angelotta C, McKoy JM, Vose JM, Bierman PJ, Kuter DJ, Trifilio SM, Devine SM, Tallman MS. Haematological malignancies developing in previously healthy individuals who received haematopoietic growth factors: report from the Research on Adverse Drug Events and Reports (RADAR) project. Br J Haematol **2006**;135: 642-50.
207. Shaw BE, Confer DL, Hwang W, Pulsipher MA. A review of the genetic and long-term effects of G-CSF injections in healthy donors: a reassuring lack of evidence for the development of haematological malignancies. Bone Marrow Transplant **2015**;50: 334-40.
208. Pulsipher MA, Chitphakdithai P, Logan BR, Navarro WH, Levine JE, Miller JP, Shaw BE, O'Donnell PV, Majhail NS, Confer DL. Lower risk for serious adverse events and no increased risk for cancer after PBSC vs BM donation. Blood **2014**;123: 3655-63.
209. Notta F, Zandi S, Takayama N, Dobson S, Gan OI, Wilson G, Kaufmann KB, McLeod J, Laurenti E, Dunant CF, McPherson JD, Stein LD, Dror Y, Dick JE. Distinct routes of lineage development reshape the human blood hierarchy across ontogeny. Science **2016**;351: aab2116.
210. Bensinger WI, Martin PJ, Storer B, Clift R, Forman SJ, Negrin R, Kashyap A, Flowers ME, Lilleby K, Chauncey TR, Storb R, Appelbaum FR. Transplantation of bone marrow as compared with peripheral-

blood cells from HLA-identical relatives in patients with hematologic cancers. N Engl J Med **2001**;344: 175-81.
211. Schmitz N, Beksac M, Hasenclever D, Bacigalupo A, Ruutu T, Nagler A, Gluckman E, Russell N, Apperley JF, Gorin NC, Szer J, Bradstock K, Buzyn A, Clark P, Borkett K, Gratwohl A, European Group for B, Marrow T. Transplantation of mobilized peripheral blood cells to HLA-identical siblings with standard-risk leukemia. Blood **2002**;100: 761-7.
212. Abrahamsen IW, Somme S, Heldal D, Egeland T, Kvale D, Tjonnfjord GE. Immune reconstitution after allogeneic stem cell transplantation: the impact of stem cell source and graft-versus-host disease. Haematologica **2005**;90: 86-93.
213. Hassan HT, Stockschlader M, Schleimer B, Kruger W, Zander AR. Comparison of the content and subpopulations of CD3 and CD34 positive cells in bone marrow harvests and G-CSF-mobilized peripheral blood leukapheresis products from healthy adult donors. Transpl Immunol **1996**;4: 319-23.
214. Ottinger HD, Beelen DW, Scheulen B, Schaefer UW, Grosse-Wilde H. Improved immune reconstitution after allotransplantation of peripheral blood stem cells instead of bone marrow. Blood **1996**;88: 2775-9.
215. Abo T, Kawate T, Itoh K, Kumagai K. Studies on the bioperiodicity of the immune response. I. Circadian rhythms of human T, B, and K cell traffic in the peripheral blood. J Immunol **1981**;126: 1360-3.
216. Athens JW, Raab SO, Haab OP, Mauer AM, Ashenbrucker H, Cartwright GE, Wintrobe MM. Leukokinetic studies. III. The distribution of granulocytes in the blood of normal subjects. J Clin Invest **1961**;40: 159-64.
217. Trepel F. Number and distribution of lymphocytes in man. A critical analysis. Klin Wochenschr **1974**;52: 511-5.
218. Giebel S, Miszczyk L, Slosarek K, Moukhtari L, Ciceri F, Esteve J, Gorin NC, Labopin M, Nagler A, Schmid C, Mohty M. Extreme heterogeneity of myeloablative total body irradiation techniques in clinical practice: a survey of the Acute Leukemia Working Party of the European Group for Blood and Marrow Transplantation. Cancer **2014**;120: 2760-5.
219. Mohty M. Mechanisms of action of antithymocyte globulin: T-cell depletion and beyond. Leukemia **2007**;21: 1387-94.
220. Servais S, Menten-Dedoyart C, Beguin Y, Seidel L, Gothot A, Daulne C, Willems E, Delens L, Humblet-Baron S, Hannon M, Baron F. Impact of Pre-Transplant Anti-T Cell Globulin (ATG) on Immune Recovery after Myeloablative Allogeneic Peripheral Blood Stem Cell Transplantation. PLoS One **2015**;10: e0130026.
221. Kottaridis PD, Milligan DW, Chopra R, Chakraverty RK, Chakrabarti S, Robinson S, Peggs K, Verfuerth S, Pettengell R, Marsh JC, Schey S, Mahendra P, Morgan GJ, Hale G, Waldmann H, de Elvira MC, Williams CD, Devereux S, Linch DC, Goldstone AH, Mackinnon S. In vivo CAMPATH-1H prevents graft-versus-host disease following nonmyeloablative stem cell transplantation. Blood **2000**;96: 2419-25.
222. Baron F, Mohty M, Blaise D, Socie G, Labopin M, Esteve J, Ciceri F, Giebel S, Gorin NC, Savani BN, Schmid C, Nagler A. Anti-thymocyte globulin as graft-versus-host disease prevention in the setting of allogeneic peripheral blood stem cell transplantation: a review from the Acute Leukemia Working Party of the European Society for Blood and Marrow Transplantation. Haematologica **2017**;102: 224-34.
223. Kim HT, Armand P. Clinical endpoints in allogeneic hematopoietic stem cell transplantation studies: the cost of freedom. Biol Blood Marrow Transplant **2013**;19: 860-6.
224. Bensinger WI, Clift R, Martin P, Appelbaum FR, Demirer T, Gooley T, Lilleby K, Rowley S, Sanders J, Storb R, Buckner CD. Allogeneic peripheral blood stem cell transplantation in patients with advanced hematologic malignancies: a retrospective comparison with marrow transplantation. Blood **1996**;88: 2794-800.
225. Shenoy S, Mohanakumar T, Todd G, Westhoff W, Dunnigan K, Adkins DR, Brown RA, DiPersio JF. Immune reconstitution following allogeneic peripheral blood stem cell transplants. Bone Marrow Transplant **1999**;23: 335-46.
226. Kim DH, Won DI, Lee NY, Sohn SK, Suh JS, Lee KB. Non-CD34+ cells, especially CD8+ cytotoxic T cells and CD56+ natural killer cells, rather than CD34 cells, predict early engraftment and better transplantation outcomes in patients with hematologic malignancies after allogeneic peripheral stem cell transplantation. Biol Blood Marrow Transplant **2006**;12: 719-28.
227. Chklovskaia E, Nowbakht P, Nissen C, Gratwohl A, Bargetzi M, Wodnar-Filipowicz A. Reconstitution of dendritic and natural killer-cell subsets after allogeneic stem cell transplantation: effects of endogenous flt3 ligand. Blood **2004**;103: 3860-8.

228. Reddy V, Iturraspe JA, Tzolas AC, Meier-Kriesche HU, Schold J, Wingard JR. Low dendritic cell count after allogeneic hematopoietic stem cell transplantation predicts relapse, death, and acute graft-versus-host disease. Blood **2004**;103: 4330-5.
229. Thoma MD, Huneke TJ, DeCook LJ, Johnson ND, Wiegand RA, Litzow MR, Hogan WJ, Porrata LF, Holtan SG. Peripheral blood lymphocyte and monocyte recovery and survival in acute leukemia postmyeloablative allogeneic hematopoietic stem cell transplant. Biol Blood Marrow Transplant **2012**;18: 600-7.
230. Ullrich E, Salzmann-Manrique E, Bakhtiar S, Bremm M, Gerstner S, Herrmann E, Bader P, Hoffmann P, Holler E, Edinger M, Wolff D. Relation between Acute GVHD and NK Cell Subset Reconstitution Following Allogeneic Stem Cell Transplantation. Front Immunol **2016**;7: 595.
231. Kheav VD, Busson M, Scieux C, Peffault de Latour R, Maki G, Haas P, Mazeron MC, Carmagnat M, Masson E, Xhaard A, Robin M, Ribaud P, Dulphy N, Loiseau P, Charron D, Socie G, Toubert A, Moins-Teisserenc H. Favorable impact of natural killer cell reconstitution on chronic graft-versus-host disease and cytomegalovirus reactivation after allogeneic hematopoietic stem cell transplantation. Haematologica **2014**;99: 1860-7.
232. Alho AC, Kim HT, Chammas MJ, Reynolds CG, Matos TR, Forcade E, Whangbo J, Nikiforow S, Cutler CS, Koreth J, Ho VT, Armand P, Antin JH, Alyea EP, Lacerda JF, Soiffer RJ, Ritz J. Unbalanced recovery of regulatory and effector T cells after allogeneic stem cell transplantation contributes to chronic GVHD. Blood **2016**;127: 646-57.
233. Kim DH, Sohn SK, Won DI, Lee NY, Suh JS, Lee KB. Rapid helper T-cell recovery above 200 x 10 6/l at 3 months correlates to successful transplant outcomes after allogeneic stem cell transplantation. Bone Marrow Transplant **2006**;37: 1119-28.
234. Fedele R, Martino M, Garreffa C, Messina G, Console G, Princi D, Dattola A, Moscato T, Massara E, Spiniello E, Irrera G, Iacopino P. The impact of early CD4+ lymphocyte recovery on the outcome of patients who undergo allogeneic bone marrow or peripheral blood stem cell transplantation. Blood Transfus **2012**;10: 174-80.
235. Storek J, Dawson MA, Storer B, Stevens-Ayers T, Maloney DG, Marr KA, Witherspoon RP, Bensinger W, Flowers ME, Martin P, Storb R, Appelbaum FR, Boeckh M. Immune reconstitution after allogeneic marrow transplantation compared with blood stem cell transplantation. Blood **2001**;97: 3380-9.
236. Ravens S, Schultze-Florey C, Raha S, Sandrock I, Drenker M, Oberdorfer L, Reinhardt A, Ravens I, Beck M, Geffers R, von Kaisenberg C, Heuser M, Thol F, Ganser A, Forster R, Koenecke C, Prinz I. Human gammadelta T cells are quickly reconstituted after stem-cell transplantation and show adaptive clonal expansion in response to viral infection. Nat Immunol **2017**;18: 393-401.
237. Haraguchi K, Takahashi T, Hiruma K, Kanda Y, Tanaka Y, Ogawa S, Chiba S, Miura O, Sakamaki H, Hirai H. Recovery of Valpha24+ NKT cells after hematopoietic stem cell transplantation. Bone Marrow Transplant **2004**;34: 595-602.
238. Storek J, Wells D, Dawson MA, Storer B, Maloney DG. Factors influencing B lymphopoiesis after allogeneic hematopoietic cell transplantation. Blood **2001**;98: 489-91.
239. Majka M, Janowska-Wieczorek A, Ratajczak J, Ehrenman K, Pietrzkowski Z, Kowalska MA, Gewirtz AM, Emerson SG, Ratajczak MZ. Numerous growth factors, cytokines, and chemokines are secreted by human CD34(+) cells, myeloblasts, erythroblasts, and megakaryoblasts and regulate normal hematopoiesis in an autocrine/paracrine manner. Blood **2001**;97: 3075-85.
240. van Heijst JW, Ceberio I, Lipuma LB, Samilo DW, Wasilewski GD, Gonzales AM, Nieves JL, van den Brink MR, Perales MA, Pamer EG. Quantitative assessment of T cell repertoire recovery after hematopoietic stem cell transplantation. Nat Med **2013**;19: 372-7.
241. Baron F, Maris MB, Storer BE, Sandmaier BM, Panse JP, Chauncey TR, Sorror M, Little MT, Maloney DG, Storb R, Heimfeld S. High doses of transplanted CD34+ cells are associated with rapid T-cell engraftment and lessened risk of graft rejection, but not more graft-versus-host disease after nonmyeloablative conditioning and unrelated hematopoietic cell transplantation. Leukemia **2005**;19: 822-8.
242. Kurian TJ, Gatell VI, Adams W, Tsai SB, Smith SE, Stiff PJ, Hagen P. Impact of Graft Composition on Graft-Versus-Host Disease in Peripheral Blood HLA-Identical Sibling Transplants: Protective Role of CD8 Cell Dose. Blood **2018**;132: 5719-.
243. Zaucha JM, Gooley T, Bensinger WI, Heimfeld S, Chauncey TR, Zaucha R, Martin PJ, Flowers ME, Storek J, Georges G, Storb R, Torok-Storb B. CD34 cell dose in granulocyte colony-stimulating factor-mobilized peripheral blood mononuclear cell grafts affects engraftment kinetics and development of extensive chronic graft-versus-host disease after human leukocyte antigen-identical sibling transplantation. Blood **2001**;98: 3221-7.

244. Cao TM, Wong RM, Sheehan K, Laport GG, Stockerl-Goldstein KE, Johnston LJ, Shizuru JA, Negrin RS, Lowsky R. CD34, CD4, and CD8 cell doses do not influence engraftment, graft-versus-host disease, or survival following myeloablative human leukocyte antigen-identical peripheral blood allografting for hematologic malignancies. Exp Hematol **2005**;33: 279-85.
245. Salzmann-Manrique E, Bremm M, Huenecke S, Stech M, Orth A, Eyrich M, Schulz A, Esser R, Klingebiel T, Bader P, Herrmann E, Koehl U. Joint Modeling of Immune Reconstitution Post Haploidentical Stem Cell Transplantation in Pediatric Patients With Acute Leukemia Comparing CD34(+)-Selected to CD3/CD19-Depleted Grafts in a Retrospective Multicenter Study. Front Immunol **2018**;9: 1841.
246. Collins NH, Gee AP, Durett AG, Kan F, Zhang MJ, Champlin RE, Confer D, Eapen M, Howard A, King R, Laughlin MJ, Plante RJ, Setterholm M, Spellman S, Keever-Taylor C, Wagner JE, Weisdorf DJ. The effect of the composition of unrelated donor bone marrow and peripheral blood progenitor cell grafts on transplantation outcomes. Biol Blood Marrow Transplant **2010**;16: 253-62.
247. Bertaina A, Zecca M, Buldini B, Sacchi N, Algeri M, Saglio F, Perotti C, Gallina AM, Bertaina V, Lanino E, Prete A, Barberi W, Tumino M, Favre C, Cesaro S, Del Bufalo F, Ripaldi M, Boghen S, Casazza G, Rabusin M, Balduzzi A, Fagioli F, Pagliara D, Locatelli F. Unrelated donor vs HLA-haploidentical alpha/beta T-cell- and B-cell-depleted HSCT in children with acute leukemia. Blood **2018**;132: 2594-607.
248. Kim DH, Sohn SK, Lee NY, Baek JH, Kim JG, Won DI, Suh JS, Lee KB, Shin IH. Transplantation with higher dose of natural killer cells associated with better outcomes in terms of non-relapse mortality and infectious events after allogeneic peripheral blood stem cell transplantation from HLA-matched sibling donors. Eur J Haematol **2005**;75: 299-308.
249. Ferrà C, Sanz J, Díaz-Pérez M-A, Morgades M, Gayoso J, Cabrera J-R, Villaescusa T, Sampol M-A, Fernández-Avilés F, Solano C, Ortín M, Duarte R, Cuesta M, Heras I, de la Serna J, Moraleda J-M, Barrenetxea C, González-Vicent M, Sanz G, Ribera J-M. Outcome of graft failure after allogeneic stem cell transplant: study of 89 patients. Leukemia & Lymphoma **2015**;56: 656-62.
250. Olsson R, Remberger M, Schaffer M, Berggren DM, Svahn BM, Mattsson J, Ringden O. Graft failure in the modern era of allogeneic hematopoietic SCT. Bone Marrow Transplant **2013**;48: 537-43.
251. Masouridi-Levrat S, Simonetta F, Chalandon Y. Immunological Basis of Bone Marrow Failure after Allogeneic Hematopoietic Stem Cell Transplantation. Front Immunol **2016**;7: 362.
252. Remberger M, Watz E, Ringden O, Mattsson J, Shanwell A, Wikman A. Major ABO blood group mismatch increases the risk for graft failure after unrelated donor hematopoietic stem cell transplantation. Biol Blood Marrow Transplant **2007**;13: 675-82.
253. Paczesny S, Hanauer D, Sun Y, Reddy P. New perspectives on the biology of acute GVHD. Bone Marrow Transplant **2010**;45: 1-11.
254. Bleakley M, Riddell SR. Molecules and mechanisms of the graft-versus-leukaemia effect. Nat Rev Cancer **2004**;4: 371-80.
255. Kolb HJ. Graft-versus-leukemia effects of transplantation and donor lymphocytes. Blood **2008**;112: 4371-83.
256. MacDonald KP, Blazar BR, Hill GR. Cytokine mediators of chronic graft-versus-host disease. J Clin Invest **2017**;127: 2452-63.
257. Jagasia MH, Greinix HT, Arora M, Williams KM, Wolff D, Cowen EW, Palmer J, Weisdorf D, Treister NS, Cheng GS, Kerr H, Stratton P, Duarte RF, McDonald GB, Inamoto Y, Vigorito A, Arai S, Datiles MB, Jacobsohn D, Heller T, Kitko CL, Mitchell SA, Martin PJ, Shulman H, Wu RS, Cutler CS, Vogelsang GB, Lee SJ, Pavletic SZ, Flowers ME. National Institutes of Health Consensus Development Project on Criteria for Clinical Trials in Chronic Graft-versus-Host Disease: I. The 2014 Diagnosis and Staging Working Group report. Biol Blood Marrow Transplant **2015**;21: 389-401 e1.
258. Suessmuth Y, Mukherjee R, Watkins B, Koura DT, Finstermeier K, Desmarais C, Stempora L, Horan JT, Langston A, Qayed M, Khoury HJ, Grizzle A, Cheeseman JA, Conger JA, Robertson J, Garrett A, Kirk AD, Waller EK, Blazar BR, Mehta AK, Robins HS, Kean LS. CMV reactivation drives posttransplant T-cell reconstitution and results in defects in the underlying TCRbeta repertoire. Blood **2015**;125: 3835-50.
259. Bjorklund AT, Clancy T, Goodridge JP, Beziat V, Schaffer M, Hovig E, Ljunggren HG, Ljungman PT, Malmberg KJ. Naive Donor NK Cell Repertoires Associated with Less Leukemia Relapse after Allogeneic Hematopoietic Stem Cell Transplantation. J Immunol **2016**;196: 1400-11.
260. Meier J, Roberts C, Avent K, Hazlett A, Berrie J, Payne K, Hamm D, Desmarais C, Sanders C, Hogan KT, Archer KJ, Manjili MH, Toor AA. Fractal organization of the human T cell repertoire in health and after stem cell transplantation. Biol Blood Marrow Transplant **2013**;19: 366-77.

261. Sethi MK, Thol F, Stadler M, Heuser M, Ganser A, Koenecke C, Pabst O. VH1 Family Immunoglobulin Repertoire Sequencing after Allogeneic Hematopoietic Stem Cell Transplantation. PLoS One **2017**;12: e0168096.
262. Hofer E, Koehl U. Natural Killer Cell-Based Cancer Immunotherapies: From Immune Evasion to Promising Targeted Cellular Therapies. Front Immunol **2017**;8: 745.
263. Simonetta F, Alvarez M, Negrin RS. Natural Killer Cells in Graft-versus-Host-Disease after Allogeneic Hematopoietic Cell Transplantation. Front Immunol **2017**;8: 465.
264. Blazar BR, MacDonald KPA, Hill GR. Immune regulatory cell infusion for graft-versus-host disease prevention and therapy. Blood **2018**;131: 2651-60.
265. Handgretinger R, Schilbach K. The potential role of gammadelta T cells after allogeneic HCT for leukemia. Blood **2018**;131: 1063-72.
266. Zhao Y, Niu C, Cui J. Gamma-delta (gammadelta) T cells: friend or foe in cancer development? J Transl Med **2018**;16: 3.
267. Pabst C, Schirutschke H, Ehninger G, Bornhauser M, Platzbecker U. The graft content of donor T cells expressing gamma delta TCR+ and CD4+foxp3+ predicts the risk of acute graft versus host disease after transplantation of allogeneic peripheral blood stem cells from unrelated donors. Clin Cancer Res **2007**;13: 2916-22.
268. Gaballa A, Stikvoort A, Onfelt B, Mattsson J, Sundin M, Watz E, Uhlin M. T-cell frequencies of CD8(+) gammadelta and CD27(+) gammadelta cells in the stem cell graft predict the outcome after allogeneic hematopoietic cell transplantation. Bone Marrow Transplant **2019**;54: 1562-74.
269. Czerw T, Labopin M, Schmid C, Cornelissen JJ, Chevallier P, Blaise D, Kuball J, Vigouroux S, Garban F, Lioure B, Fegueux N, Clement L, Sandstedt A, Maertens J, Guillerm G, Bordessoule D, Mohty M, Nagler A. High CD3+ and CD34+ peripheral blood stem cell grafts content is associated with increased risk of graft-versus-host disease without beneficial effect on disease control after reduced-intensity conditioning allogeneic transplantation from matched unrelated donors for acute myeloid leukemia - an analysis from the Acute Leukemia Working Party of the European Society for Blood and Marrow Transplantation. Oncotarget **2016**;7: 27255-66.
270. Mohty M, Bagattini S, Chabannon C, Faucher C, Bardou VJ, Bilger K, Vey N, Gaugler B, Stoppa AM, Coso D, Ladaique P, Olive D, Viens P, Blaise D. CD8+ T cell dose affects development of acute graft-vs-host disease following reduced-intensity conditioning allogeneic peripheral blood stem cell transplantation. Exp Hematol **2004**;32: 1097-102.
271. Fisher SA, Lamikanra A, Doree C, Gration B, Tsang P, Danby RD, Roberts DJ. Increased regulatory T cell graft content is associated with improved outcome in haematopoietic stem cell transplantation: a systematic review. Br J Haematol **2017**;176: 448-63.
272. Chaidos A, Patterson S, Szydlo R, Chaudhry MS, Dazzi F, Kanfer E, McDonald D, Marin D, Milojkovic D, Pavlu J, Davis J, Rahemtulla A, Rezvani K, Goldman J, Roberts I, Apperley J, Karadimitris A. Graft invariant natural killer T-cell dose predicts risk of acute graft-versus-host disease in allogeneic hematopoietic stem cell transplantation. Blood **2012**;119: 5030-6.
273. Rubio MT, Bouillie M, Bouazza N, Coman T, Trebeden-Negre H, Gomez A, Suarez F, Sibon D, Brignier A, Paubelle E, Nguyen-Khoc S, Cavazzana M, Lantz O, Mohty M, Urien S, Hermine O. Pre-transplant donor CD4(-) invariant NKT cell expansion capacity predicts the occurrence of acute graft-versus-host disease. Leukemia **2017**;31: 903-12.
274. Ho VT, Soiffer RJ. The history and future of T-cell depletion as graft-versus-host disease prophylaxis for allogeneic hematopoietic stem cell transplantation. Blood **2001**;98: 3192-204.
275. Ni X, Song Q, Cassady K, Deng R, Jin H, Zhang M, Dong H, Forman S, Martin PJ, Chen YZ, Wang J, Zeng D. PD-L1 interacts with CD80 to regulate graft-versus-leukemia activity of donor CD8+ T cells. J Clin Invest **2017**;127: 1960-77.
276. Svenberg P, Wang T, Uhlin M, Watz E, Remberger M, Ringden O, Mattsson J, Uzunel M. The importance of graft cell composition in outcome after allogeneic stem cell transplantation in patients with malignant disease. Clin Transplant **2019**;33: e13537.
277. Heimfeld S, Gooley T, Flowers M, Baron F, Martin PJ, Linenberger ML, Storb RF, Appelbaum FR, Sandmaier BM. Effect of Peripheral Blood Stem Cell (PBSC) Graft Composition on Graft Versus Host Disease (GVHD) and Mortality After Allogeneic Transplantation **2010**;116: 676-.
278. Ho VT, Kim HT, Li S, Hochberg EP, Cutler C, Lee SJ, Fisher DC, Milford E, Kao G, Daley H, Levin J, Ng A, Mauch P, Alyea EP, Antin JH, Soiffer RJ. Partial CD8+ T-cell depletion of allogeneic peripheral blood stem cell transplantation is insufficient to prevent graft-versus-host disease. Bone Marrow Transplant **2004**;34: 987-94.
279. Reshef R, Huffman AP, Gao A, Luskin MR, Frey NV, Gill SI, Hexner EO, Kambayashi T, Loren AW, Luger SM, Mangan JK, Nasta SD, Richman LP, Sell M, Stadtmauer EA, Vonderheide RH, Mick R, Porter DL. High Graft CD8 Cell Dose Predicts Improved Survival and Enables Better Donor

Selection in Allogeneic Stem-Cell Transplantation With Reduced-Intensity Conditioning. J Clin Oncol **2015**;33: 2392-8.
280. Zheng H, Matte-Martone C, Li H, Anderson BE, Venketesan S, Sheng Tan H, Jain D, McNiff J, Shlomchik WD. Effector memory CD4+ T cells mediate graft-versus-leukemia without inducing graft-versus-host disease. Blood **2008**;111: 2476-84.
281. Zheng H, Matte-Martone C, Jain D, McNiff J, Shlomchik WD. Central memory CD8+ T cells induce graft-versus-host disease and mediate graft-versus-leukemia. J Immunol **2009**;182: 5938-48.
282. Bleakley M, Heimfeld S, Loeb KR, Jones LA, Chaney C, Seropian S, Gooley TA, Sommermeyer F, Riddell SR, Shlomchik WD. Outcomes of acute leukemia patients transplanted with naive T cell-depleted stem cell grafts. J Clin Invest **2015**;125: 2677-89.
283. Muffly L, Sheehan K, Armstrong R, Jensen K, Tate K, Rezvani AR, Miklos D, Arai S, Shizuru J, Johnston L, Meyer E, Weng WK, Laport GG, Negrin RS, Strober S, Lowsky R. Infusion of donor-derived CD8(+) memory T cells for relapse following allogeneic hematopoietic cell transplantation. Blood Adv **2018**;2: 681-90.
284. Edinger M, Hoffmann P, Ermann J, Drago K, Fathman CG, Strober S, Negrin RS. CD4+CD25+ regulatory T cells preserve graft-versus-tumor activity while inhibiting graft-versus-host disease after bone marrow transplantation. Nat Med **2003**;9: 1144-50.
285. Zhang P, Tey SK, Koyama M, Kuns RD, Olver SD, Lineburg KE, Lor M, Teal BE, Raffelt NC, Raju J, Leveque L, Markey KA, Varelias A, Clouston AD, Lane SW, MacDonald KP, Hill GR. Induced regulatory T cells promote tolerance when stabilized by rapamycin and IL-2 in vivo. J Immunol **2013**;191: 5291-303.
286. Zhang P, Lee JS, Gartlan KH, Schuster IS, Comerford I, Varelias A, Ullah MA, Vuckovic S, Koyama M, Kuns RD, Locke KR, Beckett KJ, Olver SD, Samson LD, Montes de Oca M, de Labastida Rivera F, Clouston AD, Belz GT, Blazar BR, MacDonald KP, McColl SR, Thomas R, Engwerda CR, Degli-Esposti MA, Kallies A, Tey SK, Hill GR. Eomesodermin promotes the development of type 1 regulatory T (TR1) cells. Sci Immunol **2017**;2.
287. Bacchetta R, Lucarelli B, Sartirana C, Gregori S, Lupo Stanghellini MT, Miqueu P, Tomiuk S, Hernandez-Fuentes M, Gianolini ME, Greco R, Bernardi M, Zappone E, Rossini S, Janssen U, Ambrosi A, Salomoni M, Peccatori J, Ciceri F, Roncarolo MG. Immunological Outcome in Haploidentical-HSC Transplanted Patients Treated with IL-10-Anergized Donor T Cells. Front Immunol **2014**;5: 16.
288. Lamb LS, Jr., Musk P, Ye Z, van Rhee F, Geier SS, Tong JJ, King KM, Henslee-Downey PJ. Human gammadelta(+) T lymphocytes have in vitro graft vs leukemia activity in the absence of an allogeneic response. Bone Marrow Transplant **2001**;27: 601-6.
289. Drobyski WR, Vodanovic-Jankovic S, Klein J. Adoptively transferred gamma delta T cells indirectly regulate murine graft-versus-host reactivity following donor leukocyte infusion therapy in mice. J Immunol **2000**;165: 1634-40.
290. Godder KT, Henslee-Downey PJ, Mehta J, Park BS, Chiang KY, Abhyankar S, Lamb LS. Long term disease-free survival in acute leukemia patients recovering with increased gammadelta T cells after partially mismatched related donor bone marrow transplantation. Bone Marrow Transplant **2007**;39: 751-7.
291. Du J, Paz K, Thangavelu G, Schneidawind D, Baker J, Flynn R, Duramad O, Feser C, Panoskaltsis-Mortari A, Negrin RS, Blazar BR. Invariant natural killer T cells ameliorate murine chronic GVHD by expanding donor regulatory T cells. Blood **2017**;129: 3121-5.
292. Malard F, Labopin M, Chevallier P, Guillaume T, Duquesne A, Rialland F, Derenne S, Peterlin P, Leaute AG, Brissot E, Gregoire M, Moreau P, Saas P, Gaugler B, Mohty M. Larger number of invariant natural killer T cells in PBSC allografts correlates with improved GVHD-free and progression-free survival. Blood **2016**;127: 1828-35.
293. Ruggeri L, Capanni M, Urbani E, Perruccio K, Shlomchik WD, Tosti A, Posati S, Rogaia D, Frassoni F, Aversa F, Martelli MF, Velardi A. Effectiveness of donor natural killer cell alloreactivity in mismatched hematopoietic transplants. Science **2002**;295: 2097-100.
294. Olson JA, Leveson-Gower DB, Gill S, Baker J, Beilhack A, Negrin RS. NK cells mediate reduction of GVHD by inhibiting activated, alloreactive T cells while retaining GVT effects. Blood **2010**;115: 4293-301.
295. Xun C, Brown SA, Jennings CD, Henslee-Downey PJ, Thompson JS. Acute graft-versus-host-like disease induced by transplantation of human activated natural killer cells into SCID mice. Transplantation **1993**;56: 409-17.
296. Shah NN, Baird K, Delbrook CP, Fleisher TA, Kohler ME, Rampertaap S, Lemberg K, Hurley CK, Kleiner DE, Merchant MS, Pittaluga S, Sabatino M, Stroncek DF, Wayne AS, Zhang H, Fry TJ,

Mackall CL. Acute GVHD in patients receiving IL-15/4-1BBL activated NK cells following T-cell-depleted stem cell transplantation. Blood **2015**;125: 784-92.
297. Jaiswal SR, Zaman S, Nedunchezhian M, Chakrabarti A, Bhakuni P, Ahmed M, Sharma K, Rawat S, O'Donnell P, Chakrabarti S. CD56-enriched donor cell infusion after post-transplantation cyclophosphamide for haploidentical transplantation of advanced myeloid malignancies is associated with prompt reconstitution of mature natural killer cells and regulatory T cells with reduced incidence of acute graft versus host disease: A pilot study. Cytotherapy **2017**;19: 531-42.
298. Landgren O, Gilbert ES, Rizzo JD, Socie G, Banks PM, Sobocinski KA, Horowitz MM, Jaffe ES, Kingma DW, Travis LB, Flowers ME, Martin PJ, Deeg HJ, Curtis RE. Risk factors for lymphoproliferative disorders after allogeneic hematopoietic cell transplantation. Blood **2009**;113: 4992-5001.
299. Carpenter B, Haque T, Dimopoulou M, Atkinson C, Roughton M, Grace S, Denovan S, Fielding A, Kottaridis PD, Griffiths P, Mackinnon S, Emery V, Chakraverty R. Incidence and dynamics of Epstein-Barr virus reactivation after alemtuzumab-based conditioning for allogeneic hematopoietic stem-cell transplantation. Transplantation **2010**;90: 564-70.
300. Abbas AK, Benoist C, Bluestone JA, Campbell DJ, Ghosh S, Hori S, Jiang S, Kuchroo VK, Mathis D, Roncarolo MG, Rudensky A, Sakaguchi S, Shevach EM, Vignali DA, Ziegler SF. Regulatory T cells: recommendations to simplify the nomenclature. Nat Immunol **2013**;14: 307-8.
301. Lupo-Stanghellini MT, Peczynski C, Greinix HT, Polge E, Mohty M, Yakoub-Agha I, Montoto S, Kröger N, Peric Z, Penack O, Duarte RF, Basak GW. From Ex-Vivo T-Cell Depletion to Post-Transplant Cyclophosphamide: Improved GvHD-Free & Relapse-Free Survival but Comparable Chronic GvHD Incidence in Haploidentical Transplantation. A 15 Years EBMT Registry Analysis on Behalf of the TCWP-EBMT. Blood **2019**;134: 876-.
302. Battipaglia G, Labopin M, Kroger N, Vitek A, Afanasyev B, Hilgendorf I, Schetelig J, Ganser A, Blaise D, Itala-Remes M, Passweg JR, Bonifazi F, Finke J, Ruggeri A, Nagler A, Mohty M. Posttransplant cyclophosphamide vs antithymocyte globulin in HLA-mismatched unrelated donor transplantation. Blood **2019**;134: 892-9.
303. Vadakekolathu J, Rutella S. T-Cell Manipulation Strategies to Prevent Graft-Versus-Host Disease in Haploidentical Stem Cell Transplantation. Biomedicines **2017**;5.
304. Bertaina A, Merli P, Rutella S, Pagliara D, Bernardo ME, Masetti R, Pende D, Falco M, Handgretinger R, Moretta F, Lucarelli B, Brescia LP, Li Pira G, Testi M, Cancrini C, Kabbara N, Carsetti R, Finocchi A, Moretta A, Moretta L, Locatelli F. HLA-haploidentical stem cell transplantation after removal of alphabeta+ T and B cells in children with nonmalignant disorders. Blood **2014**;124: 822-6.
305. Bertaina A, Roncarolo MG. Graft Engineering and Adoptive Immunotherapy: New Approaches to Promote Immune Tolerance After Hematopoietic Stem Cell Transplantation. Front Immunol **2019**;10: 1342.
306. Schmid C, Labopin M, Schaap N, Veelken H, Schleuning M, Stadler M, Finke J, Hurst E, Baron F, Ringden O, Bug G, Blaise D, Tischer J, Bloor A, Esteve J, Giebel S, Savani B, Gorin NC, Ciceri F, Mohty M, Nagler A, Party EALW. Prophylactic donor lymphocyte infusion after allogeneic stem cell transplantation in acute leukaemia - a matched pair analysis by the Acute Leukaemia Working Party of EBMT. Br J Haematol **2019**;184: 782-7.
307. Grant ML, Bollard CM. Cell therapies for hematological malignancies: don't forget non-gene-modified t cells! Blood Rev **2018**;32: 203-24.
308. Martelli MF, Di Ianni M, Ruggeri L, Falzetti F, Carotti A, Terenzi A, Pierini A, Massei MS, Amico L, Urbani E, Del Papa B, Zei T, Iacucci Ostini R, Cecchini D, Tognellini R, Reisner Y, Aversa F, Falini B, Velardi A. HLA-haploidentical transplantation with regulatory and conventional T-cell adoptive immunotherapy prevents acute leukemia relapse. Blood **2014**;124: 638-44.
309. Greco R, Oliveira G, Stanghellini MT, Vago L, Bondanza A, Peccatori J, Cieri N, Marktel S, Mastaglio S, Bordignon C, Bonini C, Ciceri F. Improving the safety of cell therapy with the TK-suicide gene. Front Pharmacol **2015**;6: 95.
310. Zilberberg J, Feinman R, Korngold R. Strategies for the identification of T cell-recognized tumor antigens in hematological malignancies for improved graft-versus-tumor responses after allogeneic blood and marrow transplantation. Biol Blood Marrow Transplant **2015**;21: 1000-7.
311. Huang XJ, Wang Y, Liu DH, Xu LP, Chen H, Chen YH, Han W, Shi HX, Liu KY. Modified donor lymphocyte infusion (DLI) for the prophylaxis of leukemia relapse after hematopoietic stem cell transplantation in patients with advanced leukemia--feasibility and safety study. J Clin Immunol **2008**;28: 390-7.
312. Kebriaei P, Singh H, Huls MH, Figliola MJ, Bassett R, Olivares S, Jena B, Dawson MJ, Kumaresan PR, Su S, Maiti S, Dai J, Moriarity B, Forget MA, Senyukov V, Orozco A, Liu T, McCarty J, Jackson

RN, Moyes JS, Rondon G, Qazilbash M, Ciurea S, Alousi A, Nieto Y, Rezvani K, Marin D, Popat U, Hosing C, Shpall EJ, Kantarjian H, Keating M, Wierda W, Do KA, Largaespada DA, Lee DA, Hackett PB, Champlin RE, Cooper LJ. Phase I trials using Sleeping Beauty to generate CD19-specific CAR T cells. J Clin Invest **2016**;126: 3363-76.

313. Qasim W, Zhan H, Samarasinghe S, Adams S, Amrolia P, Stafford S, Butler K, Rivat C, Wright G, Somana K, Ghorashian S, Pinner D, Ahsan G, Gilmour K, Lucchini G, Inglott S, Mifsud W, Chiesa R, Peggs KS, Chan L, Farzeneh F, Thrasher AJ, Vora A, Pule M, Veys P. Molecular remission of infant B-ALL after infusion of universal TALEN gene-edited CAR T cells. Sci Transl Med **2017**;9.
314. Di Ianni M, Falzetti F, Carotti A, Terenzi A, Castellino F, Bonifacio E, Del Papa B, Zei T, Ostini RI, Cecchini D, Aloisi T, Perruccio K, Ruggeri L, Balucani C, Pierini A, Sportoletti P, Aristei C, Falini B, Reisner Y, Velardi A, Aversa F, Martelli MF. Tregs prevent GVHD and promote immune reconstitution in HLA-haploidentical transplantation. Blood **2011**;117: 3921-8.
315. Kennedy-Nasser AA, Ku S, Castillo-Caro P, Hazrat Y, Wu MF, Liu H, Melenhorst J, Barrett AJ, Ito S, Foster A, Savoldo B, Yvon E, Carrum G, Ramos CA, Krance RA, Leung K, Heslop HE, Brenner MK, Bollard CM. Ultra low-dose IL-2 for GVHD prophylaxis after allogeneic hematopoietic stem cell transplantation mediates expansion of regulatory T cells without diminishing antiviral and antileukemic activity. Clin Cancer Res **2014**;20: 2215-25.
316. Thangavelu G, Blazar BR. Achievement of Tolerance Induction to Prevent Acute Graft-vs.-Host Disease. Front Immunol **2019**;10: 309.
317. Akdis M, Aab A, Altunbulakli C, Azkur K, Costa RA, Crameri R, Duan S, Eiwegger T, Eljaszewicz A, Ferstl R, Frei R, Garbani M, Globinska A, Hess L, Huitema C, Kubo T, Komlosi Z, Konieczna P, Kovacs N, Kucuksezer UC, Meyer N, Morita H, Olzhausen J, O'Mahony L, Pezer M, Prati M, Rebane A, Rhyner C, Rinaldi A, Sokolowska M, Stanic B, Sugita K, Treis A, van de Veen W, Wanke K, Wawrzyniak M, Wawrzyniak P, Wirz OF, Zakzuk JS, Akdis CA. Interleukins (from IL-1 to IL-38), interferons, transforming growth factor beta, and TNF-alpha: Receptors, functions, and roles in diseases. J Allergy Clin Immunol **2016**;138: 984-1010.
318. Sokol CL, Luster AD. The chemokine system in innate immunity. Cold Spring Harb Perspect Biol **2015**;7.
319. Bruserud O, Kittang AO. The chemokine system in experimental and clinical hematology. Curr Top Microbiol Immunol **2010**;341: 3-12.
320. Ozaki K, Leonard WJ. Cytokine and cytokine receptor pleiotropy and redundancy. J Biol Chem **2002**;277: 29355-8.
321. Zhang Y, Cheng G, Yang K, Fan R, Xu Z, Chen L, Li Q, Yang A, Jin B. A novel function of granulocyte colony-stimulating factor in mobilization of human hematopoietic progenitor cells. Immunol Cell Biol **2009**;87: 428-32.
322. Ringden O, Labopin M, Gorin NC, Le Blanc K, Rocha V, Gluckman E, Reiffers J, Arcese W, Vossen JM, Jouet JP, Cordonnier C, Frassoni F. Treatment with granulocyte colony-stimulating factor after allogeneic bone marrow transplantation for acute leukemia increases the risk of graft-versus-host disease and death: a study from the Acute Leukemia Working Party of the European Group for Blood and Marrow Transplantation. J Clin Oncol **2004**;22: 416-23.
323. Remberger M, Naseh N, Aschan J, Barkholt L, LeBlanc K, Svennberg P, Ringden O. G-CSF given after haematopoietic stem cell transplantation using HLA-identical sibling donors is associated to a higher incidence of acute GVHD II-IV. Bone Marrow Transplant **2003**;32: 217-23.
324. Lysak D, Hrabetova M, Vrzalova J, Koza V, Navratilova J, Svoboda T, Jungova A, Topolcan O. Changes of cytokine levels during granulocyte-colony-stimulating factor stem cell mobilization in healthy donors: association with mobilization efficiency and potential predictive significance. Transfusion **2011**;51: 319-27.
325. Cecyn KZ, Kimura EYS, Lima D, Yamamoto M, Bordin JO, de Oliveira JSR. Expression of adhesion molecules on CD34+ cells from steady-state bone marrow before and after mobilization and their association with the yield of CD34+ cells. Blood Res **2018**;53: 61-70.
326. Perusina Lanfranca M, Lin Y, Fang J, Zou W, Frankel T. Biological and pathological activities of interleukin-22. J Mol Med (Berl) **2016**;94: 523-34.
327. Nagasawa M, Zhu Y, Isoda T, Tomizawa D, Itoh S, Kajiwara M, Morio T, Nonoyama S, Shimizu N, Mizutani S. Analysis of serum soluble CD40 ligand (sCD40L) in the patients undergoing allogeneic stem cell transplantation: platelet is a major source of serum sCD40L. Eur J Haematol **2005**;74: 54-60.
328. Locksley RM, Killeen N, Lenardo MJ. The TNF and TNF receptor superfamilies: integrating mammalian biology. Cell **2001**;104: 487-501.
329. Eberlein J, Nguyen TT, Victorino F, Golden-Mason L, Rosen HR, Homann D. Comprehensive assessment of chemokine expression profiles by flow cytometry. J Clin Invest **2010**;120: 907-23.
330. Cao Y. Angiogenesis modulates adipogenesis and obesity. J Clin Invest **2007**;117: 2362-8.

331. Ono-Uruga Y, Tozawa K, Horiuchi T, Murata M, Okamoto S, Ikeda Y, Suda T, Matsubara Y. Human adipose tissue-derived stromal cells can differentiate into megakaryocytes and platelets by secreting endogenous thrombopoietin. J Thromb Haemost **2016**;14: 1285-97.
332. Richter R, Forssmann W, Henschler R. Current Developments in Mobilization of Hematopoietic Stem and Progenitor Cells and Their Interaction with Niches in Bone Marrow. Transfus Med Hemother **2017**;44: 151-64.
333. Brooks AJ, Dehkhoda F, Kragelund BB. Cytokine Receptors. . In: A. B, D. L, eds. Principles of Endocrinology and Hormone Action. Endocrinology. : Springer, Cham, 2017.
334. Tamayo E, Alvarez P, Merino R. TGFbeta Superfamily Members as Regulators of B Cell Development and Function-Implications for Autoimmunity. Int J Mol Sci **2018**;19.
335. Boulay JL, O'Shea JJ, Paul WE. Molecular phylogeny within type I cytokines and their cognate receptors. Immunity **2003**;19: 159-63.
336. Griffith JW, Sokol CL, Luster AD. Chemokines and chemokine receptors: positioning cells for host defense and immunity. Annu Rev Immunol **2014**;32: 659-702.
337. Bachelerie F, Ben-Baruch A, Burkhardt AM, Combadiere C, Farber JM, Graham GJ, Horuk R, Sparre-Ulrich AH, Locati M, Luster AD, Mantovani A, Matsushima K, Murphy PM, Nibbs R, Nomiyama H, Power CA, Proudfoot AE, Rosenkilde MM, Rot A, Sozzani S, Thelen M, Yoshie O, Zlotnik A. International Union of Basic and Clinical Pharmacology. [corrected]. LXXXIX. Update on the extended family of chemokine receptors and introducing a new nomenclature for atypical chemokine receptors. Pharmacol Rev **2014**;66: 1-79.
338. Mehta HM, Malandra M, Corey SJ. G-CSF and GM-CSF in Neutropenia. J Immunol **2015**;195: 1341-9.
339. Naylor C, Petri WA, Jr. Leptin Regulation of Immune Responses. Trends Mol Med **2016**;22: 88-98.
340. Henden AS, Hill GR. Cytokines in Graft-versus-Host Disease. J Immunol **2015**;194: 4604-12.
341. Melve GK, Ersvssr E, Kittang AO, Bruserud O. The chemokine system in allogeneic stem-cell transplantation: a possible therapeutic target? Expert Rev Hematol **2011**;4: 563-76.
342. Harris AC, Ferrara JL, Levine JE. Advances in predicting acute GVHD. Br J Haematol **2013**;160: 288-302.
343. Betts BC, Pidala J, Kim J, Mishra A, Nishihori T, Perez L, Ochoa-Bayona JL, Khimani F, Walton K, Bookout R, Nieder M, Khaira DK, Davila M, Alsina M, Field T, Ayala E, Locke FL, Riches M, Kharfan-Dabaja M, Fernandez H, Anasetti C. IL-2 promotes early Treg reconstitution after allogeneic hematopoietic cell transplantation. Haematologica **2017**;102: 948-57.
344. Hirakawa M, Matos TR, Liu H, Koreth J, Kim HT, Paul NE, Murase K, Whangbo J, Alho AC, Nikiforow S, Cutler C, Ho VT, Armand P, Alyea EP, Antin JH, Blazar BR, Lacerda JF, Soiffer RJ, Ritz J. Low-dose IL-2 selectively activates subsets of CD4(+) Tregs and NK cells. JCI Insight **2016**;1: e89278.
345. Givon T, Revel M, Slavin S. Potential use of interleukin-6 in bone marrow transplantation: effects of recombinant human interleukin-6 after syngeneic and semiallogeneic bone marrow transplantation in mice. Blood **1994**;83: 1690-7.
346. Kielsen K, Jordan KK, Uhlving HH, Pontoppidan PL, Shamim Z, Ifversen M, Heilmann C, Nielsen CH, Sengelov H, Ryder LP, Muller KG. T cell reconstitution in allogeneic haematopoietic stem cell transplantation: prognostic significance of plasma interleukin-7. Scand J Immunol **2015**;81: 72-80.
347. Azevedo RI, Soares AS, Espada E, Camacho N, Martins CV, Lourenco F, Juncal C, Moreno R, Carmo JA, Lacerda JF. IL-7 and IL-10 Serum Levels Are Potential Immune Biomarkers for Acute Graft-Versus-Host Disease Following Unrelated Hematopoietic Stem Cell Transplantation. Blood **2014**;124: 5865-.
348. Paczesny S. Biomarkers for posttransplantation outcomes. Blood **2018**;131: 2193-204.
349. de Koning C, Plantinga M, Besseling P, Boelens JJ, Nierkens S. Immune Reconstitution after Allogeneic Hematopoietic Cell Transplantation in Children. Biol Blood Marrow Transplant **2016**;22: 195-206.
350. Pang N, Xu J, Qu J, Duan X, Yuan H, Chen G, Jiang M, Ding J. Peripheral blood Th9 cells reconstitution and its relationship with acute graft-versus-host disease after matched-sibling peripheral blood hematopoietic stem cell transplantation. Am J Transl Res **2017**;9: 3623-32.
351. Chan YLT, Zuo J, Inman C, Croft W, Begum J, Croudace J, Kinsella F, Maggs L, Nagra S, Nunnick J, Abbotts B, Craddock C, Malladi R, Moss P. NK cells produce high levels of IL-10 early after allogeneic stem cell transplantation and suppress development of acute GVHD. Eur J Immunol **2018**;48: 316-29.
352. Yang YG, Sykes M. The role of interleukin-12 in preserving the graft-versus-leukemia effect of allogeneic CD8 T cells independently of GVHD. Leuk Lymphoma **1999**;33: 409-20.

353. Alpdogan O, Eng JM, Muriglan SJ, Willis LM, Hubbard VM, Tjoe KH, Terwey TH, Kochman A, van den Brink MR. Interleukin-15 enhances immune reconstitution after allogeneic bone marrow transplantation. Blood **2005**;105: 865-73.
354. Pratt LM, Liu Y, Ugarte-Torres A, Hoegh-Petersen M, Podgorny PJ, Lyon AW, Williamson TS, Khan FM, Chaudhry MA, Daly A, Stewart DA, Russell JA, Grigg A, Ritchie D, Storek J. IL15 levels on day 7 after hematopoietic cell transplantation predict chronic GVHD. Bone Marrow Transplant **2013**;48: 722-8.
355. Lamarthee B, Malard F, Saas P, Mohty M, Gaugler B. Interleukin-22 in Graft-Versus-Host Disease after Allogeneic Stem Cell Transplantation. Front Immunol **2016**;7: 148.
356. Fan X, Guo D, Cheung AMS, Poon ZY, Yap CS, Goh SE, Guo D, Li H, Bari S, Li S, Lim KH, Hwang WYK. Mesenchymal Stromal Cell (MSC)-Derived Combination of CXCL5 and Anti-CCL24 Is Synergistic and Superior to MSC and Cyclosporine for the Treatment of Graft-versus-Host Disease. Biol Blood Marrow Transplant **2018**;24: 1971-80.
357. Kuba A, Raida L. Graft versus Host Disease: From Basic Pathogenic Principles to DNA Damage Response and Cellular Senescence. Mediators Inflamm **2018**;2018: 9451950.
358. Gam R, Shah P, Crossland RE, Norden J, Dickinson AM, Dressel R. Genetic Association of Hematopoietic Stem Cell Transplantation Outcome beyond Histocompatibility Genes. Front Immunol **2017**;8: 380.
359. Sun YQ, Kong Y, Zhang XH, Wang Y, Shi MM, Song Y, Kong J, Fu HX, Yan CH, Xu LP, Liu KY, Huang XJ. A novel recombinant human thrombopoietin for treating prolonged isolated thrombocytopenia after allogeneic stem cell transplantation. Platelets **2018**: 1-7.
360. Holtan SG, Arora M. Angiogenic factors and inflammation in steroid-refractory acute graft-vs-host disease. Transl Res **2016**;167: 80-7.
361. Shirvaikar N, Marquez-Curtis LA, Janowska-Wieczorek A. Hematopoietic Stem Cell Mobilization and Homing after Transplantation: The Role of MMP-2, MMP-9, and MT1-MMP. Biochem Res Int **2012**;2012: 685267.
362. Okamoto T, Takatsuka H, Fujimori Y, Wada H, Iwasaki T, Kakishita E. Increased hepatocyte growth factor in serum in acute graft-versus-host disease. Bone Marrow Transplant **2001**;28: 197-200.
363. Schwarzbich MA, Dai H, Kordelas L, Beelen DW, Radujkovic A, Muller-Tidow C, Dreger P, Luft T. Pre-Conditioning Serum Leptin Levels Predict Early Relapse Rates of High Risk AML after Allogeneic Stem Cell Transplantation. Blood **2018**;132: 3419-.
364. Tauchmanova L, Matarese G, Carella C, De Rosa G, Serio B, Ricci P, Lombardi G, Rotoli B, Colao A, Selleri C. High serum leptin in patients with chronic graft-versus-host disease after hematopoietic stem cell transplantation. Transplantation **2004**;78: 1376-83.
365. Kim SW, Fuji S, Fukuda T, Mori S, Kamiya S, Furuta K, Yokoyama H, Kurosawa S, Saito B, Kuwahara S, Heike Y, Tanosaki R. Elevated Serum Leptin Level In Patients With Persistent Anorexia After Allogeneic Hematopoietic Stem Cell Transplantation (Allo-HSCT), 2010.
366. Arcese W, De Angelis G, Cerretti R. Granulocyte-mobilized bone marrow. Curr Opin Hematol **2012**;19: 448-53.
367. Goetzl EJ, Banda MJ, Leppert D. Matrix metalloproteinases in immunity. J Immunol **1996**;156: 1-4.
368. Tervahartiala T, Pirila E, Ceponis A, Maisi P, Salo T, Tuter G, Kallio P, Tornwall J, Srinivas R, Konttinen YT, Sorsa T. The in vivo expression of the collagenolytic matrix metalloproteinases (MMP-2, -8, -13, and -14) and matrilysin (MMP-7) in adult and localized juvenile periodontitis. J Dent Res **2000**;79: 1969-77.
369. Handsley MM, Edwards DR. Metalloproteinases and their inhibitors in tumor angiogenesis. Int J Cancer **2005**;115: 849-60.
370. Brew K, Nagase H. The tissue inhibitors of metalloproteinases (TIMPs): an ancient family with structural and functional diversity. Biochim Biophys Acta **2010**;1803: 55-71.
371. Melendez-Zajgla J, Del Pozo L, Ceballos G, Maldonado V. Tissue inhibitor of metalloproteinases-4. The road less traveled. Mol Cancer **2008**;7: 85.
372. Liu YF, Zhang SY, Chen YY, Shi K, Zou B, Liu J, Yang Q, Jiang H, Wei L, Li CZ, Zhao M, Gabrilovich DI, Zhang H, Zhou J. ICAM-1 Deficiency in the Bone Marrow Niche Impairs Quiescence and Repopulation of Hematopoietic Stem Cells. Stem Cell Reports **2018**;11: 258-73.
373. Salmela MT, Karjalainen-Lindsberg ML, Jeskanen L, Saarialho-Kere U. Overexpression of tissue inhibitor of metalloproteinases-3 in intestinal and cutaneous lesions of graft-versus-host disease. Mod Pathol **2003**;16: 108-14.
374. Yu J, Storer BE, Kushekhar K, Abu Zaid M, Zhang Q, Gafken PR, Ogata Y, Martin PJ, Flowers ME, Hansen JA, Arora M, Cutler C, Jagasia M, Pidala J, Hamilton BK, Chen GL, Pusic I, Lee SJ, Paczesny S. Biomarker Panel for Chronic Graft-Versus-Host Disease. J Clin Oncol **2016**;34: 2583-90.

375. Klein G, Schmal O, Aicher WK. Matrix metalloproteinases in stem cell mobilization. Matrix Biol **2015**;44-46: 175-83.
376. Tagami K, Yujiri T, Takahashi T, Kizuki N, Tanaka Y, Mitani N, Nakamura Y, Ariyoshi K, Ando T, Gondo T, Tanizawa Y. Increased serum levels of matrix metalloproteinase-9 in acute graft-versus-host disease after allogeneic haematopoietic stem cell transplantation. Int J Hematol **2009**;90: 248-52.
377. England KA, Price AP, Tram KV, Shapiro SD, Blazar BR, Panoskaltsis-Mortari A. Evidence for early fibrosis and increased airway resistance in bone marrow transplant recipient mice deficient in MMP12. Am J Physiol Lung Cell Mol Physiol **2011**;301: L519-26.
378. Janowska-Wieczorek A, Marquez LA, Dobrowsky A, Ratajczak MZ, Cabuhat ML. Differential MMP and TIMP production by human marrow and peripheral blood CD34(+) cells in response to chemokines. Exp Hematol **2000**;28: 1274-85.
379. Hussein K, Stucki-Koch A, Alchalby H, Triviai I, Kroger N, Kreipe H. Cytokine Expression Pattern in Bone Marrow Microenvironment after Allogeneic Stem Cell Transplantation in Primary Myelofibrosis. Biol Blood Marrow Transplant **2016**;22: 644-50.
380. Paczesny S, Krijanovski OI, Braun TM, Choi SW, Clouthier SG, Kuick R, Misek DE, Cooke KR, Kitko CL, Weyand A, Bickley D, Jones D, Whitfield J, Reddy P, Levine JE, Hanash SM, Ferrara JL. A biomarker panel for acute graft-versus-host disease. Blood **2009**;113: 273-8.
381. Shen Y, Winkler IG, Barbier V, Sims NA, Hendy J, Levesque JP. Tissue inhibitor of metalloproteinase-3 (TIMP-3) regulates hematopoiesis and bone formation in vivo. PLoS One **2010**;5.
382. De Greef GE, Braakman E, Alemayehu WG, Graaf LD, van Geel P, Grootes M, Engelman C, Petersen E, Visser O, Vucinic V, Niederwieser D, Cornelissen JJ. Lymphocyte and Hematopoietic Stem and Progenitor Cell (HSPC) Subsets Mobilized By Either Plerixafor or G-GCSF: A Retrospective Comparison of Grafts Harvested in Healthy Allogeneic Stem Cell Donors **2014**;124: 2451-.
383. Wong PPC, Kariminia A, Jones D, Eaves CJ, Foley R, Ivison S, Couban S, Schultz KR. Plerixafor effectively mobilizes CD56(bright) NK cells in blood, providing an allograft predicted to protect against GVHD. Blood **2018**;131: 2863-6.
384. Fidyk W, Mitrus I, Ciomber A, Smagur A, Chwieduk A, Glowala-Kosinska M, Giebel S. Evaluation of proinflammatory and immunosuppressive cytokines in blood and bone marrow of healthy hematopoietic stem cell donors. Cytokine **2017**.
385. Marquez-Curtis LA, Turner AR, Sridharan S, Ratajczak MZ, Janowska-Wieczorek A. The ins and outs of hematopoietic stem cells: studies to improve transplantation outcomes. Stem Cell Rev **2011**;7: 590-607.
386. Christ O, Kronenwett R, Haas R, Zoller M. Combining G-CSF with a blockade of adhesion strongly improves the reconstitutive capacity of mobilized hematopoietic progenitor cells. Exp Hematol **2001**;29: 380-90.
387. Hoggatt J, Singh P, Tate TA, Chou BK, Datari SR, Fukuda S, Liu L, Kharchenko PV, Schajnovitz A, Baryawno N, Mercier FE, Boyer J, Gardner J, Morrow DM, Scadden DT, Pelus LM. Rapid Mobilization Reveals a Highly Engraftable Hematopoietic Stem Cell. Cell **2018**;172: 191-204.e10.
388. Thiant S, Moutuou MM, Leboeuf D, Guimond M. Homeostatic cytokines in immune reconstitution and graft-versus-host disease. Cytokine **2016**;82: 24-32.
389. Seggewiss R, Einsele H. Immune reconstitution after allogeneic transplantation and expanding options for immunomodulation: an update. Blood **2010**;115: 3861-8.
390. Liu Z, Fan H, Jiang S. CD4(+) T-cell subsets in transplantation. Immunol Rev **2013**;252: 183-91.
391. Buzzeo MP, Yang J, Casella G, Reddy V. Hematopoietic stem cell mobilization with G-CSF induces innate inflammation yet suppresses adaptive immune gene expression as revealed by microarray analysis. Exp Hematol **2007**;35: 1456-65.
392. Hernandez JM, Castilla C, Gutierrez NC, Isidro IM, Delgado M, de las Rivas J, Ferminan E, Garcia JL, Ocio EM, del Canizo MC, San Miguel JF. Mobilisation with G-CSF in healthy donors promotes a high but temporal deregulation of genes. Leukemia **2005**;19: 1088-91.
393. Mohty M, Blaise D, Faucher C, Vey N, Bouabdallah R, Stoppa AM, Viret F, Gravis G, Olive D, Gaugler B. Inflammatory cytokines and acute graft-versus-host disease after reduced-intensity conditioning allogeneic stem cell transplantation. Blood **2005**;106: 4407-11.
394. Asavaroengchai W, Wang H, Wang S, Wang L, Bronson R, Sykes M, Yang YG. An essential role for IFN-gamma in regulation of alloreactive CD8 T cells following allogeneic hematopoietic cell transplantation. Biol Blood Marrow Transplant **2007**;13: 46-55.
395. Wang H, Yang YG. The complex and central role of interferon-gamma in graft-versus-host disease and graft-versus-tumor activity. Immunol Rev **2014**;258: 30-44.
396. Holler E, Roncarolo MG, Hintermeier-Knabe R, Eissner G, Ertl B, Schulz U, Knabe H, Kolb HJ, Andreesen R, Wilmanns W. Prognostic significance of increased IL-10 production in patients prior to allogeneic bone marrow transplantation. Bone Marrow Transplant **2000**;25: 237-41.

397. Remberger M, Jaksch M, Uzunel M, Mattsson J. Serum levels of cytokines correlate to donor chimerism and acute graft-vs.-host disease after haematopoietic stem cell transplantation. Eur J Haematol **2003**;70: 384-91.
398. Abraham S, Choi JG, Ye C, Manjunath N, Shankar P. IL-10 exacerbates xenogeneic GVHD by inducing massive human T cell expansion. Clin Immunol **2015**;156: 58-64.
399. Sykes M, Szot GL, Nguyen PL, Pearson DA. Interleukin-12 inhibits murine graft-versus-host disease. Blood **1995**;86: 2429-38.
400. Yang YG, Sergio JJ, Pearson DA, Szot GL, Shimizu A, Sykes M. Interleukin-12 preserves the graft-versus-leukemia effect of allogeneic CD8 T cells while inhibiting CD4-dependent graft-versus-host disease in mice. Blood **1997**;90: 4651-60.
401. Reddy P, Teshima T, Kukuruga M, Ordemann R, Liu C, Lowler K, Ferrara JL. Interleukin-18 regulates acute graft-versus-host disease by enhancing Fas-mediated donor T cell apoptosis. J Exp Med **2001**;194: 1433-40.
402. Yi T, Chen Y, Wang L, Du G, Huang D, Zhao D, Johnston H, Young J, Todorov I, Umetsu DT, Chen L, Iwakura Y, Kandeel F, Forman S, Zeng D. Reciprocal differentiation and tissue-specific pathogenesis of Th1, Th2, and Th17 cells in graft-versus-host disease. Blood **2009**;114: 3101-12.
403. Nikolic B, Lee S, Bronson RT, Grusby MJ, Sykes M. Th1 and Th2 mediate acute graft-versus-host disease, each with distinct end-organ targets. J Clin Invest **2000**;105: 1289-98.
404. Carlson MJ, West ML, Coghill JM, Panoskaltsis-Mortari A, Blazar BR, Serody JS. In vitro-differentiated TH17 cells mediate lethal acute graft-versus-host disease with severe cutaneous and pulmonary pathologic manifestations. Blood **2009**;113: 1365-74.
405. Loeffler J, Ok M, Morton OC, Mezger M, Einsele H. Genetic polymorphisms in the cytokine and chemokine system: their possible importance in allogeneic stem cell transplantation. Curr Top Microbiol Immunol **2010**;341: 83-96.
406. Fisher LW, Torchia DA, Fohr B, Young MF, Fedarko NS. Flexible structures of SIBLING proteins, bone sialoprotein, and osteopontin. Biochem Biophys Res Commun **2001**;280: 460-5.
407. Kazanecki CC, Uzwiak DJ, Denhardt DT. Control of osteopontin signaling and function by post-translational phosphorylation and protein folding. J Cell Biochem **2007**;102: 912-24.
408. Mazzali M, Kipari T, Ophascharoensuk V, Wesson JA, Johnson R, Hughes J. Osteopontin--a molecule for all seasons. QJM **2002**;95: 3-13.
409. Yokosaki Y, Tanaka K, Higashikawa F, Yamashita K, Eboshida A. Distinct structural requirements for binding of the integrins alphavbeta6, alphavbeta3, alphavbeta5, alpha5beta1 and alpha9beta1 to osteopontin. Matrix Biol **2005**;24: 418-27.
410. Wang KX, Denhardt DT. Osteopontin: role in immune regulation and stress responses. Cytokine Growth Factor Rev **2008**;19: 333-45.
411. Ponta H, Sherman L, Herrlich PA. CD44: from adhesion molecules to signalling regulators. Nat Rev Mol Cell Biol **2003**;4: 33-45.
412. Zohar R, Suzuki N, Suzuki K, Arora P, Glogauer M, McCulloch CA, Sodek J. Intracellular osteopontin is an integral component of the CD44-ERM complex involved in cell migration. J Cell Physiol **2000**;184: 118-30.
413. Castello LM, Raineri D, Salmi L, Clemente N, Vaschetto R, Quaglia M, Garzaro M, Gentilli S, Navalesi P, Cantaluppi V, Dianzani U, Aspesi A, Chiocchetti A. Osteopontin at the Crossroads of Inflammation and Tumor Progression. Mediators Inflamm **2017**;2017: 4049098.
414. Inoue M, Shinohara ML. Intracellular osteopontin (iOPN) and immunity. Immunol Res **2011**;49: 160-72.
415. Denhardt DT, Noda M, O'Regan AW, Pavlin D, Berman JS. Osteopontin as a means to cope with environmental insults: regulation of inflammation, tissue remodeling, and cell survival. J Clin Invest **2001**;107: 1055-61.
416. Rangaswami H, Bulbule A, Kundu GC. Osteopontin: role in cell signaling and cancer progression. Trends Cell Biol **2006**;16: 79-87.
417. Stier S, Ko Y, Forkert R, Lutz C, Neuhaus T, Grunewald E, Cheng T, Dombkowski D, Calvi LM, Rittling SR, Scadden DT. Osteopontin is a hematopoietic stem cell niche component that negatively regulates stem cell pool size. J Exp Med **2005**;201: 1781-91.
418. Nilsson SK, Johnston HM, Whitty GA, Williams B, Webb RJ, Denhardt DT, Bertoncello I, Bendall LJ, Simmons PJ, Haylock DN. Osteopontin, a key component of the hematopoietic stem cell niche and regulator of primitive hematopoietic progenitor cells. Blood **2005**;106: 1232-9.
419. Li S, Zhai Q, Zou D, Meng H, Xie Z, Li C, Wang Y, Qi J, Cheng T, Qiu L. A pivotal role of bone remodeling in granulocyte colony stimulating factor induced hematopoietic stem/progenitor cells mobilization. J Cell Physiol **2013**;228: 1002-9.

420. Guidi N, Sacma M, Standker L, Soller K, Marka G, Eiwen K, Weiss JM, Kirchhoff F, Weil T, Cancelas JA, Florian MC, Geiger H. Osteopontin attenuates aging-associated phenotypes of hematopoietic stem cells. EMBO J **2017**;36: 840-53.
421. Weber GF, Zawaideh S, Hikita S, Kumar VA, Cantor H, Ashkar S. Phosphorylation-dependent interaction of osteopontin with its receptors regulates macrophage migration and activation. J Leukoc Biol **2002**;72: 752-61.
422. Koh A, da Silva AP, Bansal AK, Bansal M, Sun C, Lee H, Glogauer M, Sodek J, Zohar R. Role of osteopontin in neutrophil function. Immunology **2007**;122: 466-75.
423. Renkl AC, Wussler J, Ahrens T, Thoma K, Kon S, Uede T, Martin SF, Simon JC, Weiss JM. Osteopontin functionally activates dendritic cells and induces their differentiation toward a Th1-polarizing phenotype. Blood **2005**;106: 946-55.
424. Zhang ZX, Shek K, Wang S, Huang X, Lau A, Yin Z, Sun H, Liu W, Garcia B, Rittling S, Jevnikar AM. Osteopontin expressed in tubular epithelial cells regulates NK cell-mediated kidney ischemia reperfusion injury. J Immunol **2010**;185: 967-73.
425. O'Regan AW, Chupp GL, Lowry JA, Goetschkes M, Mulligan N, Berman JS. Osteopontin is associated with T cells in sarcoid granulomas and has T cell adhesive and cytokine-like properties in vitro. J Immunol **1999**;162: 1024-31.
426. Ashkar S, Weber GF, Panoutsakopoulou V, Sanchirico ME, Jansson M, Zawaideh S, Rittling SR, Denhardt DT, Glimcher MJ, Cantor H. Eta-1 (osteopontin): an early component of type-1 (cell-mediated) immunity. Science **2000**;287: 860-4.
427. Higuchi Y, Tamura Y, Uchida T, Matsuura K, Hijiya N, Yamamoto S. The roles of soluble osteopontin using osteopontin-transgenic mice in vivo: proliferation of CD4+ T lymphocytes and the enhancement of cell-mediated immune responses. Pathobiology **2004**;71: 1-11.
428. Shinohara ML, Jansson M, Hwang ES, Werneck MB, Glimcher LH, Cantor H. T-bet-dependent expression of osteopontin contributes to T cell polarization. Proc Natl Acad Sci U S A **2005**;102: 17101-6.
429. Cantor H, Shinohara ML. Regulation of T-helper-cell lineage development by osteopontin: the inside story. Nat Rev Immunol **2009**;9: 137-41.
430. Murugaiyan G, Mittal A, Weiner HL. Increased osteopontin expression in dendritic cells amplifies IL-17 production by CD4+ T cells in experimental autoimmune encephalomyelitis and in multiple sclerosis. J Immunol **2008**;181: 7480-8.
431. Murugaiyan G, Mittal A, Weiner HL. Identification of an IL-27/osteopontin axis in dendritic cells and its modulation by IFN-gamma limits IL-17-mediated autoimmune inflammation. Proc Natl Acad Sci U S A **2010**;107: 11495-500.
432. Guan H, Nagarkatti PS, Nagarkatti M. Role of CD44 in the differentiation of Th1 and Th2 cells: CD44-deficiency enhances the development of Th2 effectors in response to sheep RBC and chicken ovalbumin. J Immunol **2009**;183: 172-80.
433. Guo B, Tumang JR, Rothstein TL. B cell receptor crosstalk: B cells express osteopontin through the combined action of the alternate and classical BCR signaling pathways. Mol Immunol **2009**;46: 587-91.
434. Leavenworth JW, Verbinnen B, Yin J, Huang H, Cantor H. A p85alpha-osteopontin axis couples the receptor ICOS to sustained Bcl-6 expression by follicular helper and regulatory T cells. Nat Immunol **2015**;16: 96-106.
435. Lampe MA, Patarca R, Iregui MV, Cantor H. Polyclonal B cell activation by the Eta-1 cytokine and the development of systemic autoimmune disease. J Immunol **1991**;147: 2902-6.
436. Rittling SR, Singh R. Osteopontin in Immune-mediated Diseases. J Dent Res **2015**;94: 1638-45.
437. Leavenworth JW, Verbinnen B, Wang Q, Shen E, Cantor H. Intracellular osteopontin regulates homeostasis and function of natural killer cells. Proc Natl Acad Sci U S A **2015**;112: 494-9.
438. Heilmann K, Hoffmann U, Witte E, Loddenkemper C, Sina C, Schreiber S, Hayford C, Holzlohner P, Wolk K, Tchatchou E, Moos V, Zeitz M, Sabat R, Gunthert U, Wittig BM. Osteopontin as two-sided mediator of intestinal inflammation. J Cell Mol Med **2009**;13: 1162-74.
439. Goncalves DaSilva A, Liaw L, Yong VW. Cleavage of osteopontin by matrix metalloproteinase-12 modulates experimental autoimmune encephalomyelitis disease in C57BL/6 mice. Am J Pathol **2010**;177: 1448-58.
440. Zhao F, Zhang Y, Wang H, Jin M, He S, Shi Y, Guo Y, Zhang Y. Blockade of osteopontin reduces alloreactive CD8+ T cell-mediated graft-versus-host disease. Blood **2011**;117: 1723-33.
441. Kawakami K, Minami N, Matsuura M, Iida T, Toyonaga T, Nagaishi K, Arimura Y, Fujimiya M, Uede T, Nakase H. Osteopontin attenuates acute gastrointestinal graft-versus-host disease by preventing apoptosis of intestinal epithelial cells. Biochem Biophys Res Commun **2017**;485: 468-75.

442. Fiehn O. Combining genomics, metabolome analysis, and biochemical modelling to understand metabolic networks. Comp Funct Genomics **2001**;2: 155-68.
443. Klassen A, Faccio AT, Canuto GA, da Cruz PL, Ribeiro HC, Tavares MF, Sussulini A. Metabolomics: Definitions and Significance in Systems Biology. Adv Exp Med Biol **2017**;965: 3-17.
444. Bantug GR, Galluzzi L, Kroemer G, Hess C. The spectrum of T cell metabolism in health and disease. Nat Rev Immunol **2018**;18: 19-34.
445. Zmora N, Bashiardes S, Levy M, Elinav E. The Role of the Immune System in Metabolic Health and Disease. Cell Metab **2017**;25: 506-21.
446. Ganeshan K, Chawla A. Metabolic regulation of immune responses. Annu Rev Immunol **2014**;32: 609-34.
447. Hino S, Nagaoka K, Nakao M. Metabolism-epigenome crosstalk in physiology and diseases. J Hum Genet **2013**;58: 410-5.
448. Buck MD, O'Sullivan D, Pearce EL. T cell metabolism drives immunity. J Exp Med **2015**;212: 1345-60.
449. Chi H. Regulation and function of mTOR signalling in T cell fate decisions. Nat Rev Immunol **2012**;12: 325-38.
450. Wang R, Dillon CP, Shi LZ, Milasta S, Carter R, Finkelstein D, McCormick LL, Fitzgerald P, Chi H, Munger J, Green DR. The transcription factor Myc controls metabolic reprogramming upon T lymphocyte activation. Immunity **2011**;35: 871-82.
451. Gnanaprakasam JNR, Sherman JW, Wang R. MYC and HIF in shaping immune response and immune metabolism. Cytokine Growth Factor Rev **2017**;35: 63-70.
452. Gerriets VA, Rathmell JC. Metabolic pathways in T cell fate and function. Trends Immunol **2012**;33: 168-73.
453. Vander Heiden MG, Cantley LC, Thompson CB. Understanding the Warburg effect: the metabolic requirements of cell proliferation. Science **2009**;324: 1029-33.
454. Warburg O. On the origin of cancer cells. Science **1956**;123: 309-14.
455. Hui S, Ghergurovich JM, Morscher RJ, Jang C, Teng X, Lu W, Esparza LA, Reya T, Le Z, Yanxiang Guo J, White E, Rabinowitz JD. Glucose feeds the TCA cycle via circulating lactate. Nature **2017**;551: 115-8.
456. Chang CH, Curtis JD, Maggi LB, Jr., Faubert B, Villarino AV, O'Sullivan D, Huang SC, van der Windt GJ, Blagih J, Qiu J, Weber JD, Pearce EJ, Jones RG, Pearce EL. Posttranscriptional control of T cell effector function by aerobic glycolysis. Cell **2013**;153: 1239-51.
457. Haas R, Smith J, Rocher-Ros V, Nadkarni S, Montero-Melendez T, D'Acquisto F, Bland EJ, Bombardieri M, Pitzalis C, Perretti M, Marelli-Berg FM, Mauro C. Lactate Regulates Metabolic and Pro-inflammatory Circuits in Control of T Cell Migration and Effector Functions. PLoS Biol **2015**;13: e1002202.
458. Jacobs SR, Herman CE, Maciver NJ, Wofford JA, Wieman HL, Hammen JJ, Rathmell JC. Glucose uptake is limiting in T cell activation and requires CD28-mediated Akt-dependent and independent pathways. J Immunol **2008**;180: 4476-86.
459. De Rosa V, Galgani M, Porcellini A, Colamatteo A, Santopaolo M, Zuchegna C, Romano A, De Simone S, Procaccini C, La Rocca C, Carrieri PB, Maniscalco GT, Salvetti M, Buscarinu MC, Franzese A, Mozzillo E, La Cava A, Matarese G. Glycolysis controls the induction of human regulatory T cells by modulating the expression of FOXP3 exon 2 splicing variants. Nat Immunol **2015**;16: 1174-84.
460. Berod L, Friedrich C, Nandan A, Freitag J, Hagemann S, Harmrolfs K, Sandouk A, Hesse C, Castro CN, Bahre H, Tschirner SK, Gorinski N, Gohmert M, Mayer CT, Huehn J, Ponimaskin E, Abraham WR, Muller R, Lochner M, Sparwasser T. De novo fatty acid synthesis controls the fate between regulatory T and T helper 17 cells. Nat Med **2014**;20: 1327-33.
461. Klysz D, Tai X, Robert PA, Craveiro M, Cretenet G, Oburoglu L, Mongellaz C, Floess S, Fritz V, Matias MI, Yong C, Surh N, Marie JC, Huehn J, Zimmermann V, Kinet S, Dardalhon V, Taylor N. Glutamine-dependent alpha-ketoglutarate production regulates the balance between T helper 1 cell and regulatory T cell generation. Sci Signal **2015**;8: ra97.
462. Jones N, Cronin JG, Dolton G, Panetti S, Schauenburg AJ, Galloway SAE, Sewell AK, Cole DK, Thornton CA, Francis NJ. Metabolic Adaptation of Human CD4(+) and CD8(+) T-Cells to T-Cell Receptor-Mediated Stimulation. Front Immunol **2017**;8: 1516.
463. Johnson MO, Wolf MM, Madden MZ, Andrejeva G, Sugiura A, Contreras DC, Maseda D, Liberti MV, Paz K, Kishton RJ, Johnson ME, de Cubas AA, Wu P, Li G, Zhang Y, Newcomb DC, Wells AD, Restifo NP, Rathmell WK, Locasale JW, Davila ML, Blazar BR, Rathmell JC. Distinct Regulation of Th17 and Th1 Cell Differentiation by Glutaminase-Dependent Metabolism. Cell **2018**;175: 1780-95 e19.

464. Pearce EL, Walsh MC, Cejas PJ, Harms GM, Shen H, Wang LS, Jones RG, Choi Y. Enhancing CD8 T-cell memory by modulating fatty acid metabolism. Nature **2009**;460: 103-7.
465. Buck MD, O'Sullivan D, Klein Geltink RI, Curtis JD, Chang CH, Sanin DE, Qiu J, Kretz O, Braas D, van der Windt GJ, Chen Q, Huang SC, O'Neill CM, Edelson BT, Pearce EJ, Sesaki H, Huber TB, Rambold AS, Pearce EL. Mitochondrial Dynamics Controls T Cell Fate through Metabolic Programming. Cell **2016**;166: 63-76.
466. Doughty CA, Bleiman BF, Wagner DJ, Dufort FJ, Mataraza JM, Roberts MF, Chiles TC. Antigen receptor-mediated changes in glucose metabolism in B lymphocytes: role of phosphatidylinositol 3-kinase signaling in the glycolytic control of growth. Blood **2006**;107: 4458-65.
467. Cho SH, Ahn AK, Bhargava P, Lee CH, Eischen CM, McGuinness O, Boothby M. Glycolytic rate and lymphomagenesis depend on PARP14, an ADP ribosyltransferase of the B aggressive lymphoma (BAL) family. Proc Natl Acad Sci U S A **2011**;108: 15972-7.
468. Waters LR, Ahsan FM, Wolf DM, Shirihai O, Teitell MA. Initial B Cell Activation Induces Metabolic Reprogramming and Mitochondrial Remodeling. iScience **2018**;5: 99-109.
469. Shi Z, Mirza M, Wang B, Kennedy MA, Weber GF. Osteopontin-a alters glucose homeostasis in anchorage-independent breast cancer cells. Cancer Lett **2014**;344: 47-53.
470. Nguyen HD, Chatterjee S, Haarberg KM, Wu Y, Bastian D, Heinrichs J, Fu J, Daenthanasanmak A, Schutt S, Shrestha S, Liu C, Wang H, Chi H, Mehrotra S, Yu XZ. Metabolic reprogramming of alloantigen-activated T cells after hematopoietic cell transplantation. J Clin Invest **2016**;126: 1337-52.
471. Bettonville M, d'Aria S, Weatherly K, Porporato PE, Zhang J, Bousbata S, Sonveaux P, Braun MY. Long-term antigen exposure irreversibly modifies metabolic requirements for T cell function. Elife **2018**;7.
472. Glick GD, Rossignol R, Lyssiotis CA, Wahl D, Lesch C, Sanchez B, Liu X, Hao LY, Taylor C, Hurd A, Ferrara JL, Tkachev V, Byersdorfer CA, Boros L, Opipari AW. Anaplerotic metabolism of alloreactive T cells provides a metabolic approach to treat graft-versus-host disease. J Pharmacol Exp Ther **2014**;351: 298-307.
473. Gatza E, Wahl DR, Opipari AW, Sundberg TB, Reddy P, Liu C, Glick GD, Ferrara JL. Manipulating the bioenergetics of alloreactive T cells causes their selective apoptosis and arrests graft-versus-host disease. Sci Transl Med **2011**;3: 67ra8.
474. Byersdorfer CA, Tkachev V, Opipari AW, Goodell S, Swanson J, Sandquist S, Glick GD, Ferrara JL. Effector T cells require fatty acid metabolism during murine graft-versus-host disease. Blood **2013**;122: 3230-7.
475. sMathewson ND, Jenq R, Mathew AV, Koenigsknecht M, Hanash A, Toubai T, Oravecz-Wilson K, Wu SR, Sun Y, Rossi C, Fujiwara H, Byun J, Shono Y, Lindemans C, Calafiore M, Schmidt TM, Honda K, Young VB, Pennathur S, van den Brink M, Reddy P. Gut microbiome-derived metabolites modulate intestinal epithelial cell damage and mitigate graft-versus-host disease. Nat Immunol **2016**;17: 505-13.
476. Patel CH, Powell JD. Targeting T cell metabolism to regulate T cell activation, differentiation and function in disease. Curr Opin Immunol **2017**;46: 82-8.
477. Chang CH, Pearce EL. Emerging concepts of T cell metabolism as a target of immunotherapy. Nat Immunol **2016**;17: 364-8.
478. Tanimine N, Turka LA, Priyadharshini B. Navigating T-Cell Immunometabolism in Transplantation. Transplantation **2018**;102: 230-9.
479. Lee CF, Lo YC, Cheng CH, Furtmuller GJ, Oh B, Andrade-Oliveira V, Thomas AG, Bowman CE, Slusher BS, Wolfgang MJ, Brandacher G, Powell JD. Preventing Allograft Rejection by Targeting Immune Metabolism. Cell Rep **2015**;13: 760-70.
480. Galluzzi L, Kepp O, Vander Heiden MG, Kroemer G. Metabolic targets for cancer therapy. Nat Rev Drug Discov **2013**;12: 829-46.
481. Sukumar M, Liu J, Ji Y, Subramanian M, Crompton JG, Yu Z, Roychoudhuri R, Palmer DC, Muranski P, Karoly ED, Mohney RP, Klebanoff CA, Lal A, Finkel T, Restifo NP, Gattinoni L. Inhibiting glycolytic metabolism enhances CD8+ T cell memory and antitumor function. J Clin Invest **2013**;123: 4479-88.
482. Sukumar M, Kishton RJ, Restifo NP. Metabolic reprograming of anti-tumor immunity. Curr Opin Immunol **2017**;46: 14-22.
483. Raha S, Raud B, Oberdorfer L, Castro CN, Schreder A, Freitag J, Longerich T, Lochner M, Sparwasser T, Berod L, Koenecke C, Prinz I. Disruption of de novo fatty acid synthesis via acetyl-CoA carboxylase 1 inhibition prevents acute graft-versus-host disease. Eur J Immunol **2016**;46: 2233-8.

484. Sofi MH, Heinrichs J, Dany M, Nguyen H, Dai M, Bastian D, Schutt S, Wu Y, Daenthanasanmak A, Gencer S, Zivkovic A, Szulc Z, Stark H, Liu C, Chang YJ, Ogretmen B, Yu XZ. Ceramide synthesis regulates T cell activity and GVHD development. JCI Insight **2017**;2.
485. Rutella S, Rumi C, Pierelli L, Morosetti R, Sica S, Bonanno G, Scambia G, Leone G. Granulocyte colony-stimulating factor perturbs lymphocyte mitochondrial function and inhibits cell cycle progression. Exp Hematol **2000**;28: 612-25.
486. Dietrich S, Radujkovic A, Stolzel F, Falk CS, Benner A, Schaich M, Bornhauser M, Ehninger G, Kramer A, Hegenbart U, Ho AD, Dreger P, Luft T. Pretransplant metabolic distress predicts relapse of acute myeloid leukemia after allogeneic stem cell transplantation. Transplantation **2015**;99: 1065-71.
487. Lippi G, Guidi GC, Mattiuzzi C, Plebani M. Preanalytical variability: the dark side of the moon in laboratory testing. Clin Chem Lab Med **2006**;44: 358-65.
488. Bull M, Lee D, Stucky J, Chiu YL, Rubin A, Horton H, McElrath MJ. Defining blood processing parameters for optimal detection of cryopreserved antigen-specific responses for HIV vaccine trials. J Immunol Methods **2007**;322: 57-69.
489. Rundgren IM, Bruserud O, Ryningen A, Ersvaer E. Standardization of sampling and sample preparation for analysis of human monocyte subsets in peripheral blood. J Immunol Methods **2018**;461: 53-62.
490. Thornthwaite JT, Rosenthal PK, Vazquez DA, Seckinger D. The effects of anticoagulant and temperature on the measurements of helper and suppressor cells. Diagn Immunol **1984**;ge2: 167-74.
491. Gao C, Boylan B, Fang J, Wilcox DA, Newman DK, Newman PJ. Heparin promotes platelet responsiveness by potentiating alphaIIbbeta3-mediated outside-in signaling. Blood **2011**;117: 4946-52.
492. Wong HL, Pfeiffer RM, Fears TR, Vermeulen R, Ji S, Rabkin CS. Reproducibility and correlations of multiplex cytokine levels in asymptomatic persons. Cancer Epidemiol Biomarkers Prev **2008**;17: 3450-6.
493. Tvedt TH, Rye KP, Reikvam H, Brenner AK, Bruserud O. The importance of sample collection when using single cytokine levels and systemic cytokine profiles as biomarkers--a comparative study of serum versus plasma samples. J Immunol Methods **2015**;418: 19-28.
494. Yu Z, Kastenmuller G, He Y, Belcredi P, Moller G, Prehn C, Mendes J, Wahl S, Roemisch-Margl W, Ceglarek U, Polonikov A, Dahmen N, Prokisch H, Xie L, Li Y, Wichmann HE, Peters A, Kronenberg F, Suhre K, Adamski J, Illig T, Wang-Sattler R. Differences between human plasma and serum metabolite profiles. PLoS One **2011**;6: e21230.
495. Suarez-Diez M, Adam J, Adamski J, Chasapi SA, Luchinat C, Peters A, Prehn C, Santucci C, Spyridonidis A, Spyroulias GA, Tenori L, Wang-Sattler R, Saccenti E. Plasma and Serum Metabolite Association Networks: Comparability within and between Studies Using NMR and MS Profiling. J Proteome Res **2017**;16: 2547-59.
496. Boyum A. Separation of White Blood Cells. Nature **1964**;204: 793-4.
497. Boyum A. Separation of leukocytes from blood and bone marrow. Introduction. Scand J Clin Lab Invest Suppl **1968**;97: 7.
498. Maecker HT, Rinfret A, D'Souza P, Darden J, Roig E, Landry C, Hayes P, Birungi J, Anzala O, Garcia M, Harari A, Frank I, Baydo R, Baker M, Holbrook J, Ottinger J, Lamoreaux L, Epling CL, Sinclair E, Suni MA, Punt K, Calarota S, El-Bahi S, Alter G, Maila H, Kuta E, Cox J, Gray C, Altfeld M, Nougarede N, Boyer J, Tussey L, Tobery T, Bredt B, Roederer M, Koup R, Maino VC, Weinhold K, Pantaleo G, Gilmour J, Horton H, Sekaly RP. Standardization of cytokine flow cytometry assays. BMC Immunol **2005**;6: 13.
499. Lemieux J, Jobin C, Simard C, Neron S. A global look into human T cell subsets before and after cryopreservation using multiparametric flow cytometry and two-dimensional visualization analysis. J Immunol Methods **2016**;434: 73-82.
500. Sasnoor LM, Kale VP, Limaye LS. Supplementation of conventional freezing medium with a combination of catalase and trehalose results in better protection of surface molecules and functionality of hematopoietic cells. J Hematother Stem Cell Res **2003**;12: 553-64.
501. Sasnoor LM, Kale VP, Limaye LS. A combination of catalase and trehalose as additives to conventional freezing medium results in improved cryoprotection of human hematopoietic cells with reference to in vitro migration and adhesion properties. Transfusion **2005**;45: 622-33.
502. Lioznov M, Dellbrugger C, Sputtek A, Fehse B, Kroger N, Zander AR. Transportation and cryopreservation may impair haematopoietic stem cell function and engraftment of allogeneic PBSCs, but not BM. Bone Marrow Transplant **2008**;42: 121-8.
503. Liseth K, Ersvaer E, Abrahamsen JF, Nesthus I, Ryningen A, Bruserud O. Long-term cryopreservation of autologous stem cell grafts: a clinical and experimental study of hematopoietic and immunocompetent cells [monograph on the internet]. 2009.

504. Fisher V, Khuu H, David-Ocampo V, Byrne K, Pavletic S, Bishop M, Fowler DH, Barrett AJ, Stroncek DF. Analysis of the recovery of cryopreserved and thawed CD34+ and CD3+ cells collected for hematopoietic transplantation. Transfusion **2014**;54: 1088-92.
505. Berens C, Heine A, Muller J, Held SA, Mayer K, Brossart P, Oldenburg J, Potzsch B, Wolf D, Ruhl H. Variable resistance to freezing and thawing of CD34-positive stem cells and lymphocyte subpopulations in leukapheresis products. Cytotherapy **2016**;18: 1325-31.
506. Watz E, Remberger M, Ringden O, Ljungman P, Sundin M, Mattsson J, Uhlin M. Quality of the hematopoietic stem cell graft affects the clinical outcome of allogeneic stem cell transplantation. Transfusion **2015**;55: 2339-50.
507. Kim DH, Jamal N, Saragosa R, Loach D, Wright J, Gupta V, Kuruvilla J, Lipton JH, Minden M, Messner HA. Similar outcomes of cryopreserved allogeneic peripheral stem cell transplants (PBSCT) compared to fresh allografts. Biol Blood Marrow Transplant **2007**;13: 1233-43.
508. Parody R, Caballero D, Marquez-Malaver FJ, Vazquez L, Saldana R, Madrigal MD, Calderon C, Carrillo E, Lopez-Corral L, Espigado I, Carmona M, Lopez-Villar O, Perez-Simon JA. To freeze or not to freeze peripheral blood stem cells prior to allogeneic transplantation from matched related donors. Eur J Haematol **2013**;91: 448-55.
509. Medd P, Nagra S, Hollyman D, Craddock C, Malladi R. Cryopreservation of allogeneic PBSC from related and unrelated donors is associated with delayed platelet engraftment but has no impact on survival. Bone Marrow Transplant **2013**;48: 243-8.
510. Florek M, Schneidawind D, Pierini A, Baker J, Armstrong R, Pan Y, Leveson-Gower D, Negrin R, Meyer E. Freeze and Thaw of CD4+CD25+Foxp3+ Regulatory T Cells Results in Loss of CD62L Expression and a Reduced Capacity to Protect against Graft-versus-Host Disease. PLoS One **2015**;10: e0145763.
511. Hattori Y, Kato H, Nitta M, Takamoto S. Decrease of L-selectin expression on human CD34+ cells on freeze-thawing and rapid recovery with short-term incubation. Exp Hematol **2001**;29: 114-22.
512. de Jager W, Bourcier K, Rijkers GT, Prakken BJ, Seyfert-Margolis V. Prerequisites for cytokine measurements in clinical trials with multiplex immunoassays. BMC Immunol **2009**;10: 52.
513. Roederer M, De Rosa S, Gerstein R, Anderson M, Bigos M, Stovel R, Nozaki T, Parks D, Herzenberg L, Herzenberg L. 8 color, 10-parameter flow cytometry to elucidate complex leukocyte heterogeneity. Cytometry **1997**;29: 328-39.
514. Roederer M. Spectral compensation for flow cytometry: visualization artifacts, limitations, and caveats. Cytometry **2001**;45: 194-205.
515. Appay V, van Lier RA, Sallusto F, Roederer M. Phenotype and function of human T lymphocyte subsets: consensus and issues. Cytometry A **2008**;73: 975-83.
516. Romano M, Fanelli G, Albany CJ, Giganti G, Lombardi G. Past, Present, and Future of Regulatory T Cell Therapy in Transplantation and Autoimmunity. Front Immunol **2019**;10: 43.
517. Baumgarth N, Roederer M. A practical approach to multicolor flow cytometry for immunophenotyping. J Immunol Methods **2000**;243: 77-97.
518. Finak G, Perez JM, Weng A, Gottardo R. Optimizing transformations for automated, high throughput analysis of flow cytometry data. BMC Bioinformatics **2010**;11: 546.
519. Finak G, Jiang W, Krouse K, Wei C, Sanz I, Phippard D, Asare A, De Rosa SC, Self S, Gottardo R. High-throughput flow cytometry data normalization for clinical trials. Cytometry A **2014**;85: 277-86.
520. Hsiao C, Liu M, Stanton R, McGee M, Qian Y, Scheuermann RH. Mapping cell populations in flow cytometry data for cross-sample comparison using the Friedman-Rafsky test statistic as a distance measure. Cytometry A **2016**;89: 71-88.
521. Bendall SC, Simonds EF, Qiu P, Amir el AD, Krutzik PO, Finck R, Bruggner RV, Melamed R, Trejo A, Ornatsky OI, Balderas RS, Plevritis SK, Sachs K, Pe'er D, Tanner SD, Nolan GP. Single-cell mass cytometry of differential immune and drug responses across a human hematopoietic continuum. Science **2011**;332: 687-96.
522. Stikvoort A, Chen Y, Radestad E, Torlen J, Lakshmikanth T, Bjorklund A, Mikes J, Achour A, Gertow J, Sundberg B, Remberger M, Sundin M, Mattsson J, Brodin P, Uhlin M. Combining Flow and Mass Cytometry in the Search for Biomarkers in Chronic Graft-versus-Host Disease. Front Immunol **2017**;8: 717.
523. Burd EM. Validation of laboratory-developed molecular assays for infectious diseases. Clin Microbiol Rev **2010**;23: 550-76.
524. Engvall E, Perlmann P. Enzyme-linked immunosorbent assay (ELISA). Quantitative assay of immunoglobulin G. Immunochemistry **1971**;8: 871-4.
525. Horan PK, Wheeless LL, Jr. Quantitative single cell analysis and sorting. Science **1977**;198: 149-57.
526. Fulton RJ, McDade RL, Smith PL, Kienker LJ, Kettman JR, Jr. Advanced multiplexed analysis with the FlowMetrix system. Clin Chem **1997**;43: 1749-56.

527. Khan SS, Smith MS, Reda D, Suffredini AF, McCoy JP, Jr. Multiplex bead array assays for detection of soluble cytokines: comparisons of sensitivity and quantitative values among kits from multiple manufacturers. Cytometry B Clin Cytom **2004**;61: 35-9.
528. Martins TB, Pasi BM, Pickering JW, Jaskowski TD, Litwin CM, Hill HR. Determination of cytokine responses using a multiplexed fluorescent microsphere immunoassay. Am J Clin Pathol **2002**;118: 346-53.
529. Alonso A, Marsal S, Julia A. Analytical methods in untargeted metabolomics: state of the art in 2015. Front Bioeng Biotechnol **2015**;3: 23.
530. Evans AM; Bridgewater BR; Liu Q; Mitchell MW; Robinson RJ ea. High Resolution Mass Spectrometry Improves Data Quantity and Quality as Compared to Unit Mass Resolution Mass Spectrometry in High-Throughput Profiling Metabolomics. . Metabolomics **2014**;4.
531. Baker M. Metabolomics: from small molecules to big ideas. Nature Methods **2011**;8: 117-21.
532. Cui L, Lu H, Lee YH. Challenges and emergent solutions for LC-MS/MS based untargeted metabolomics in diseases. Mass Spectrom Rev **2018**;37: 772-92.
533. Wishart DS, Tzur D, Knox C, Eisner R, Guo AC, Young N, Cheng D, Jewell K, Arndt D, Sawhney S, Fung C, Nikolai L, Lewis M, Coutouly MA, Forsythe I, Tang P, Shrivastava S, Jeroncic K, Stothard P, Amegbey G, Block D, Hau DD, Wagner J, Miniaci J, Clements M, Gebremedhin M, Guo N, Zhang Y, Duggan GE, Macinnis GD, Weljie AM, Dowlatabadi R, Bamforth F, Clive D, Greiner R, Li L, Marrie T, Sykes BD, Vogel HJ, Querengesser L. HMDB: the Human Metabolome Database. Nucleic Acids Res **2007**;35: D521-6.
534. Horai H, Arita M, Kanaya S, Nihei Y, Ikeda T, Suwa K, Ojima Y, Tanaka K, Tanaka S, Aoshima K, Oda Y, Kakazu Y, Kusano M, Tohge T, Matsuda F, Sawada Y, Hirai MY, Nakanishi H, Ikeda K, Akimoto N, Maoka T, Takahashi H, Ara T, Sakurai N, Suzuki H, Shibata D, Neumann S, Iida T, Tanaka K, Funatsu K, Matsuura F, Soga T, Taguchi R, Saito K, Nishioka T. MassBank: a public repository for sharing mass spectral data for life sciences. J Mass Spectrom **2010**;45: 703-14.
535. Tautenhahn R, Cho K, Uritboonthai W, Zhu Z, Patti GJ, Siuzdak G. An accelerated workflow for untargeted metabolomics using the METLIN database. Nat Biotechnol **2012**;30: 826-8.
536. Fahy E, Sud M, Cotter D, Subramaniam S. LIPID MAPS online tools for lipid research. Nucleic Acids Res **2007**;35: W606-12.
537. Phinney KW, Ballihaut G, Bedner M, Benford BS, Camara JE, Christopher SJ, Davis WC, Dodder NG, Eppe G, Lang BE, Long SE, Lowenthal MS, McGaw EA, Murphy KE, Nelson BC, Prendergast JL, Reiner JL, Rimmer CA, Sander LC, Schantz MM, Sharpless KE, Sniegoski LT, Tai SS, Thomas JB, Vetter TW, Welch MJ, Wise SA, Wood LJ, Guthrie WF, Hagwood CR, Leigh SD, Yen JH, Zhang NF, Chaudhary-Webb M, Chen H, Fazili Z, LaVoie DJ, McCoy LF, Momin SS, Paladugula N, Pendergrast EC, Pfeiffer CM, Powers CD, Rabinowitz D, Rybak ME, Schleicher RL, Toombs BM, Xu M, Zhang M, Castle AL. Development of a Standard Reference Material for metabolomics research. Anal Chem **2013**;85: 11732-8.
538. Kind T, Fiehn O. Seven Golden Rules for heuristic filtering of molecular formulas obtained by accurate mass spectrometry. BMC Bioinformatics **2007**;8: 105.
539. van der Greef J, Hankemeier T, McBurney RN. Metabolomics-based systems biology and personalized medicine: moving towards n = 1 clinical trials? Pharmacogenomics **2006**;7: 1087-94.
540. Jain AK, Murty MN, Flynn PJ. Data clustering: A review. . ACM Comput. Surv. **1999**;31: 264-323.
541. Ronan T, Qi Z, Naegle KM. Avoiding common pitfalls when clustering biological data. Sci Signal **2016**;9: re6.
542. Oyelade J, Isewon I, Oladipupo F, Aromolaran O, Uwoghiren E, Ameh F, Achas M, Adebiyi E. Clustering Algorithms: Their Application to Gene Expression Data. Bioinform Biol Insights **2016**;10: 237-53.
543. Stavrum AK, Petersen K, Jonassen I, Dysvik B. Analysis of gene-expression data using J-Express. Curr Protoc Bioinformatics **2008**;Chapter 7: Unit 7 3.
544. Rothman KJ. No adjustments are needed for multiple comparisons. Epidemiology **1990**;1: 43-6.
545. Straka C, Oduncu F, Hinke A, Einsele H, Drexler E, Schnabel B, Arseniev L, Walther J, Konig A, Emmerich B. Responsiveness to G-CSF before leukopenia predicts defense to infection in high-dose chemotherapy recipients. Blood **2004**;104: 1989-94.
546. Straka C, Sandherr M, Salwender H, Wandt H, Metzner B, Hubel K, Silling G, Hentrich M, Franke D, Schwerdtfeger R, Freund M, Sezer O, Giagounidis A, Ehninger G, Grimminger W, Engert A, Schlimok G, Scheid C, Hellmann P, Heinisch H, Einsele H, Hinke A, Emmerich B. Testing G-CSF responsiveness predicts the individual susceptibility to infection and consecutive treatment in recipients of high-dose chemotherapy. Blood **2011**;117: 2121-8.

547. Chatta GS, Andrews RG, Rodger E, Schrag M, Hammond WP, Dale DC. Hematopoietic progenitors and aging: alterations in granulocytic precursors and responsiveness to recombinant human G-CSF, GM-CSF, and IL-3. J Gerontol **1993**;48: M207-12.
548. Liongue C, Ward AC. Granulocyte colony-stimulating factor receptor mutations in myeloid malignancy. Front Oncol **2014**;4: 93.
549. McLemore ML, Poursine-Laurent J, Link DC. Increased granulocyte colony-stimulating factor responsiveness but normal resting granulopoiesis in mice carrying a targeted granulocyte colony-stimulating factor receptor mutation derived from a patient with severe congenital neutropenia. J Clin Invest **1998**;102: 483-92.
550. Hermans MH, Antonissen C, Ward AC, Mayen AE, Ploemacher RE, Touw IP. Sustained receptor activation and hyperproliferation in response to granulocyte colony-stimulating factor (G-CSF) in mice with a severe congenital neutropenia/acute myeloid leukemia-derived mutation in the G-CSF receptor gene. J Exp Med **1999**;189: 683-92.
551. Bogunia-Kubik K, Gieryng A, Gebura K, Lange A. Genetic variant of the G-CSF receptor gene is associated with lower mobilization potential and slower recovery of granulocytes after transplantation of autologous peripheral blood progenitor cells. Cytokine **2012**;60: 463-7.
552. Camurdanoglu BZ, Esendagli G, Ozdemir E, Canpinar H, Guc D, Kansu E. The effect of granulocyte colony stimulating factor receptor gene missense single nucleotide polymorphisms on peripheral blood stem cell enrichment. Cytokine **2013**;61: 572-7.
553. Bunse CE, Tischer S, Lahrberg J, Oelke M, Figueiredo C, Blasczyk R, Eiz-Vesper B. Granulocyte colony-stimulating factor impairs CD8(+) T cell functionality by interfering with central activation elements. Clin Exp Immunol **2016**;185: 107-18.
554. Boyle K, Egan P, Rakar S, Willson TA, Wicks IP, Metcalf D, Hilton DJ, Nicola NA, Alexander WS, Roberts AW, Robb L. The SOCS box of suppressor of cytokine signaling-3 contributes to the control of G-CSF responsiveness in vivo. Blood **2007**;110: 1466-74.
555. Croker BA, Metcalf D, Robb L, Wei W, Mifsud S, DiRago L, Cluse LA, Sutherland KD, Hartley L, Williams E, Zhang JG, Hilton DJ, Nicola NA, Alexander WS, Roberts AW. SOCS3 is a critical physiological negative regulator of G-CSF signaling and emergency granulopoiesis. Immunity **2004**;20: 153-65.
556. Benboubker L, Watier H, Carion A, Georget MT, Desbois I, Colombat P, Bardos P, Binet C, Domenech J. Association between the SDF1-3'A allele and high levels of CD34(+) progenitor cells mobilized into peripheral blood in humans. Br J Haematol **2001**;113: 247-50.
557. Nguyen-Jackson H, Panopoulos AD, Zhang H, Li HS, Watowich SS. STAT3 controls the neutrophil migratory response to CXCR2 ligands by direct activation of G-CSF-induced CXCR2 expression and via modulation of CXCR2 signal transduction. Blood **2010**;115: 3354-63.
558. Thunström Salzer A, Niemiec M, Hosseinzadeh A, Stylianou M, Åström F, Röhm M, Ahlm C, Wahlin A, Ermert D, Urban C. Assessment of Neutrophil Chemotaxis Upon G-CSF Treatment of Healthy Stem Cell Donors and in Allogeneic Transplant Recipients, 2018.
559. Weihua C, Fei W, Meng L, Haiyan Z, Wanming D, Chunji G. The effect of rhG-CSF on the conformation of LFA-1 on CD4+ T cells in hemopoietic stem cell transplantation. Immunopharmacol Immunotoxicol **2009**;31: 267-73.
560. Zhao S, Gu Z, Wang L, Guan L, Wang F, Yang N, Luo L, Gao Z, Song Y, Wang L, Liu D, Gao C. G-CSF inhibits LFA-1-mediated CD4(+) T cell functions by inhibiting Lck and ZAP-70. Oncotarget **2017**;8: 51578-90.
561. Nacher M, Blazquez AB, Shao B, Matesanz A, Prophete C, Berin MC, Frenette PS, Hidalgo A. Physiological contribution of CD44 as a ligand for E-Selectin during inflammatory T-cell recruitment. Am J Pathol **2011**;178: 2437-46.
562. Bonder CS, Clark SR, Norman MU, Johnson P, Kubes P. Use of CD44 by CD4+ Th1 and Th2 lymphocytes to roll and adhere. Blood **2006**;107: 4798-806.
563. Ali AJ, Abuelela AF, Merzaban JS. An Analysis of Trafficking Receptors Shows that CD44 and P-Selectin Glycoprotein Ligand-1 Collectively Control the Migration of Activated Human T-Cells. Front Immunol **2017**;8: 492.
564. Nandi A, Estess P, Siegelman M. Bimolecular complex between rolling and firm adhesion receptors required for cell arrest; CD44 association with VLA-4 in T cell extravasation. Immunity **2004**;20: 455-65.
565. Baaten BJ, Li CR, Deiro MF, Lin MM, Linton PJ, Bradley LM. CD44 regulates survival and memory development in Th1 cells. Immunity **2010**;32: 104-15.
566. Murakami S, Miyake K, Kincade PW, Hodes RJ. Functional role of CD44 (Pgp-1) on activated B cells. Immunol Res **1991**;10: 15-27.

567. Murakami S, Miyake K, Abe R, Kincade PW, Hodes RJ. Characterization of autoantibody-secreting B cells in mice undergoing stimulatory (chronic) graft-versus-host reactions. Identification of a CD44hi population that binds specifically to hyaluronate. J Immunol **1991**;146: 1422-7.
568. Kurz J, Barthelmes J, Blum L, Ulshofer T, Wegner MS, Ferreiros N, Roser L, Geisslinger G, Grosch S, Schiffmann S. Role of ceramide synthase 2 in G-CSF signaling and G-CSF-R translocation into detergent-resistant membranes. Sci Rep **2019**;9: 747.
569. Drezet A, Granata A, Lemarie C, Calmels B, Chabannon C. An intra-patient comparison of blood cell separators Spectra and Optia in patients and donors undergoing blood mononuclear cell collections at a single institution for subsequent autologous or allogeneic hematopoietic cell transplantation reveals comparable collection efficiencies. Bone Marrow Transplant **2016**;51: 1007-9.
570. Reinhardt P, Brauninger S, Bialleck H, Thorausch K, Smith R, Schrezenmeier H, Seifried E, Wiesneth M, Bonig H. Automatic interface-controlled apheresis collection of stem/progenitor cells: results from an autologous donor validation trial of a novel stem cell apheresis device. Transfusion **2011**;51: 1321-30.
571. Katipamula R, Porrata LF, Gastineau DA, Markovic SN, Moore SB, Greiner C, Burgstaler EA, Padley DJ, Winters JL. Apheresis instrument settings influence infused absolute lymphocyte count affecting survival following autologous peripheral hematopoietic stem cell transplantation in non-Hodgkin's lymphoma: the need to optimize instrument setting and define a lymphocyte collection target. Bone Marrow Transplant **2006**;37: 811-7.
572. Burgstaler EA, Porrata LF, Markovic SN, Winters JL. Use of various offset settings in the Fenwal Amicus during hematopoietic progenitor cell collection to increase lymphocyte yield and reduce cross-cellular contamination. J Clin Apher **2010**;25: 301-9.
573. Sorsa T, Tervahartiala T, Leppilahti J, Hernandez M, Gamonal J, Tuomainen AM, Lauhio A, Pussinen PJ, Mantyla P. Collagenase-2 (MMP-8) as a point-of-care biomarker in periodontitis and cardiovascular diseases. Therapeutic response to non-antimicrobial properties of tetracyclines. Pharmacol Res **2011**;63: 108-13.
574. Laitinen A, Hagstrom J, Mustonen H, Kokkola A, Tervahartiala T, Sorsa T, Bockelman C, Haglund C. Serum MMP-8 and TIMP-1 as prognostic biomarkers in gastric cancer. Tumour Biol **2018**;40: 1010428318799266.
575. Tvedt THA, Melve GK, Tsykunova G, Ahmed AB, Brenner AK, Bruserud O. Immunological Heterogeneity of Healthy Peripheral Blood Stem Cell Donors-Effects of Granulocyte Colony-Stimulating Factor on Inflammatory Responses. Int J Mol Sci **2018**;19.
576. Carstanjen D, Regenfus M, Muller C, Salama A. Interleukin-6 is a major effector molecule of short-term G-CSF treatment inducing bone metabolism and an acute-phase response. Exp Hematol **2001**;29: 812-21.
577. Ilhan C, Suyani E, Sucak GT, Pasaoglu OT, Aki SZ, Pasaoglu H. Inflammatory markers, oxidative stress, and antioxidant capacity in healthy allo-HSCT donors during hematopoietic stem cell mobilization. J Clin Apher **2015**;30: 197-203.
578. Bruserud O, Melve GK, Gedde-Dahl T, Tvedt THA. Immunological heterogeneity of healthy peripheral blood stem cell donors - preharvesting donor characteristics, additional heterogeneity induced by granulocyte colony-stimulating factor and possible importance for outcome after allotransplantation. Expert Rev Hematol **2018**: 1-3.
579. Martinez-Laperche C, Buces E, Aguilera-Morillo MC, Picornell A, Gonzalez-Rivera M, Lillo R, Santos N, Martin-Antonio B, Guillem V, Nieto JB, Gonzalez M, de la Camara R, Brunet S, Jimenez-Velasco A, Espigado I, Vallejo C, Sampol A, Bellon JM, Serrano D, Kwon M, Gayoso J, Balsalobre P, Urbano-Izpizua A, Solano C, Gallardo D, Diez-Martin JL, Romo J, Buno I, Transplantation GVICotSGfH. A novel predictive approach for GVHD after allogeneic SCT based on clinical variables and cytokine gene polymorphisms. Blood Adv **2018**;2: 1719-37.
580. Tang H, Hahn T, Karaesmen E, Rizvi AA, Wang J, Paczesny S, Wang T, Preus L, Zhu Q, Wang Y, Haiman CA, Stram D, Pooler L, Sheng X, Van Den Berg D, Brock G, Webb A, Pasquini MC, McCarthy PL, Spellman SR, Sucheston-Campbell LE. Validation of genetic associations with acute GVHD and nonrelapse mortality in DISCOVeRY-BMT. Blood Adv **2019**;3: 2337-41.
581. McCurdy SR, Zhang MJ, St Martin A, Al Malki MM, Bashey A, Gaballa S, Keesler DA, Hamadani M, Norkin M, Perales MA, Reshef R, Rocha V, Romee R, Solh M, Urbano-Ispizua A, Waller EK, Fuchs EJ, Eapen M. Effect of donor characteristics on haploidentical transplantation with posttransplantation cyclophosphamide. Blood Adv **2018**;2: 299-307.
582. Rotta M, Storer BE, Storb RF, Martin PJ, Heimfeld S, Peffer A, Maloney DG, Deeg HJ, Sandmaier BM, Appelbaum FR, Mielcarek M. Donor statin treatment protects against severe acute graft-versus-host disease after related allogeneic hematopoietic cell transplantation. Blood **2010**;115: 1288-95.

583. Szmigielska-Kaplon A, Szemraj J, Hamara K, Robak M, Wolska A, Pluta A, Czemerska M, Krawczynska A, Jamroziak K, Szmigielska K, Robak T, Wierzbowska A. Polymorphism of CD44 influences the efficacy of CD34(+) cells mobilization in patients with hematological malignancies. Biol Blood Marrow Transplant **2014**;20: 986-91.
584. Huet S, Groux H, Caillou B, Valentin H, Prieur AM, Bernard A. CD44 contributes to T cell activation. J Immunol **1989**;143: 798-801.
585. DeGrendele HC, Estess P, Siegelman MH. Requirement for CD44 in activated T cell extravasation into an inflammatory site. Science **1997**;278: 672-5.
586. Schumann J, Stanko K, Schliesser U, Appelt C, Sawitzki B. Differences in CD44 Surface Expression Levels and Function Discriminates IL-17 and IFN-gamma Producing Helper T Cells. PLoS One **2015**;10: e0132479.
587. Milinkovic M, Antin JH, Hergrueter CA, Underhill CB, Sackstein R. CD44-hyaluronic acid interactions mediate shear-resistant binding of lymphocytes to dermal endothelium in acute cutaneous GVHD. Blood **2004**;103: 740-2.
588. Zhang Y, Joe G, Hexner E, Zhu J, Emerson SG. Alloreactive memory T cells are responsible for the persistence of graft-versus-host disease. J Immunol **2005**;174: 3051-8.
589. McDonald B, Kubes P. Interactions between CD44 and Hyaluronan in Leukocyte Trafficking. Front Immunol **2015**;6: 68.
590. Singer K, DelProposto J, Morris DL, Zamarron B, Mergian T, Maley N, Cho KW, Geletka L, Subbaiah P, Muir L, Martinez-Santibanez G, Lumeng CN. Diet-induced obesity promotes myelopoiesis in hematopoietic stem cells. Mol Metab **2014**;3: 664-75.

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Peripheral Blood Stem Cell Mobilization in Healthy Donors by Granulocyte Colony-Stimulating Factor Causes Preferential Mobilization of Lymphocyte Subsets

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Background: Allogeneic hematopoietic stem cell transplantation is associated with a high risk of immune-mediated post-transplant complications. Graft depletion of immunocompetent cell subsets is regarded as a possible strategy to reduce this risk without reducing antileukemic immune reactivity.

Study design and methods: We investigated the effect of hematopoietic stem cell mobilization with granulocyte colony-stimulating factor (G-CSF) on peripheral blood and stem cell graft levels of various T, B, and NK cell subsets in healthy donors. The results from flow cytometric cell quantification were examined by bioinformatics analyses.

Results: The G-CSF-induced mobilization of lymphocytes was a non-random process with preferential mobilization of naïve $CD4^+$ and $CD8^+$ T cells together with T cell receptor $\alpha\beta^+$ T cells, naïve T regulatory cells, type 1 T regulatory cells, mature and memory B cells, and cytokine-producing NK cells. Analysis of circulating lymphoid cell capacity to release various cytokines (IFNγ, IL10, TGFβ, IL4, IL9, IL17, and IL22) showed preferential mobilization of IL10 releasing $CD4^+$ T cells and $CD3^-19^-$ cells. During G-CSF treatment, the healthy donors formed two subsets with generally strong and weaker mobilization of immunocompetent cells, respectively; hence the donors differed in their G-CSF responsiveness with regard to mobilization of immunocompetent cells. The different responsiveness was not reflected in the graft levels of various immunocompetent cell subsets. Furthermore, differences in donor G-CSF responsiveness were associated with time until platelet engraftment. Finally, strong G-CSF-induced mobilization of various T cell subsets seemed to increase the risk of recipient acute graft versus host disease, and this was independent of the graft T cell levels.

Conclusion: Healthy donors differ in their G-CSF responsiveness and preferential mobilization of immunocompetent cells. This difference seems to influence post-transplant recipient outcomes.

Keywords: apheresis, graft versus host disease, granulocyte colony-stimulating factor, hematopoietic stem cell mobilization, hematopoietic stem cell transplantation, immune reconstitution, living donors, peripheral blood stem cells

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INTRODUCTION

Allogeneic hematopoietic stem cell transplantation is increasingly used in the treatment of several diseases, especially hematological malignancies and disorders characterized by severe bone marrow failure (1–4). The treatment is associated with a risk of early death mainly due to treatment toxicity, severe early immunological complications [i.e., acute graft versus host disease (aGVHD)], and a risk of long-term morbidity mainly caused by chronic GVHD (5). Various strategies of graft manipulation have been tried to reduce the frequencies of these immunological complications, including CD34 enrichment by positive or negative selection, general T cell depletion, depletion of T cell subsets, or combined B/T cell depletion (5). The early studies showed that general T cell depletion was associated with a reduced risk of severe GVHD but an increased risk of leukemia relapse and graft failure (5), whereas more recent studies based on depletion of immunocompetent cell subsets are more promising (6–10). However, the effects of depleting subsets of immunocompetent cells from the graft will probably be influenced by the frequencies of various remaining subsets of immunocompetent cells.

Treatment with granulocyte colony-stimulating factor (G-CSF) is commonly used for mobilization of peripheral blood stem cells in healthy donors (11, 12). G-CSF has several immunomodulatory effects, and for a detailed discussion and additional references we refer to a recent review (13). First, among the important effects on T cells are G-CSF-induced preferential mobilization of naïve T cells, decreased expression of T cell activation markers as well as adhesion molecules and chemokine receptors, and finally Th2 polarization with reduced production of Th1 cytokines. The levels of regulatory T cells are increased. Second, effects on NK cells and NK cell subsets are less well characterized, but there seems to be a decreased release of pro-inflammatory cytokines. Third, the differentiation status of monocytes is altered with reduced production of pro-inflammatory cytokines and increased release of immunosuppressive IL-10. These effects seem to favor an immunosuppressive effect of G-CSF administration to healthy stem cell donors, but it should be emphasized that the question of donor heterogeneity has not been investigated in detail previously.

The aim of this study was to characterize more in detail the effects of G-CSF on the mobilization of various subsets of immunocompetent cells and to have a focus on donor heterogeneity and differences in donor response to G-CSF. Hereafter, we will use the term "G-CSF responsiveness" to express the heterogeneous changes in donor peripheral blood levels of various lymphoid cell subsets during G-CSF treatment. We have characterized in detail the peripheral blood levels of various T, B, and NK cell subsets after G-CSF stem cell mobilization for an unselected group of healthy stem cell donors. Our results showed that G-CSF treatment of healthy donors caused a preferential mobilization of immunocompetent cell subsets, donors could be classified as either strong or weak mobilizers of immunocompetent cells, and this difference in G-CSF responsiveness seemed to affect the post-transplant recipient outcomes.

MATERIALS AND METHODS

Stem Cell Donors and Allotransplant Recipients

The following participants were included: (i) 22 consecutive healthy HLA-matched related allogeneic stem cell donors, 14 males and 8 females, median age 52.5 years (25–73) and (ii) 13 male and 7 female allogeneic stem cell recipients with hematological diseases, median age 47 years (35–69). 11 patients were diagnosed with acute myeloid leukemia (AML), 4 with aplastic anemia, 2 with chronic myeloid leukemia, 2 with myelofibrosis, and 1 with chronic lymphatic leukemia. A more detailed characterization of the allotransplant recipients is given in Table S1 in Supplementary Material. The patients represent all allotransplanted patients from a defined area in Norway (the Western, Middle, and Northern Regions) during a defined time period and receiving stem cell grafts from matched family donors; i.e., this study should be regarded as a population-based study.

Stem Cell Mobilization and Apheresis

The donors received stem cell mobilization with the human non-glycosylated G-CSF analog Filgrastim (Neupogen; Amgen, Thousand Oaks, CA, USA) or Tevagrastim (biosimilar Filgrastim; Petah Tiqwa, Israel). The donors received a median dose of 5.4 μg/kg (range 4.1–6.7 μg/kg) twice daily. Stem cell harvest was performed when the peripheral blood stem cell count exceeded $15–20 \times 10^3$/mL after 4 or 5 days with either large volume apheresis using Cobe Spectra cell separator version 7 (Terumo BCT Inc., Lakewood, CO, USA; 8 donors) or automated large volume MNC procedure using Spectra Optia cell separator version 9 (Terumo BCT Inc., Lakewood, CO, USA; 14 donors).

Allogeneic Stem Cell Transplantation

At the time of transplantation 11 patients were in their first complete hematological remission, 2 patients were in their second complete remission and 7 patients had detectable disease (Table S1 in Supplementary Material). 10 patients received myeloablative conditioning with intravenous busulfan plus cyclophosphamide (i) and 10 patients received reduced intensity conditioning with intravenous fludarabine plus busulfan (ii). After transplantation, all patients received GVHD prophylaxis with cyclosporine A plus methotrexate.

Sample Collection and Preparation

Blood and Allograft Sampling

Venous blood samples from the allogeneic stem cell donors were collected (I) prior to G-CSF treatment at the time of the pre-transplant evaluation (median 20.5 days before apheresis). Blood samples were also drawn (II) in the morning immediately before apheresis, (III) immediately after apheresis, and (IV) approximately 24 h after start of apheresis. Samples for cell preparation were collected into ACD-A tubes with sodium citrate and acid-citrate-dextrose solution A as anticoagulants (Greiner Bio-One GmbH, Kremsmünster, Austria). Samples

from stem cell allografts were transferred to plastic tubes without additives.

Cryopreservation of PBMC Samples

After isolation by density-gradient centrifugation (Lymphoprep, AXIS-SHIELD PoC AS, Oslo, Norway; specific density: 1.077 g/mL), PBMCs were dissolved in RPMI 1640 medium supplemented with 2 mmol/L L-glutamine, penicillin 100 IE/mL, streptomycin 0.1 mg/mL (Sigma-Aldrich, St. Louis, MO, USA), and 20% inactivated fetal bovine serum (Biowest, Nuaillé, France). Dimethyl sulfoxide 10% (Sigma-Aldrich, St. Louis, MO, USA) was used as a cryoprotectant, and the vials were stored in liquid nitrogen at −150°C after gradual cooling to −80°C in a Mr. Frosty Freezing Container (Thermo Fisher Scientific, Waltham, MA, USA).

Preparation and Flow Cytometry Analyses of Peripheral Blood Mononuclear Cells

(a) All PBMC samples were thawed in a 37°C water bath, dissolved in supplemented RPMI 1640 medium and incubated for 1 h (37°C, a humidified atmosphere of 5% CO_2) before incubation with Near-IR fluorescent reactive dye (LIVE/DEAD Fixable Dead Cell Stain Kit, Molecular Probes, Eugene, OR, USA) for 30 min. After washing in phosphate-buffered saline with 1% bovine serum albumin fraction V (Roche Diagnostics GmbH, Mannheim, Germany), the cells were incubated for 20 min with the following mouse anti-human monoclonal antibodies: CD3-PE-Cy7 (SK7), CD3-V450 (UCHT1), CD4-PerCP-Cy5.5 (RPA-T4), CD8-V500 (RPA-T8), CD16-Ax647 (3G8), CD19-PerCP-Cy5.5 (SJ25C1), CD24-PE-Cy7 (ML5), CD25-PE (M-A251), CD26-FITC (M-A261), CD27-FITC (M-T271), CD45-RA-V450 (HI100), CD56-PE (B159), CD45-RO-PE (UCHL), CD197/CCR7-FITC (150503), CD197/CCR7-Ax647 (150503), T cell receptor (TCR)αβ-BV510 (T10B91.A), TCRγδ-PE-Cy7 (11F2), iNKT(Vα24)-FITC (6b11) (all from Becton Dickinson Biosciences; BD Pharmingen, San Diego, CA, USA), CD49b-FITC (AK7; BioLegend, San Diego, CA, USA), LAG-3-PE (FAB2319P; R&D systems, Minneapolis, MN, USA), and mouse anti-human CD38-PB (HIT2; EXBIO, Prague, the Czech republic).

(b) Samples for quantification of Treg cells were thawed and surface stained as described earlier before fixation and permeabilization using eBioscience Staining Buffer Set (00-5523) as recommended by the manufacturer (eBioscience, San Diego, CA, USA). Intracellular staining was performed by incubating the cells for 30 min with mouse anti-human FoxP3-Ax647 (236A/E7; Becton Dickinson Biosciences).

(c) Samples for intracellular cytokine analyses were thawed as described in (a). The cell concentration was adjusted to 10^6 cells/mL before stimulation for 5 h with leukocyte activation cocktail with BD GolgiPlug 2 µL/mL (PMA, Ionomycin and Brefeldin A) from Becton Dickinson Biosciences at 37°C in a humified atmosphere of 5% CO_2. The cells were surface stained as described in (a) before fixation and permeabilization as described in (b) and finally incubated for 30 min with the following mouse anti-human monoclonal antibodies: IL4-Ax488 (8D4-8), IL9-Ax647 (MH9A3), IL10-APC (JES3-19F), IL17-A Ax488 (N49-653), IFNγ-V450 (B27), TGFβ (LAP)-PE (TW4-2F8) (all from Becton Dickinson Biosciences), and mouse anti-human monoclonal IL22-PE (142928) from R&D Systems (Abingdon, UK).

Flow cytometry analysis was performed using a FACS Canto II flow cytometer (Immunocytometry Systems; Becton Dickinson Biosciences, San Jose, CA, USA). Acquisition of 30,000 $CD3^+$ T cells or 10,000 $CD19^+$ B cells per sample was endeavored, and cytometer performance was monitored daily with Cytometer Setup and Tracking Beads (Becton Dickinson Biosciences). The data were analyzed with FlowJo software version 10.2 (FlowJo LLC, Ashland, OR, USA). The detailed gating strategy is shown in Figure S1 in Supplementary Material, and the main lymphoid cell subsets identified are presented in Table S2 in Supplementary Material together with detailed description of monoclonal antibodies. The identification of various cell subsets are also shown in **Tables 1** and **2**.

White blood differential counts were performed at Laboratory of Clinical Biochemistry, Haukeland University Hospital, Bergen, Norway by multi-angle polarized scatter separation optical flow cytometry using the Cell-Dyn Sapphire analyzer (Abbot Diagnostics, Santa Clara, CA, USA).

Statistical and Bioinformatics Analyses

Descriptive statistics are given as median and range for non-normally distributed variables. The Wilcoxon's test for paired samples was used for analyses of paired observations, and the independent-samples Mann–Whitney *U* test and the Chi Square test for comparison of unpaired groups. Correlations between

TABLE 1 | Effect of granulocyte colony-stimulating factor (G-CSF) on peripheral blood and graft concentrations of various leukocyte subsets ($n = 22$) presented as median levels ($\times 10^9$/L) with variation ranges in parentheses.

Leukocyte subset	Prior to G-CSF	During G-CSF	*p*	Stem cell graft	*R/p*
Neutrophils	3.4 (2.4–11.0)	36.8 (21.0–65.5)	<0.00005	100.6 (29.6–234.0)	0.182/0.193
Monocytes	0.5 (0.2–0.7)	1.9 (0.9–3.9)	<0.00005	35.1 (5.5–75.6)	0.062/0.659
Lymphocytes	1.7 (0.9–2.8)	3.9 (2.4–6.5)	<0.00005	78.1 (42.2–182.6)	0.195/0.170
T cells	1.25 (0.60–2.26)	2.92 (1.29–4.17)	<0.00005	53.92 (23.72–145.71)	0.316/0.052
B cells	0.15 (0.03–0.33)	0.50 (0.21–1.77)	<0.00005	13.50 (3.12–26.46)	0.357/0.033*
NK cells	0.22 (0.05–0.50)	0.25 (0.07–0.68)	NS	4.46 (1.74–14.47)	0.421/0.009**

The Wilcoxon's test for paired samples was used for comparison of pre-treatment and G-CSF-treated concentrations (fourth column). The correlations between pre-apheresis and graft concentrations were analyzed with Kendall's tau-b test and the correlation coefficients (R) and corresponding p-values are presented in the rightmost column.
**p < 0.05 and **p < 0.01.*

TABLE 2 | Effect of granulocyte colony-stimulating factor (G-CSF) on peripheral blood and graft concentrations of T cell subsets ($n = 22$) presented as median levels ($\times 10^9$/L) with variation ranges in parentheses.

T cell subsets	Prior to G-CSF	During G-CSF	*p*	Stem cell graft	*R/p*
T helper cells (T_H) ($CD4^+$)	0.83 (0.39–1.37)	2.11 (0.92–3.47)	0.00004	41.10 (17.85–107.76)	0.337/0.038*
Cytotoxic T cells (T_C) ($CD8^+$)	0.29 (0.09–0.79)	0.58 (0.14–1.08)	0.0003	10.85 (3.37–33.15)	0.274/0.092
Naïve T_H ($CD4^+45RA^+CCR7^+$)	0.45 (0.13–0.95)	1.21 (0.34–2.05)	0.00004	21.82 (7.30–60.24)	0.474/0.004**
Central memory cells (T_{CM}) ($CD4^+45RA^-CCR7^+$)	0.20 (0.09–0.39)	0.37 (0.13–0.87)	0.00007	7.38 (2.79–24.35)	0.442/0.006**
Effector memory cells (T_{EM}) ($CD4^+45RA^-CCR7^-$)	0.14 (0.06–0.28)	0.29 (0.08–0.72)	0.00004	5.44 (4.01–13.36)	0.326/0.044*
Terminally differentiated (T_{TD}) ($CD4^+45RA^+CCR7^-$)	0.05 (0.02–0.18)	0.11 (0.05–0.38)	0.00008	3.09 (1.12–8.05)	0.463/0.004**
($CD4^+45RO^+CD26^{++}$)	0.02 (0.01–0.07)	0.05 (0.02–0.20)	0.00004	0.82 (0.31–3.25)	0.474/0.004**
Naïve T_C ($CD8^+45RA^+CCR7^+$)	0.13 (0.04–0.36)	0.24 (0.06–0.66)	0.0002	5.77 (1.63–12.46)	0.316/0.052
Central memory ($CD8^+45RA^-CCR7^+$)	0.023 (0.003–0.080)	0.030 (0.007–0.137)	0.004	0.67 (0.08–3.03)	0.567/0.001**
Effector memory ($CD8^+45RA^-CCR7^-$)	0.03 (0.01–0.10)	0.06 (0.01–0.17)	0.0002	1.05 (0.52–3.85)	0.442/0.006**
Effector (TEMRA) ($CD8^+45RA^+CCR7^-$)	0.08 (0.02–0.41)	0.12 (0.02–0.36)	0.036	2.93 (0.88–14.35)	0.537/0.001**
($CD8^+45RO^+CD26^{++}$)	0.011 (0.001–0.088)	0.016 (0.002–0.101)	NS	0.24 (0.02–1.83)	0.637/0.0001***
$\alpha\beta$ T cells ($CD3^+$T cell receptor (TCR)$\alpha\beta^+$)	1.18 (0.58–2.14)	2.76 (1.21–4.04)	0.00005	52.60 (20.63–140.47)	0.316/0.052
$\gamma\delta$ T cells ($CD3^+4^-8^-TCR\gamma\delta^+$)	0.048 (0.004–0.118)	0.046 (0.009–0.178)	0.017	1.15 (0.30–4.20)	0.484/0.003**
Naïve T regulatory cells ($CD4^+25^+45RA^+FOXP3^+$)	0.010 (0.003–0.042)	0.019 (0.007–0.124)	0.00008	0.457 (0.165–1.817)	0.453/0.005**
Effector T regulatory cells ($CD4^+25^+45RA^-FOXP3^+$)	0.030 (0.016–0.068)	0.071 (0.027–0.178)	0.00004	1.268 (0.541–4.207)	0.453/0.005**
Type 1 regulatory (Tr1) ($CD4^+45RA^-49b^+LAG3^+$)	0.006 (0.002–0.018)	0.011 (0.004–0.064)	0.003	0.217 (<0.001–0.920)	0.211/0.194

The Wilcoxon's test for paired samples was used for comparison of pre-treatment and G-CSF-treated concentrations (fourth column). The correlations between pre-apheresis and graft concentrations were analyzed with Kendall's tau-b test and the correlation coefficients (R) and corresponding p-values are presented in the rightmost column.
**p < 0.05, **p < 0.01, and ***p < 0.001.*

continuous variables are given as the Kendall's tau-b coefficient with corresponding *p*-value. J-Express (MolMine AS, Bergen, Norway) was applied for bioinformatics analyses (14). Time to reconstitution was analyzed with the Kaplan–Meier survival method with the log-rank test, Cox regression with backward selection, and competing risks analysis. All statistical analyses were performed in the standard computer software package IBM SPSS Statistics 22 (IBM Corporate, New York, NY, USA) except for the competing risks analyses that were done using Stata (StataCorp, Lakeway Drive College Station, Texas, USA).

RESULTS

G-CSF Treatment of Healthy Stem Cell Donors Increased Peripheral Blood Levels Especially of Neutrophils but Also Monocytes and Total Lymphocytes

Granulocyte colony-stimulating factor treatment induced a five- to tenfold increase in the total peripheral blood leukocyte counts from a median level of 6.0×10^9/L (range $4.4–13.4 \times 10^9$/L) to 44.9×10^9/L (range $26.0–71.2 \times 10^9$/L). The absolute levels of virtually all leukocyte subpopulations increased (see below, **Table 1**; **Figure 1**). The increase in the proportion of neutrophils corresponded to a median fold change of 8.6, whereas the median fold change for monocytes was 5.0 and for total lymphocytes 2.1 (**Figure 2**; Table S3 in Supplementary Material).

G-CSF Treatment Resulted in an Increased B Cell Fraction and Decreased NK Cell Fraction Whereas the T Cell Fraction Was Not Altered

As can be seen from **Table 1** and **Figures 1** and **2**, there was a twofold rise in median peripheral blood T cell concentration and a threefold increase in median B cell concentration during G-CSF administration. However, the median NK cell concentration was not significantly affected by G-CSF. Consequently, there was a significant decrease in the NK cell percentage among lymphoid cells during G-CSF treatment from median 11.7 to 6.4% ($p = 0.00006$) and an increase in lymphocyte B cell percentage from median 8.4 to 10.8% ($p = 0.0001$). The change in T cell percentage from a median value of 73.3 to 69.4% was not statistically significant (Table S3 in Supplementary Material; **Figure 1**).

G-CSF Increased the CD4/CD8 Ratio and the Proportion of Naïve T Regulatory Cells but Reduced the Fraction of Cytotoxic Terminally Differentiated Effector T Cells and $TCR\gamma\delta^+$ T Cells

There was a significant increase in the fraction of $CD4^+$ T helper cells (T_H) in peripheral blood and an equivalent decrease in $CD8^+$ T cytotoxic cells (T_C) during G-CSF therapy (**Table 2**; **Figure 1**). The median CD4/CD8 ratio thereby increased from 2.6 (range 1.1–7.3) to 2.9 (range 1.3–7.4, $p = 0.001$) during treatment. The increased fraction of $CD4^+$ cells was mainly due to an increased mobilization of naïve $CD4^+$ T cells (T_N) with a significantly reduced fraction of central memory cells.

Cytotoxic $CD8^+$ T cells can be divided into at least four subsets (15, 16). G-CSF caused a preferential mobilization of naïve $CD8^+$ cytotoxic T cells, and we now observed significantly reduced fractions of terminally differentiated cytotoxic $CD45RA^+$ effector cell (TEMRA) and cytotoxic $CD45RA^-RO^+$ $CD26^{hi}$ cells with unchanged central and effector memory T_C levels (**Table 2**; **Figure 1**; Table S4 in Supplementary Material).

Granulocyte colony-stimulating factor therapy preferentially increased the levels of circulating $TCR\alpha\beta^+$ T cells, leading to significantly reduced proportion of $TCR\gamma\delta^+$ T cells. Finally,

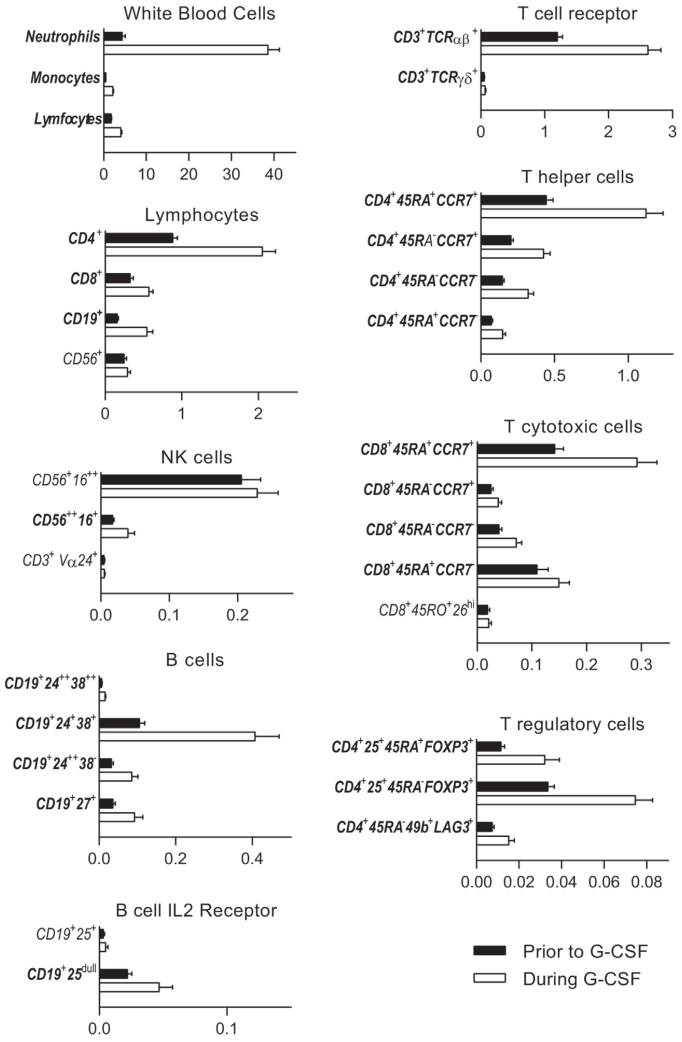

FIGURE 1 | Untreated healthy donor peripheral blood immune cell concentrations (black columns) are compared to levels after granulocyte colony-stimulating factor (G-CSF) treatment (white columns). Subsets with significantly changed concentrations during G-CSF treatment are indicated with bold fonts. The peripheral blood concentrations ($\times 10^9$/L) are given on the *x*-axes. The following subpopulations are presented: neutrophils, monocytes, and lymphocytes ($CD4^+$ T helper cells, $CD8^+$ T cytotoxic cells, $CD19^+$ B cells, and $CD56^+$ NK cells), NK cells [$CD56^+16^{++}$ cytolytic NK cells, $CD56^{++}$ 16^+ cytokine producing NK cells, and $CD3^+V\alpha24^+$ invariant NKT (iNKT) cells], B cells [$CD19^+24^{++}38^{++}$ transitional B cells, $CD19^+24^+38^+$ mature B cells, $CD19^+24^{++}38^-$, and $CD19^+27^+$ memory B cells and B cell IL-2 receptor (CD25) expression], $CD3^+$ T cell expression of T cell receptor $\alpha\beta$ and $\gamma\delta$, $CD4^+$ and $CD8^+$ memory subsets (naïve $CD45RA^+$ $CCR7^+$, central memory $CD45RA^-CCR7^+$, effector memory $CD45RA^-CCR7^-$, and terminally differentiated $CD45RA^+CCR7^-$), and T regulatory cells [naïve $CD4^+25^+45RA^+FOXP3^+$ T regulatory cells, effector $CD4^+25^+45RA^-FOXP3^+$ T regulatory cells, and $CD4^+45RA^-49b^+LAG3^+$ type 1 T regulatory cells (Tr1)]. All immune subsets with corresponding immunophenotypes and concentrations before and after G-CSF treatment are also listed in **Table 2** and in Table S4 in Supplementary Material. The percentages from all flow cytometry analyses are presented in Table S4 in Supplementary Material.

especially the levels of circulating naïve but also effector T regulatory cells and type 1 T regulatory cells (Tr1) increased during G-CSF therapy (**Table 2**; **Figures 1** and **2**).

Taken together, these observations demonstrate that G-CSF-induced T cell mobilization is not a random process with a similar effect on all T cell subsets, but rather a more selective process

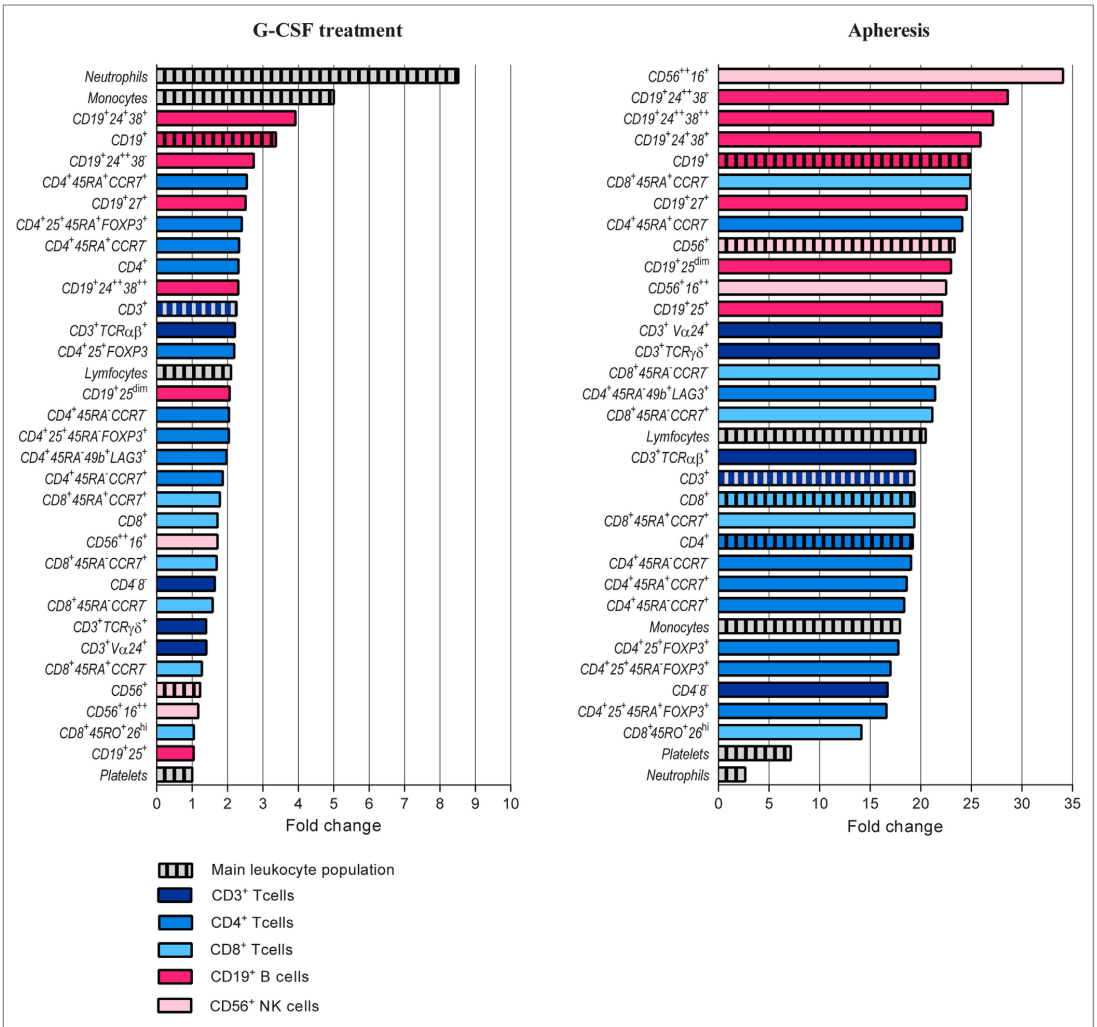

FIGURE 2 | Comparison of granulocyte colony-stimulating factor (G-CSF) induced peripheral blood increments of different immune cell populations and blood platelets. T, B, and NK cell subsets are presented with different colors. The peripheral blood concentration of each subset was calculated before and after G-CSF treatment and relative change calculated. Please see **Table 2** and Table S2 in Supplementary Material for classification of all immunophenotypes presented.

with preferential mobilization of naïve CD4+ and CD8+ T cells together with TCR$\alpha\beta^+$ T cells and various subsets of regulatory T cells.

G-CSF Therapy Caused a Strong Mobilization of Mature and Memory B Cells and Decreased B Cell Expression of the IL-2 Receptor

Peripheral blood CD19+ B cells can be divided into the three subsets transitional, mature, and memory B cells based on the coexpression of CD24 and CD38 (17). Mature and memory B cells showed the highest fold change during G-CSF treatment of all lymphoid cell subsets examined (**Figure 2**). Thus, B cell mobilization is not a random process either but represents a preferential increase of certain subsets similar to the T cell mobilization. Finally, the expression of IL2 receptor on human B cells is reported to be important for their antigen presentation and T cell activation (18). During G-CSF treatment, the B cell expression of the IL2 receptor decreased, and particularly the fraction of B cells with high IL2-R expression was reduced (**Figures 1** and **2**; **Table 3**; Table S4 in Supplementary Material).

TABLE 3 | Effect of granulocyte colony-stimulating factor (G-CSF) on peripheral blood and graft concentrations of B and NK cell subsets ($n = 22$) presented as median levels ($\times 10^9$/L) with variation ranges in parentheses.

Lymphoid cell subsets	Prior to G-CSF	During G-CSF	*p*	Stem cell graft	*R/p*
Transitional B ($CD19^+24^{++}38^{++}$)	0.005 (0.001–0.021)	0.013 (0.005–0.034)	0.00004	0.311 (0.087–1.045)	0.310/0.064
Mature B ($CD19^+24^+38^+$)	0.094 (0.022–0.274)	0.352 (0.147–1.471)	0.00004	7.61 (1.52–19.09)	0.462/0.006**
Memory B ($CD19^+24^{++}38^-$)	0.023 (0.002–0.097)	0.059 (0.016–0.295)	0.00004	1.898 (0.319–9.011)	0.427/0.011*
($CD19^+27^+$)	0.027 (0.003–0.131)	0.067 (0.018–0.459)	0.00004	1.055 (0.535–13.742)	0.462/0.006**
IL-$2R^+$ B ($CD19^+25^+$)	0.002 (<0.001–0.017)	0.002 (0.001–0.043)	NS	0.065 (0.012–1.028)	0.661/0.00008****
IL-$2R^{dull}$ B ($CD19^+25^{dull}$)	0.017 (0.002–0.070)	0.031 (0.007–0.228)	0.0001	0.671 (0.196–6.422)	0.322/0.054
Cytolytic NK ($CD56^+16^{++}$)	0.191 (0.025–0.447)	0.201 (0.025–0.521)	NS	3.901 (0.882–11.986)	0.379/0.019*
Cytokine producing NK ($CD56^{++}16^+$)	0.018 (0.006–0.038)	0.029 (0.005–0.230)	0.001	0.619 (0.261–2.117)	0.200/0.218
Invariant NKT ($CD3^+V\alpha24^+$)	0.003 (0.001–0.022)	0.003 (0.001–0.023)	NS	0.089 (0.006–2.147)	0.295/0.069

The Wilcoxon's test for paired samples was used for comparison of pre-treatment and G-CSF-treated concentrations (fourth column). The correlations between pre-apheresis and graft concentrations were analyzed with Kendall's tau-b test, and the correlation coefficients (R) and corresponding p-values are presented in the rightmost column.
**p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001.*

Only Cytokine-Producing NK Cells Increased During G-CSF Therapy Whereas the Levels of Other Circulating NK Cell Subsets Were Not Altered

The peripheral blood concentrations of cytokine-producing $CD56^{++}CD16^+$ NK cells increased only weakly (**Table 2**, $p = 0.001$) during G-CSF treatment, whereas neither the level of cytolytic $CD56^+CD16^{++}$ NK cells nor invariant NKT (iNKT) cells showed any significant changes. Consequently, the fractions of these subsets were decreased during G-CSF therapy [i.e., immediately before stem cell apheresis (**Table 3**; **Figure 1** and **2**; Table S4 in Supplementary Material)].

G-CSF Treatment of Healthy Donors Caused Preferential Mobilization of Certain Cytokine-Producing Lymphoid Cell Subsets

We investigated the intracellular levels of IFNγ, IL10, TGFβ, IL4, IL9, IL17, and IL22 in circulating main lymphoid subsets, and generally we found increased levels of cytokine-producing cells during mobilization with G-CSF (**Figure 3**; **Tables 4** and **5**). In addition to $CD3^+$, $CD4^+$, and $CD8^+$ T cells and $CD19^+$ B cells, we analyzed the cytokine production in the PB $CD3^-19^-$ and $CD3^+4^-8^-$ populations. The $CD3^-19^-$ compartment is mainly composed of NK cells and innate lymphoid cells and the $CD3^+4^-8^-$ subset primarily contains γδ T cells in addition to NKT cells. As shown in Table S5 in Supplementary Material, the percentage distribution of various cytokines was characteristic of each lymphoid subset, and the essential cytokine profile of each subset was conserved during G-CSF treatment. The fractions of IFNγ- and TGFβ-producing cells were high in all subsets except B cells, which showed low IFNγ and high IL10 production, and $CD3^-19^-$ cells with low TGFβ-production and high IL9 expression.

Both prior to and during G-CSF administration, the highest fractions of IFNγ expressing cells were observed for T_C. G-CSF treatment led to reduced $IFN\gamma^+$ fractions for T_C cells, $CD4^-CD8^-$ T cells, and $CD3^-19^-$ cells. Furthermore, G-CSF increased the fractions of IL10 expressing T_H, T_C, and $CD3^-19^-$ cells. Finally, TGFβ was expressed in a large fraction of most investigated lymphoid subsets before and during mobilization, but only B cells and $CD3^-19^-$ cells showed significantly reduced $TGF\beta^+$ fractions during G-CSF therapy. There were generally low fractions of IL4, IL17, and IL22 expressing cells for all lymphoid subsets and these fractions remained small after G-CSF therapy, whereas for IL9 we noticed relatively large fractions within $CD4^-8^-$ T cells and especially $CD3^-19^-$ cells (**Figure 3**; Table S5 in Supplementary Material).

Taken together, these observations suggest that the preferential mobilization alters the overall cytokine release capacity of circulating immunocompetent cells.

Graft Levels of Lymphoid Cell Subsets Were Increased but Reflected the Peripheral Blood Levels of Immunocompetent Cells Immediately Before Harvesting

As expected, the graft concentrations of various lymphoid cell subpopulations were generally higher than the peripheral blood levels tested immediately before apheresis, and for most lymphoid cell subsets the graft concentration represents at least a 20-fold enrichment (**Figure 2**; **Tables 1**–**3**). The median lymphocyte percentage corresponded to only 9.3% of circulating viable white blood cells immediately before stem cell apheresis, but increased to 36.6% in the stem cell graft. The median monocyte percentage increased to 16.2%, whereas the neutrophil percentage decreased to 42.4% (**Figure 2**; **Table 1**; Table S3 in Supplementary Material).

The fractions of B cells and monocytes among total PB leukocytes increased during G-CSF treatment, and there was an up-concentration of these two cell subsets in the grafts (corresponding to 90-fold and almost 60-fold, respectively) compared to the blood level before G-CSF administration. The T cell and especially neutrophil fractions also increased during mobilization, and the up-concentration in the graft corresponded to 45-fold and 30-fold increments compared with the pre-treatment levels. The NK cell fraction was reduced during mobilization and the graft levels of NK cells corresponded to a 20-fold increment compared to pre-treatment PB level (**Figure 2**; **Table 1**; Table S3 in Supplementary Material).

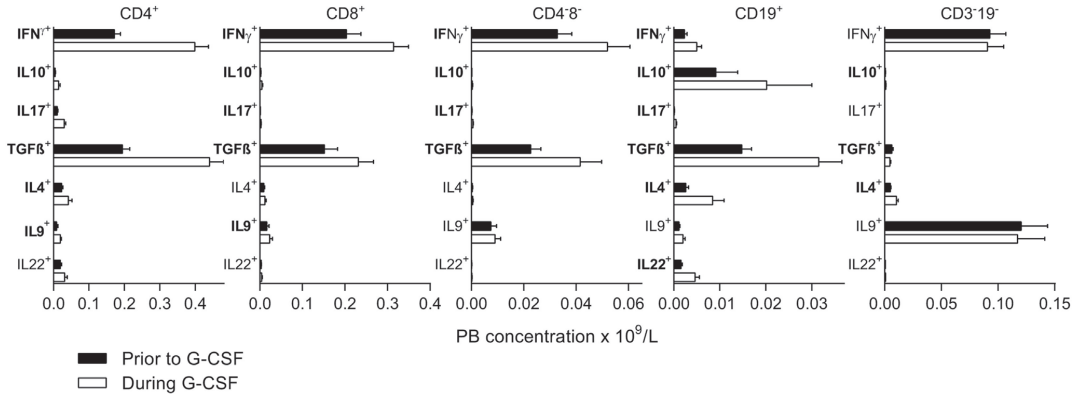

FIGURE 3 | Intracellular concentrations of immunoregulatory cytokines prior to stem cell mobilization (black columns) are compared to levels after granulocyte colony-stimulating factor (G-CSF) treatment (white columns). Subsets with significantly changed concentrations during G-CSF treatment are indicated with bold fonts. The peripheral blood concentrations ($\times 10^9$/L) are given on the *x*-axes. From left to right, results for $CD4^+$ T helper cells, $CD8^+$ T cytotoxic cells, $CD4^-8^-$ T cells, $CD19^+$ B cells, and $CD3^-19^-$ cells are presented. The concentrations of all subsets prior to and during G-CSF treatment are also listed in **Tables 4** and **5** and Table S5 in Supplementary Material. The percentages from all flow cytometry analyses are presented in Table S5 in Supplementary Material.

TABLE 4 | Effect of stem cell mobilization with granulocyte colony-stimulating factor (G-CSF) on healthy donor T cell intracellular cytokine production ($n = 22$).

	T_H cells		T_C cells		$CD3^+4^-8^-$ T cells	
Cytokine	**Prior to/during G-CSF**	***p***	**Prior to/during G-CSF**	***p***	**Prior to/during G-CSF**	***p***
IFNγ	0.157/0.374	0.00004 (↑)	0.154/0.302	0.001 (↑)	0.030/0.040	0.022 (↑)
IL10	0.0042/0.0100	0.00004 (↑)	0.0010/0.0019	0.000061 (↑)	0.0001/0.0003	0.001 (↑)
IL17	0.0091/0.0152	0.000046 (↑)	0.0008/0.0014	0.005 (↑)	0.0001/0.0004	0.007 (↑)
TGFβ	0.178/0.367	0.000061 (↑)	0.112/0.231	0.004 (↑)	0.0150/0.0297	0.024 (↑)
IL4	0.0175/0.0322	0.000367 (↑)	0.0053/0.0070	NS	0.0002/0.0002	NS
IL9	0.0048/0.0152	0.001 (↑)	0.0112/0.0107	0.011 (↓)	0.0025/0.0055	NS
IL22	0.0153/0.0211	NS	0.0033/0.0034	NS	0.0001/0.0001	NS

From left to right, the results for T helper cells (T_H), T cytotoxic cells (T_C), and $CD3^+4^-8^-$ T cells are presented. For each cytokine, the untreated concentrations ($\times 10^9$/L) of positive cells are shown together with the concentrations during G-CSF treatment on the line below (prior to/during G-CSF), see also **Figure 3**. *The Wilcoxon's test for paired samples was used for comparison of pre-treatment and G-CSF-treated concentrations.*
(↑), significant increased concentration; (↓), significant decreased concentration; NS, non-significant; p = p-value.

TABLE 5 | Effect of stem cell mobilization with granulocyte colony-stimulating factor (G-CSF) on healthy donor B and $CD3^-19^-$ cell intracellular cytokine production ($n = 22$).

	B cells		$CD3^-19^-$ cells	
Cytokine	**Prior to/during G-CSF**	***p***	**Prior to/during G-CSF**	***p***
IFNγ	0.0013/0.0036	0.002 (↑)	0.0789/0.0776	NS
IL10	0.0012/0.0041	0.001 (↑)	0.0005/0.0010	0.005 (↑)
IL17	0.0001/0.0003	0.001 (↑)	0.0001/0.0004	NS
TGFβ	0.0130/0.0249	0.000295 (↑)	0.0043/0.0033	0.022 (↓)
IL4	0.0013/0.0049	0.000069 (↑)	0.0039/0.0078	0.003 (↑)
IL9	0.0011/0.0012	NS	0.0925/0.1086	NS
IL22	0.0010/0.0030	0.000187 (↑)	0.0006/0.0006	NS

For each cytokine, the pre-treatment concentrations ($\times 10^9$/L) of positive cells are shown together with the concentrations after G-CSF treatment on the line below (prior to/during G-CSF), see also **Figure 3**. *The Wilcoxon's test for paired samples was used for comparison of pre-treatment and G-CSF-treated concentrations.*
(↑), significant increased concentration; (↓), significant decreased concentration; NS, non-significant; p = p-value.

Finally, we investigated whether the PB cell subset levels immediately before apheresis showed any correlations with the corresponding graft levels (**Tables 1–3**). Significant correlations were detected for most cell subsets. Thus, the graft levels of immunocompetent cells in general reflected the corresponding peripheral blood levels at the time of harvesting.

Healthy Donors Could Be Sub-Classified Based on Both the Pre-Treatment Levels and the Increase in Circulating Lymphoid Cell Subsets in Response to G-CSF Treatment

We observed a considerable variation between stem cell donors in leukocyte subset levels in peripheral blood both prior to and during G-CSF therapy. An unsupervised hierarchical clustering analysis based on untreated B, T, and NK cell concentrations identified two donor clusters (Figure S2A in Supplementary

Material) characterized by significant and inverse differences in NK cell and B cell concentrations ($p = 0.0001$ and 0.0004, Mann–Whitney U test). Differences between donors with regard to the B/NK cell levels were maintained during G-CSF therapy (Figure S2B in Supplementary Material).

We also performed unsupervised hierarchical clustering based on concentration changes in immunocompetent cells during G-CSF therapy (i.e., the ratio between pre-harvest PB concentrations and the concentrations prior to G-CSF administration for each immune cell subset), and again we identified two main donor subsets characterized by a generally strong or weak immune cell mobilizing effect of G-CSF (**Figure 4**). The donors in the upper cluster had significantly stronger effects of G-CSF compared to the donors in the lower cluster, and a greater increase in the peripheral blood cell concentration than in the lower cluster was seen for all lymphoid cell subsets except Tr1, iNKT cells, and CD25$^+$ B cells. The most significant differences in G-CSF-induced concentration alterations were seen for TCR$\alpha\beta^+$ T cells and T cytotoxic effector memory cells (Mann–Whitney U test; $p = 0.000006$), T helper effector memory cells and CD3$^+$4$^-$8$^-$ cells ($p = 0.00002$), and T helper central memory cells ($p = 0.00004$).

We investigated whether the main clusters identified in these two analyses (i.e., pre G-CSF lymphocyte concentration and G-CSF responsiveness) differed with regard to donor age, gender, ethnicity, previous diseases (especially autoimmune diseases), G-CSF dose, peripheral blood and graft CD34$^+$ cell concentration, donor yield, infused dose of CD34$^+$ cells per kilogram to the patients and graft content of all identified cell subsets. However, no significant differences were then observed for any of these variables when comparing the two main clusters in each of the two hierarchical clustering analyses (data not shown).

Graft Levels of Immunocompetent Cell Subsets Did Not reflect the Corresponding Alterations in Circulating Lymphoid Cell Subsets During G-CSF Mobilization

We investigated whether the G-CSF-induced alteration in PB concentrations of various immunocompetent cell subsets (i.e., their cell concentration increments or G-CSF responsiveness) showed any correlations with the corresponding graft concentrations. However, these analyses did not show significant correlations for any of the cell subsets. Thus, the graft concentrations of immunocompetent cell subsets do not reflect the pre-apheresis donor responsiveness to G-CSF immune cell mobilization. Furthermore, we also compared the two donor subsets identified in

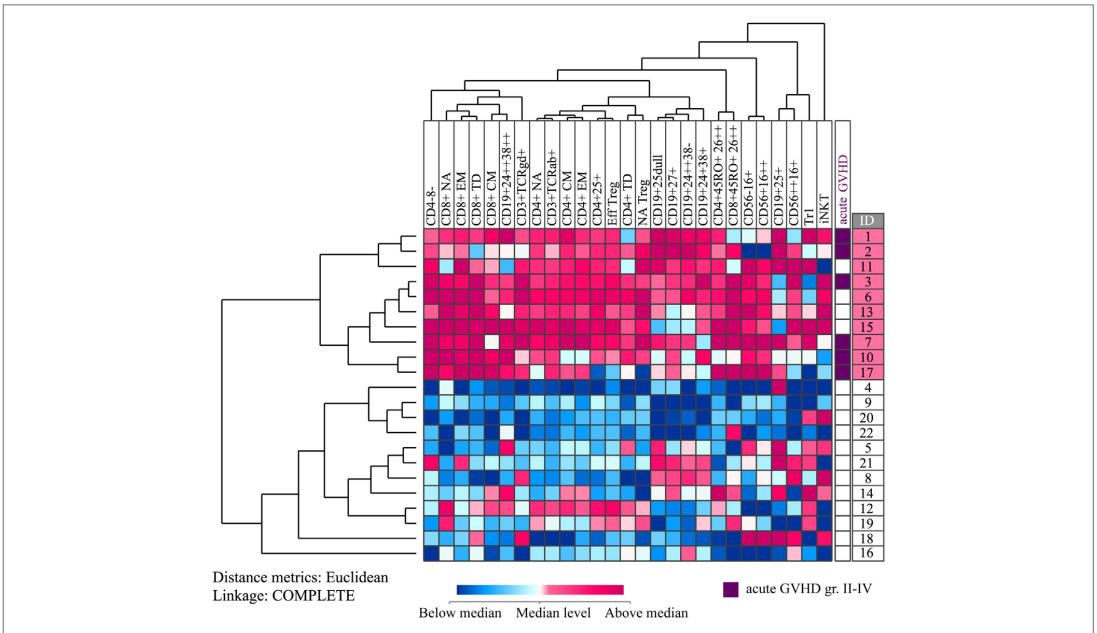

FIGURE 4 | Unsupervised hierarchical cluster analyses based on healthy donor lymphocyte subset concentration changes during granulocyte colony-stimulating factor (G-CSF) treatment. All values were median normalized and log-2 transformed before performing the unsupervised hierarchical clustering analysis, and complete linkage was used as a linkage method. Euclidian distance metrics was used for distance measure. The heat map with the corresponding dendrograms is presented. Red color indicates concentration change higher than the median, whereas blue color indicates concentration change lower than the median. The vertical donor clustering into two main clusters is presented to the left of the heat map, whereas the rightmost column presents the donor identification numbers of the two clusters marked with different colors. The prevalence of acute graft versus host disease (GVHD) grades II–IV in the recipients of the donor cells is presented in a separate column to the right of the heat map. The six donors to the recipients diagnosed with this complication are marked with purple color.

the clustering analysis of G-CSF responsiveness (**Figure 4**), and these two donor subsets did not differ significantly with regard to graft concentrations or infused cell doses of $CD34^+$ cells or of any immunocompetent cell subset or with regard to any of the donor characteristics mentioned above. Both these analyses suggest that the differences in donor responsiveness to G-CSF treatment (i.e., qualitative characteristics) are not reflected in the graft concentrations (i.e., quantitative characteristics) of immunocompetent cell subsets.

G-CSF Responsiveness in Mobilization of Various Immunocompetent Cell Subsets Was Associated With Time Until Post-Transplant Hematopoietic Reconstitution

During the first day of apheresis, the median yield of $CD34^+$ hematopoietic stem cells corresponded to 5.1×10^6 per kg donor weight (range $0.8–22.4 \times 10^6$/kg) and showed a negative correlation to donor weight ($R = -0.481$, $p = 0.001$). The stem cell products from 20 of the 22 healthy donors were transplanted to the recipients as planned, whereas the two last transplantations were canceled due to disease progression. The median total stem cell dose infused was 5.6×10^6 per kg patient's body weight (range $3.9–8.2 \times 10^6$/kg). Neutrophil reconstitution with stable peripheral blood neutrophils $>0.5 \times 10^9$/L was achieved by 18 of the 20 recipients after a mean of 17 days (range 8–26 days). Furthermore, stable platelet reconstitution with peripheral blood levels exceeding 50×10^9/L was achieved by 15 recipients after a mean of 18 days (range 12–39 days).

We investigated whether the donor subsets identified based on the pre-treatment levels of lymphocytes (Figure S2A in Supplementary Material) or the G-CSF induced concentration increase in various lymphocyte subsets (**Figure 4**) differed with respect to recipient neutrophil and platelet reconstitution. The recipients corresponding to donors in the upper cluster in Figure S2A in Supplementary Material ($n = 9$) had mean time to neutrophil reconstitution of 20 days (range 17–26 days), whereas the recipients corresponding to the lower cluster ($n = 9$) achieved neutrophil reconstitution after mean 16 days (range 8–18). Two patients died early before reconstitution. We did a multivariate analysis of predictors potentially influencing time to neutrophil reconstitution using Cox regression and including all 20 patients. Patients that died and patients without reconstitution were treated as censored observations. The potential predictors included patient age and gender, female to male transplantation, myeloablative versus reduced intensity conditioning, acute GVHD prophylaxis (completed methotrexate prophylaxis versus reduced methotrexate dose), infused stem cell dose, infused total leukocyte dose, ABO incompatibility, disease diagnosis, disease stage according to the EBMT index (19), and patient classification based on the donor clustering in Figure S2A in Supplementary Material. In the Cox regression of time to neutrophil reconstitution, the following four variables remained significant predictors after backward selection at significance level 0.05 in the likelihood ratio test: ABO incompatibility [HR = 11.74, 95% CI: (1.84, 75.70), $p = 0.004$], patient age [HR = 1.12, 95% CI: (0.99,1.26), $p = 0.037$], conditioning regimen [myeloablative or reduced intensity conditioning, HR = 7.48, 95% CI: (0.78,71.20), $p = 0.045$] and pre-transplant remission status [first complete remission, second complete remission or detectable disease [HR1 = 9.45:95% CI: (0.96,92.71), HR2 = 4.81; 95% CI: (0.46, 50.45), $p = 0.050$].

We also compared the hematopoietic reconstitution for the two donor clusters/subsets identified in **Figure 4** (G-CSF induced concentration increase in peripheral blood cell levels). These donor/patient subsets did not differ with respect to neutrophil reconstitution. Of the 15 patients who achieved platelet counts above 50×10^9/L during the first 7 weeks seven belonged to the upper donor cluster that was characterized by a generally large G-CSF induced increase in the peripheral blood levels of all immunocompetent cell subsets, and their mean time until platelet reconstitution was 21 days (range 15–39 days). Eight of the 15 patients recipients belonged to the lower donor cluster had a mean time until platelet reconstitution of 15 days (range 12–17 days). Two patients died early before reconstitution, one patient never had platelet counts below 50×10^9/L (registered as missing data), and two patients showed delayed platelet reconstitution. Similar to our analysis of neutrophil reconstitution (see above), we did a multivariate analysis of factors potentially influencing platelet reconstitution, including all the variables listed above (patient age and gender, female to male transplantation, myeloablative versus reduced intensity conditioning, acute GVHD prophylaxis, infused stem cell dose, infused total leukocyte dose, ABO incompatibility, disease diagnosis, disease stage according to the EBMT index (19), and patient classification corresponding to the donor clustering presented in **Figure 4**). In the Cox regression of time to platelet reconstitution, the following two variables remained significant predictors: ABO incompatibility [HR = 16.0, 95% CI: (1.64,156), $p = 0.002$] and overall donor G-CSF responsiveness in terms of G-CSF-induced concentration change [see **Figure 4**, HR = 4.54, 95% CI: (1.25, 16.5), $p = 0.017$]. Thus, the donor G-CSF responsiveness seems to be one of the factors important for the hematopoietic reconstitution.

Post-Transplant Outcomes Differ for Patients Receiving Allografts From Donors With Generally Strong and Weak Mobilization of Immunocompetent Cells in Response to G-CSF

After allogeneic stem cell infusion, the patients were observed until death or study closure; the median observation time was 701 days (variation range 19–1944 days). All survivors had been observed for at least 1160 days. Six recipients were diagnosed with acute GVHD grade II–IV, and all their donors belonged to the upper cluster in **Figure 4** characterized by great G-CSF-induced increase, i.e., strong G-CSF responsiveness ($p = 0.001$, Pearson Chi-Square test).

We also compared the recipient mortality for the two donor subsets identified in **Figure 4** (response to G-CSF) using the Kaplan–Meier method. The two recipient subsets corresponding to these two donor clusters did not differ significantly in overall survival. However, the causes of death differed between the two groups. For the donors/patients in the upper cluster, one patient

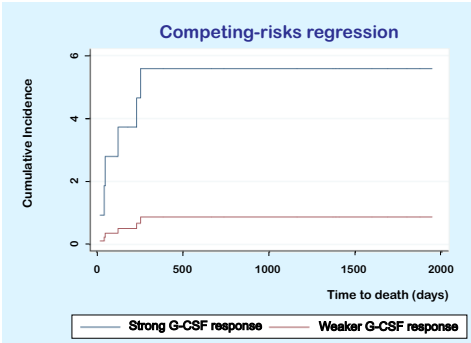

FIGURE 5 | Competing risk analysis of time to death. The figure shows the results for a competing risk analysis of time to death due to toxicity (i.e., non-relapse mortality) or relapse. Patients receiving PBMC allografts from donors with a strong granulocyte colony-stimulating factor (G-CSF) response had a higher risk of non-relapse ($p = 0.031$) but donor responsiveness did not significantly influence the risk of relapse ($p = 0.121$).

died of relapse but five patients died from other causes, whereas for the patients in the lower subset five patients died from relapse and one patient died after retransplantation for graft failure. Thus, there was a different distribution of relapse and non-relapse mortality for the patients corresponding to these two donor clusters/subsets. In a competing risk analysis of time to non-relapse or relapse death, we found that patients receiving stem cell grafts from donors with strong G-CSF responsiveness had a higher risk of non-relapse death compared to recipients of grafts from donors with weaker G-CSF responsiveness (**Figure 5**, $p = 0.031$), but the donor G-CSF responsiveness did not have any effect on time to death due to relapse ($p = 0.121$).

DISCUSSION

In the present study, we describe hematopoietic stem cell mobilization in healthy donors as a heterogeneous process both with regard to differences between donors in pre-mobilization levels of circulating immunocompetent cell subsets, the general donor responsiveness to G-CSF with respect to mobilization of immune cell subsets, and differences in mobilization between various immune cell subsets (i.e., preferential mobilization). Our observations suggest that such differences may have an impact on the post-transplant outcome of the graft recipients.

We investigated an unselected group of allotransplanted patients from a defined geographic area during a defined time period and receiving peripheral blood stem cell grafts from matched family donors; this study should therefore be regarded as population-based and including well-characterized patients with a limited heterogeneity with regard to conditioning treatment, stem cell donors, graft preparation, and posttransplant handling with regard to GVHD and antibiotic prophylaxis. We would therefore emphasize that future studies have to clarify whether our results are representative also for other allotransplant recipients, i.e., patients with matched unrelated donors, other conditioning regimens, other GVHD or antibiotic prophylaxis, or other diagnoses.

The immune system represents an interactive network of a wide range of immunocompetent cell subsets. Clustering analysis is a methodological approach to identify such network-mediated interactions and correlations/covariations, and these covariations can then be a basis for identification of patient or donor subsets showing biological similarities. In the present study, clustering analyses could be used to identify distinct donor subsets based on analysis of their responsiveness to G-CSF.

The preferential G-CSF induced mobilization of several T, B, and NK cell subsets is also reflected in the graft. Graft manipulation either as *ex vivo* positive or negative selection, *in vivo* depletion of T cells by anti-thymocyte globulin or *in vivo* donor immunomodulation prior to harvesting are now considered as possible strategies for graft manipulation of healthy donors (5–10, 20–25). This study shows that donors/grafts differ in their content of various immunocompetent cell subsets, and a detailed characterization of these cells in stem cell allografts will probably be a necessary basis for optimally designed allografts. Previous studies of immunocompetent cells in G-CSF-mobilized grafts (13, 26–28) as well as more recent studies investigating associations between graft immunocompetent cells and recipient outcome have focused on selected immunocompetent cell subsets (26, 29–34), whereas we examined a wider profile of immunocompetent cells and included a focus on their G-CSF responsiveness.

Our results suggest that G-CSF therapy induces a preferential mobilization of immunocompetent cells. Relatively weak mobilizing of certain cell subsets may be important for the post-transplant clinical course of the allotransplant recipients. First, TCRγδ$^+$ T cells and NK cells seem to be important for the risk of aGVHD (35–37). Second, high numbers of CD8$^+$ CD45RO$^+$ CD26^{++} cells in autografts are important for the risk of relapse/progression (38), whereas TEMRA is associated with a risk of cGVHD (39). Third, IL-2R-expressing B cells play a role in T cell activation and may have a role in the pathogenesis of aGVHD (18). Finally, reduced fractions of iNKT cells and preferential mobilization of naïve T_H may increase the risk of aGVHD (40, 41), but the preferential mobilization of CD4 cells also includes regulatory T cell subsets with immunosuppressive effects (42). Thus, the final effect of the reduced mobilization of these functionally different lymphoid subsets is difficult to predict but may represent an immunosuppressive effect. The effect of G-CSF on the cytokine release by immunocompetent cells has only been examined in a few previous studies (43–47); our present detailed characterization suggests that G-CSF therapy also alters the cytokine release profile of immunocompetent cells.

We did not find any associations between the infused dose of various immune cell subsets and the clinical outcome of the recipients, and results from previous studies of associations between cell subset dose and outcome are also conflicting (29, 30, 33, 48–50). Our present results support previous studies suggesting that the balance between different immunocompetent cell subsets is important (31, 32, 37, 51) and in addition

our results suggest that the broader immunocompetent cell subset profile as well as the dose-independent responsiveness to G-CSF (i.e., the increase in the concentrations of various subsets, **Figure 4**) are more important than differences in single cell subset levels. Dhedin et al. previously reported that the individual donor response to G-CSF with regard to $CD34^+$ stem cell mobilization was the best predictor of later aGVHD (52), but we could not confirm this. However, we also observed an association between donor responsiveness to G-CSF and aGVHD (**Figure 4**), i.e., a generally strong G-CSF-induced mobilization of immunocompetent cells (especially T cell subsets) in the donor was associated with increased risk of aGVHD for the recipient. The G-CSF responsiveness showed no association with the concentrations of various immunocompetent cells in the stem cell grafts, and this last observation suggests that the impact of G-CSF responsiveness is not caused simply by quantitative differences of reinfused immunocompetent cells to the transplant recipients.

The possible importance of the overall $CD34^+$ stem cells dose and T cell dose for engraftment, aGVHD, and survival is still uncertain, and results from previous studies are conflicting (53–57). One possible explanation could be that the described impact of donor responsiveness to G-CSF represents an additional and dose-independent mechanism that differs between donors and thereby between recipients. Another explanation could be differences in patient inclusion, e.g., one study included only AML patients (30), whereas our study was population-based but included only patients with family donors.

We identified two main donor clusters based on the responsiveness to G-CSF (**Figure 4**), but at the same time the grafts from these two donor subsets did not differ with regard to the amount of $CD34^+$ cells or immunocompetent cell subsets. The most likely explanation for our observed effects of donor heterogeneity on reconstitution/non-relapse mortality in the absence of quantitative differences in the number of reinfused cells is qualitative differences between the grafts. One would expect immunocompetent graft cells to exert their effects on outcome during the early post-transplant period, and several previous studies suggest that this is a critical period with regard to later complications. First, the clinical experience suggests that GVHD prophylaxis should start pre-transplant; this is true both when using prophylaxis based on anti-thymocyte globulin and cyclosporine (58). Second, post-transplant cyclophosphamide as well as methotrexate prophylaxis also start early post-transplant (58, 59). Third, the adverse effects of G-CSF treatment after allogeneic stem cell transplantation seem to depend on the biological context early after graft infusion and the use of total body irradiation in the conditioning treatment; this is supported both by clinical and experimental studies (60–62). Finally, the adverse effects of post-transplant G-CSF therapy was not seen for patients receiving G-CSF mobilized stem cell grafts, i.e., graft cells where one would expect the post-transplant effects of G-CSF to be limited because the cells had already been exposed to G-CSF before and during graft preparation. All these previous observations support our hypothesis that activation/qualitative differences between donors with regard to infused donor immunocompetent cells (i.e., their responsiveness to G-CSF) can influence the posttransplant outcome.

The immunological heterogeneity of the donors is evident both prior to and during G-CSF therapy. Platelet engraftment seems to be predicted by the intrinsic G-CSF immune cell mobilizing effect, and engraftment in the patient is influenced by both G-CSF-dependent and G-CSF-independent characteristics. The time to platelet engraftment was longer in recipients of the most G-CSF responsive donors, an apparent paradox as T cell depletion increases the risk of graft failure (63, 64). However, experience from autologous transplantation shows that T cells are less important for engraftment, when the stem cell dose is sufficient (65), and the absolute concentrations or infused doses of any immune cell subset did not differ between the G-CSF high and low responsive donor groups in this study. Furthermore, several immune cell subsets have been shown to facilitate engraftment without increasing the risk of acute GVHD through mechanisms that are not yet known (66). In line with this, intrinsic donor responsiveness to G-CSF may represent a separate mechanism that can increase the risk of recipient acute GVHD but at the same time tend to prolong time to engraftment.

Stem cell harvest by leukapheresis also contributes to the immune cell composition and activation status of the stem cell graft. Immunomodulatory effects of apheresis procedures are taken advantage of in therapeutic apheresis (67–71). Not only the mobilization but also the collection of stem cells results in a skewed distribution of different immune cell subsets that may represent a separate immunomodulatory mechanism.

In addition to detailed characterization of various lymphoid subsets, we also detected increased monocyte:lymphocyte ratio during G-CSF therapy, and stem cell mobilization with G-CSF has been shown to give preferential mobilization of $CD34^+$ regulatory monocytes as well as monocytic myeloid-derived suppressor cells (34, 72–76). Monocytic and lymphoid cells are not easily separated by leukapheresis, and consequently a large fraction of monocytic cells are infused during transplantation and probably contributes to the immunomodulatory effect the stem cell graft. Several studies have demonstrated that the levels of $CD34^+$ regulatory monocytes as well as monocytic myeloid-derived suppressor cells are associated with the risk of post-transplant GVHD (34, 75, 76). However, the immunosuppressive effect of monocytic cells is considered to be a double-edged sword (75), and in autologous stem cell transplantation high fractions of monocytes in the graft have been shown to have a negative effect on overall survival (77).

We observed a difference in post-transplant outcomes between the two patient clusters/subsets identified by the analysis of donor G-CSF responsiveness (**Figure 4**). First, for neutrophil reconstitution ABO incompatibility, patient age, conditioning regimen, and pre-transplant remission status were significant predictors after multivariate Cox regression analysis, whereas the donor differences did not have any influence. Second, for platelet reconstitution we observed an independent effect of differences in donor G-CSF responsiveness in addition to the effect of ABO incompatibility. Finally, the two clusters identified in **Figure 4** showed similar early recipient mortality and no statistically significant difference in median overall survival. However, the cause of recipient death differed significantly between the two donor

clusters; for the upper cluster only one out of six patients died from relapse, whereas for the lower cluster five out of six patients did so. Our competing risk regression analysis also showed an association of borderline significance between high G-CSF responsiveness and non-relapse mortality. Taken together, these observations suggest that immunological differences between donors with regard to G-CSF responsiveness are important for recipient outcome after allotransplantation. However, due to our low number of donors/recipients, we would emphasize that our observations need to be confirmed in larger clinical studies.

In conclusion, our study gives one of the most detailed characterizations of the immunomodulatory effects of stem cell mobilization and apheresis on the distribution of multiple lymphoid cell subsets available this far and shows that donor immune characteristics may be important for recipient outcome. Both G-CSF treatment and apheresis skew the distribution of various immune cell subsets and thereby influence graft composition, and both G-CSF dependent and independent immunological heterogeneity of the donors are reflected in the outcome of the patients. The results of our study indicate that the intrinsic effect of G-CSF on donor immune cell mobilization is associated with the reconstitution of platelets and the prevalence of acute GVHD after related HLA-matched stem cell transplantation. As this study includes relatively few participants, these results need to be confirmed in larger studies.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations in the guidelines drawn up by The Norwegian National Research Ethics Committee for medical and health research (NEM) with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the Regional Committee for Medical and Health Research Ethics of Western Norway (2011/996, 2011/1237, 2011/1241 and 2013/634).

AUTHOR CONTRIBUTIONS

ØB, EE, GM, and EK designed the study. GM performed the laboratory experiments trained by EE and with advice from EK. GM analyzed the flow cytometry data and performed basic statistical and bioinformatics analyses. GE performed the Cox regression and competing risk analyses. GM, ØB, EE, EK, and GE evaluated the results. GM and ØB wrote the paper, and EK, GE, and EE critically revised the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at https://www.frontiersin.org/articles/10.3389/fimmu.2018.00845/full#supplementary-material.

REFERENCES

1. Vadakekolathu J, Rutella S. T-cell manipulation strategies to prevent graft-versus-host disease in haploidentical stem cell transplantation. *Biomedicines* (2017) 5(2):33. doi:10.3390/biomedicines5020033
2. Devine SM, Carter S, Soiffer RJ, Pasquini MC, Hari PN, Stein A, et al. Low risk of chronic graft-versus-host disease and relapse associated with T cell-depleted peripheral blood stem cell transplantation for acute myelogenous leukemia in first remission: results of the blood and marrow transplant clinical trials network protocol 0303. *Biol Blood Marrow Transplant* (2011) 17(9):1343–51. doi:10.1016/j.bbmt.2011.02.002
3. Pasquini MC, Devine S, Mendizabal A, Baden LR, Wingard JR, Lazarus HM, et al. Comparative outcomes of donor graft CD34+ selection and immune suppressive therapy as graft-versus-host disease prophylaxis for patients with acute myeloid leukemia in complete remission undergoing HLA-matched sibling allogeneic hematopoietic cell transplantation. *J Clin Oncol* (2012) 30(26):3194–201. doi:10.1200/JCO.2012.41.7071
4. Hobbs GS, Hamdi A, Hilden PD, Goldberg JD, Poon ML, Ledesma C, et al. Comparison of outcomes at two institutions of patients with ALL receiving ex vivo T-cell-depleted or unmodified allografts. *Bone Marrow Transplant* (2015) 50(4):493–8. doi:10.1038/bmt.2014.302
5. Saad A, Lamb LS. Ex vivo T-cell depletion in allogeneic hematopoietic stem cell transplant: past, present and future. *Bone Marrow Transplant* (2017) 52(9):1241–8. doi:10.1038/bmt.2017.22
6. Huang W, Chao NJ. Memory T cells: a helpful guard for allogeneic hematopoietic stem cell transplantation without causing graft-versus-host disease. *Hematol Oncol Stem Cell Ther* (2017) 10(4):211–9. doi:10.1016/j.hemonc.2017.05.006
7. Schumm M, Lang P, Bethge W, Faul C, Feuchtinger T, Pfeiffer M, et al. Depletion of T-cell receptor alpha/beta and CD19 positive cells from apheresis products with the CliniMACS device. *Cytotherapy* (2013) 15(10):1253–8. doi:10.1016/j.jcyt.2013.05.014
8. Bader P, Soerensen J, Jarisch A, Ponstingl E, Krenn T, Faber J, et al. Rapid immune recovery and low TRM in haploidentical stem cell transplantation in children and adolescence using CD3/CD19-depleted stem cells. *Best Pract Res Clin Haematol* (2011) 24(3):331–7. doi:10.1016/j.beha.2011.04.005
9. Abdelhakim H, Abdel-Azim H, Saad A. Role of alphabeta T cell depletion in prevention of graft versus host disease. *Biomedicines* (2017) 5(3):35. doi:10.3390/biomedicines5030035
10. Diaz MA, Perez-Martinez A, Herrero B, Deltoro N, Martinez I, Ramirez M, et al. Prognostic factors and outcomes for pediatric patients receiving an haploidentical relative allogeneic transplant using CD3/CD19-depleted grafts. *Bone Marrow Transplant* (2016) 51(9):1211–6. doi:10.1038/bmt.2016.101
11. Krejci M, Janikova A, Folber F, Kral Z, Mayer J. Outcomes of 167 healthy sibling donors after peripheral blood stem cell mobilization with G-CSF 16mug/kg/day: efficacy and safety. *Neoplasma* (2015) 62(5):787–92. doi:10.4149/neo_2015_094
12. Mueller MM, Bialleck H, Bomke B, Brauninger S, Varga C, Seidl C, et al. Safety and efficacy of healthy volunteer stem cell mobilization with filgrastim G-CSF and mobilized stem cell apheresis: results of a prospective longitudinal 5-year follow-up study. *Vox Sang* (2013) 104(1):46–54. doi:10.1111/j.1423-0410.2012.01632.x

13. Melve GK, Ersvssr E, Kittang AO, Bruserud O. The chemokine system in allogeneic stem-cell transplantation: a possible therapeutic target? *Expert Rev Hematol* (2011) 4(5):563–76. doi:10.1586/ehm.11.54
14. Stavrum AK, Petersen K, Jonassen I, Dysvik B. Analysis of gene-expression data using J-express. *Curr Protoc Bioinformatics* (2008) Chapter 7:Unit73. doi:10.1002/0471250953.bi0703s21
15. Geginat J, Lanzavecchia A, Sallusto F. Proliferation and differentiation potential of human CD8+ memory T-cell subsets in response to antigen or homeostatic cytokines. *Blood* (2003) 101(11):4260–6. doi:10.1182/blood-2002-11-3577
16. Takata H, Takiguchi M. Three memory subsets of human CD8+ T cells differently expressing three cytolytic effector molecules. *J Immunol* (2006) 177(7):4330–40. doi:10.4049/jimmunol.177.7.4330
17. Carsetti R, Rosado MM, Wardmann H. Peripheral development of B cells in mouse and man. *Immunol Rev* (2004) 197:179–91. doi:10.1111/j.0105-2896.2004.0109.x
18. Brisslert M, Bokarewa M, Larsson P, Wing K, Collins LV, Tarkowski A. Phenotypic and functional characterization of human CD25+ B cells. *Immunology* (2006) 117(4):548–57. doi:10.1111/j.1365-2567.2006.02331.x
19. Gratwohl A, Stern M, Brand R, Apperley J, Baldomero H, de Witte T, et al. Risk score for outcome after allogeneic hematopoietic stem cell transplantation: a retrospective analysis. *Cancer* (2009) 115(20):4715–26. doi:10.1002/cncr.24531
20. Ito S, Bollard CM, Carlsten M, Melenhorst JJ, Biancotto A, Wang E, et al. Ultra-low dose interleukin-2 promotes immune-modulating function of regulatory T cells and natural killer cells in healthy volunteers. *Mol Ther* (2014) 22(7):1388–95. doi:10.1038/mt.2014.50
21. Bleakley M, Heimfeld S, Loeb KR, Jones LA, Chaney C, Seropian S, et al. Outcomes of acute leukemia patients transplanted with naive T cell-depleted stem cell grafts. *J Clin Invest* (2015) 125(7):2677–89. doi:10.1172/JCI81229
22. Kaynar L, Demir K, Turak EE, Ozturk CP, Zararsiz G, Gonen ZB, et al. TcRalphabeta-depleted haploidentical transplantation results in adult acute leukemia patients. *Hematology* (2017) 22(3):136–44. doi:10.1080/10245332.2016.1238182
23. Lang P, Teltschik HM, Feuchtinger T, Muller I, Pfeiffer M, Schumm M, et al. Transplantation of CD3/CD19 depleted allografts from haploidentical family donors in paediatric leukaemia. *Br J Haematol* (2014) 165(5):688–98. doi:10.1111/bjh.12810
24. Li Pira G, Malaspina D, Girolami E, Biagini S, Cicchetti E, Conflitti G, et al. Selective depletion of alphabeta t cells and b cells for human leukocyte antigen-haploidentical hematopoietic stem cell transplantation. A three-year follow-up of procedure efficiency. *Biol Blood Marrow Transplant* (2016) 22(11):2056–64. doi:10.1016/j.bbmt.2016.08.006
25. Locatelli F, Bauquet A, Palumbo G, Moretta F, Bertaina A. Negative depletion of alpha/beta+ T cells and of CD19+ B lymphocytes: a novel frontier to optimize the effect of innate immunity in HLA-mismatched hematopoietic stem cell transplantation. *Immunol Lett* (2013) 155(1–2):21–3. doi:10.1016/j.imlet.2013.09.027
26. Saraceni F, Shem-Tov N, Olivieri A, Nagler A. Mobilized peripheral blood grafts include more than hematopoietic stem cells: the immunological perspective. *Bone Marrow Transplant* (2015) 50(7):886–91. doi:10.1038/bmt.2014.330
27. Franzke A. The role of G-CSF in adaptive immunity. *Cytokine Growth Factor Rev* (2006) 17(4):235–44. doi:10.1016/j.cytogfr.2006.05.002
28. Rutella S, Zavala F, Danese S, Kared H, Leone G. Granulocyte colony-stimulating factor: a novel mediator of T cell tolerance. *J Immunol* (2005) 175(11):7085–91. doi:10.4049/jimmunol.175.11.7085
29. Vasu S, Geyer S, Bingman A, Auletta JJ, Jaglowski S, Elder P, et al. Granulocyte colony-stimulating factor-mobilized allografts contain activated immune cell subsets associated with risk of acute and chronic graft-versus-host disease. *Biol Blood Marrow Transplant* (2016) 22(4):658–68. doi:10.1016/j.bbmt.2015.12.015
30. Impola U, Larjo A, Salmenniemi U, Putkonen M, Itala-Remes M, Partanen J. Graft immune cell composition associates with clinical outcome of allogeneic hematopoietic stem cell transplantation in patients with AML. *Front Immunol* (2016) 7:523. doi:10.3389/fimmu.2016.00523
31. Pastore D, Delia M, Mestice A, Carluccio P, Perrone T, Gaudio F, et al. CD3+/Tregs ratio in donor grafts is linked to acute graft-versus-host disease and immunologic recovery after allogeneic peripheral blood stem cell transplantation. *Biol Blood Marrow Transplant* (2012) 18(6):887–93. doi:10.1016/j.bbmt.2011.10.039
32. Delia M, Pastore D, Mestice A, Carluccio P, Perrone T, Gaudio F, et al. Outcome of allogeneic peripheral blood stem cell transplantation by donor graft CD3+/Tregs ratio: a single-center experience. *Biol Blood Marrow Transplant* (2013) 19(3):495–9. doi:10.1016/j.bbmt.2012.11.015
33. Waller EK, Logan BR, Harris WA, Devine SM, Porter DL, Mineishi S, et al. Improved survival after transplantation of more donor plasmacytoid dendritic or naive T cells from unrelated-donor marrow grafts: results from BMTCTN 0201. *J Clin Oncol* (2014) 32(22):2365–72. doi:10.1200/JCO.2013.54.4577
34. Vendramin A, Gimondi S, Bermema A, Longoni P, Rizzitano S, Corradini P, et al. Graft monocytic myeloid-derived suppressor cell content predicts the risk of acute graft-versus-host disease after allogeneic transplantation of granulocyte colony-stimulating factor-mobilized peripheral blood stem cells. *Biol Blood Marrow Transplant* (2014) 20(12):2049–55. doi:10.1016/j.bbmt.2014.09.011
35. Pabst C, Schirutschke H, Ehninger G, Bornhauser M, Platzbecker U. The graft content of donor T cells expressing gamma delta TCR+ and CD4+foxp3+ predicts the risk of acute graft versus host disease after transplantation of allogeneic peripheral blood stem cells from unrelated donors. *Clin Cancer Res* (2007) 13(10):2916–22. doi:10.1158/1078-0432.CCR-06-2602
36. Maeda Y, Reddy P, Lowler KP, Liu C, Bishop DK, Ferrara JL. Critical role of host gammadelta T cells in experimental acute graft-versus-host disease. *Blood* (2005) 106(2):749–55. doi:10.1182/blood-2004-10-4087
37. Zhao XY, Chang YJ, Xu LP, Liu DH, Liu KY, Huang XJ. Association of natural killer cells in allografts with transplant outcomes in patients receiving G-CSF-mobilized PBSC grafts and G-CSF-primed BM grafts from HLA-haploidentical donors. *Bone Marrow Transplant* (2009) 44(11):721–8. doi:10.1038/bmt.2009.73
38. Hildebrandt M, Dijkstra D, Gollasch H, Daemen K, Stevanovic-Meyer M, Ludwig WD. Apheresis-related enrichment of CD26++ T lymphocytes: phenotypic characterization and correlation with unfavorable outcome in autologous hematopoietic progenitor cell transplantation. *Transfusion* (2012) 52(4):765–76. doi:10.1111/j.1537-2995.2011.03351.x
39. D'Asaro M, Dieli F, Caccamo N, Musso M, Porretto F, Salerno A. Increase of CCR7- CD45RA+ CD8 T cells (T(EMRA)) in chronic graft-versus-host disease. *Leukemia* (2006) 20(3):545–7. doi:10.1038/sj.leu.2404079
40. Mavers M, Maas-Bauer K, Negrin RS. Invariant natural killer T cells as suppressors of graft-versus-host disease in allogeneic hematopoietic stem cell transplantation. *Front Immunol* (2017) 8:900. doi:10.3389/fimmu.2017.00900
41. Li JM, Giver CR, Lu Y, Hossain MS, Akhtari M, Waller EK. Separating graft-versus-leukemia from graft-versus-host disease in allogeneic hematopoietic stem cell transplantation. *Immunotherapy* (2009) 1(4):599–621. doi:10.2217/imt.09.32
42. Hoffmann P, Ermann J, Edinger M, Fathman CG, Strober S. Donor-type CD4(+)CD25(+) regulatory T cells suppress lethal acute graft-versus-host disease after allogeneic bone marrow transplantation. *J Exp Med* (2002) 196(3):389–99. doi:10.1084/jem.20020399
43. Sloand EM, Kim S, Maciejewski JP, Van Rhee F, Chaudhuri A, Barrett J, et al. Pharmacologic doses of granulocyte colony-stimulating factor affect cytokine production by lymphocytes in vitro and in vivo. *Blood* (2000) 95(7):2269–74.
44. Tayebi H, Kuttler F, Saas P, Lienard A, Petracca B, Lapierre V, et al. Effect of granulocyte colony-stimulating factor mobilization on phenotypical and functional properties of immune cells. *Exp Hematol* (2001) 29(4):458–70. doi:10.1016/S0301-472X(01)00613-0
45. Crough T, Nieda M, Nicol AJ. Granulocyte colony-stimulating factor modulates alpha-galactosylceramide-responsive human Valpha24+Vbeta11+NKT cells. *J Immunol* (2004) 173(8):4960–6. doi:10.4049/jimmunol.173.8.4960
46. Sun LX, Ren HY, Shi YJ, Wang LH, Qiu ZX. Recombinant human granulocyte colony-stimulating factor significantly decreases the expression of CXCR3 and CCR6 on T cells and preferentially induces T helper cells to a T helper 17 phenotype in peripheral blood harvests. *Biol Blood Marrow Transplant* (2009) 15(7):835–43. doi:10.1016/j.bbmt.2009.03.016
47. Schlahsa L, Jaimes Y, Blaszczyk R, Figueiredo C. Granulocyte-colony-stimulatory factor: a strong inhibitor of natural killer cell function. *Transfusion* (2011) 51(2):293–305. doi:10.1111/j.1537-2995.2010.02820.x
48. Eissens DN, Michelo CM, Preijers FW, van Cranenbroek B, van Houwelingen K, van der Meer A, et al. Selective expansion of human natural killer cells leads to enhanced alloreactivity. *Cell Mol Immunol* (2014) 11(2):160–8. doi:10.1038/cmi.2013.56

49. Marcenaro E, Carlomagno S, Pesce S, Della Chiesa M, Moretta A, Sivori S. Role of alloreactive KIR2DS1(+) NK cells in haploidentical hematopoietic stem cell transplantation. *J Leukoc Biol* (2011) 90(4):661–7. doi:10.1189/jlb.0311137
50. Moretta L, Pietra G, Montaldo E, Vacca P, Pende D, Falco M, et al. Human NK cells: from surface receptors to the therapy of leukemias and solid tumors. *Front Immunol* (2014) 5:87. doi:10.3389/fimmu.2014.00087
51. Kalinski P, Mailliard RB, Giermasz A, Zeh HJ, Basse P, Bartlett DL, et al. Natural killer-dendritic cell cross-talk in cancer immunotherapy. *Expert Opin Biol Ther* (2005) 5(10):1303–15. doi:10.1517/14712598.5.10.1303
52. Dhedin N, Chamakhi I, Perreault C, Roy DC, Sauvageau G, Ducruet T, et al. Evidence that donor intrinsic response to G-CSF is the best predictor of acute graft-vs-host disease following allogeneic peripheral blood stem cell transplantation. *Exp Hematol* (2006) 34(1):107–14. doi:10.1016/j.exphem.2005.09.013
53. Richa EM, Kunnavakkam R, Godley LA, Kline J, Odenike O, Larson RA, et al. Influence of related donor age on outcomes after peripheral blood stem cell transplantation. *Cytotherapy* (2012) 14(6):707–15. doi:10.3109/14653249.2012.681041
54. Cao TM, Wong RM, Sheehan K, Laport GG, Stockerl-Goldstein KE, Johnston LJ, et al. CD34, CD4, and CD8 cell doses do not influence engraftment, graft-versus-host disease, or survival following myeloablative human leukocyte antigen-identical peripheral blood allografting for hematologic malignancies. *Exp Hematol* (2005) 33(3):279–85. doi:10.1016/j.exphem.2004.12.004
55. Korbling M, Huh YO, Durett A, Mirza N, Miller P, Engel H, et al. Allogeneic blood stem cell transplantation: peripheralization and yield of donor-derived primitive hematopoietic progenitor cells (CD34+ Thy-1dim) and lymphoid subsets, and possible predictors of engraftment and graft-versus-host disease. *Blood* (1995) 86(7):2842–8.
56. Gomez-Almaguer D, Gomez-Pena A, Jaime-Perez JC, Gomez-Guijosa MA, Cantu-Rodriguez O, Gutierrez-Aguirre H, et al. Higher doses of CD34+ progenitors are associated with improved overall survival without increasing GVHD in reduced intensity conditioning allogeneic transplant recipients with clinically advanced disease. *J Clin Apher* (2013) 28(5):349–55. doi:10.1002/jca.21278
57. Remberger M, Torlen J, Ringden O, Engstrom M, Watz E, Uhlin M, et al. Effect of total nucleated and CD34(+) cell dose on outcome after allogeneic hematopoietic stem cell transplantation. *Biol Blood Marrow Transplant* (2015) 21(5):889–93. doi:10.1016/j.bbmt.2015.01.025
58. Ruutu T, Gratwohl A, de Witte T, Afanasyev B, Apperley J, Bacigalupo A, et al. Prophylaxis and treatment of GVHD: EBMT-ELN working group recommendations for a standardized practice. *Bone Marrow Transplant* (2014) 49(2):168–73. doi:10.1038/bmt.2013.107
59. Farhan S, Peres E, Janakiraman N. Choice of unmanipulated T cell replete graft for haploidentical stem cell transplant and posttransplant cyclophosphamide in hematologic malignancies in adults: peripheral blood or bone marrow-review of published literature. *Adv Hematol* (2016) 2016:6950346. doi:10.1155/2016/6950346
60. Ringden O, Labopin M, Gorin NC, Le Blanc K, Rocha V, Gluckman E, et al. Treatment with granulocyte colony-stimulating factor after allogeneic bone marrow transplantation for acute leukemia increases the risk of graft-versus-host disease and death: a study from the acute leukemia working party of the European group for blood and marrow transplantation. *J Clin Oncol* (2004) 22(3):416–23. doi:10.1200/JCO.2004.06.102
61. Khoury HJ, Loberiza FR Jr, Ringden O, Barrett AJ, Bolwell BJ, Cahn JY, et al. Impact of posttransplantation G-CSF on outcomes of allogeneic hematopoietic stem cell transplantation. *Blood* (2006) 107(4):1712–6. doi:10.1182/blood-2005-07-2661
62. Morris ES, MacDonald KP, Kuns RD, Morris HM, Banovic T, Don AL, et al. Induction of natural killer T cell-dependent alloreactivity by administration of granulocyte colony-stimulating factor after bone marrow transplantation. *Nat Med* (2009) 15(4):436–41. doi:10.1038/nm.1948
63. Martin PJ, Hansen JA, Storb R, Thomas ED. Human marrow transplantation: an immunological perspective. *Adv Immunol* (1987) 40:379–438. doi:10.1016/S0065-2776(08)60243-6
64. O'Reilly RJ. Allogenic bone marrow transplantation: current status and future directions. *Blood* (1983) 62(5):941–64.
65. Russell A, Malik S, Litzow M, Gastineau D, Roy V, Zubair AC. Dual roles of autologous CD8+ T cells in hematopoietic progenitor cell mobilization and engraftment. *Transfusion* (2015) 55(7):1758–65; quiz 7. doi:10.1111/trf.13073
66. Geerman S, Nolte MA. Impact of T cells on hematopoietic stem and progenitor cell function: good guys or bad guys? *World J Stem Cells* (2017) 9(2):37–44. doi:10.4252/wjsc.v9.i2.37
67. Agishi T. Birth of the concept and the development of extracorporeal immunomodulation. *Ther Apher* (2002) 6(4):312–6. doi:10.1046/j.1526-0968.2002.00436.x
68. Ghio M, Contini P, Ansaldi F, Ubezio G, Setti M, Risso M, et al. A possible role of soluble HLA-I molecule in the immunomodulatory effects of therapeutic apheresis. *Blood Transfus* (2014) 12(Suppl1):s167–9. doi:10.2450/2013.0026-13
69. Contini P, Negrini S, Bodini G, Trucchi C, Ubezio G, Strada P, et al. Granulocytes and monocytes apheresis induces upregulation of TGFbeta1 in patients with active ulcerative colitis: a possible involvement of soluble HLA-I. *J Clin Apher* (2017) 32(1):49–55. doi:10.1002/jca.21466
70. Ghio M, Contini P, Ubezio G, Ansaldi F, Setti M, Tripodi G. Transient transforming growth factor beta1 modulation in monocytes and natural killer cells following plasma or plasma-platelet apheresis donation procedures. *Blood Transfus* (2015) 13(4):684–6. doi:10.2450/2015.0258-14
71. Ghio M, Contini P, Ansaldi F, Ubezio G, Setti M, Risso M, et al. Donor neutrophil activation and transforming growth factor-beta1 modulation induced by donor apheresis procedures. *Blood Transfus* (2014) 12(4):615–7. doi:10.2450/2014.0007-14
72. Koehn BH, Blazar BR. Role of myeloid-derived suppressor cells in allogeneic hematopoietic cell transplantation. *J Leukoc Biol* (2017) 102(2):335–41. doi:10.1189/jlb.5MR1116-464R
73. Fraser AR, Cook G, Franklin IM, Templeton JG, Campbell M, Holyoake TL, et al. Immature monocytes from G-CSF-mobilized peripheral blood stem cell collections carry surface-bound IL-10 and have the potential to modulate alloreactivity. *J Leukoc Biol* (2006) 80(4):862–9. doi:10.1189/jlb.0605297
74. Luyckx A, Schouppe E, Rutgeerts O, Lenaerts C, Koks C, Fevery S, et al. Subset characterization of myeloid-derived suppressor cells arising during induction of BM chimerism in mice. *Bone Marrow Transplant* (2012) 47(7):985–92. doi:10.1038/bmt.2011.207
75. Le Blanc K, Jitschin R, Mougiakakos D. Myeloid-derived suppressor cells in allogeneic hematopoietic stem cell transplantation: a double-edged sword? *Oncoimmunology* (2013) 2(7):e25009. doi:10.4161/onci.25009
76. D'Aveni M, Rossignol J, Coman T, Sivakumaran S, Henderson S, Manzo T, et al. G-CSF mobilizes CD34+ regulatory monocytes that inhibit graft-versus-host disease. *Sci Transl Med* (2015) 7(281):281ra42. doi:10.1126/scitranslmed.3010435
77. Porrata LF, Inwards DJ, Ansell SM, Micallef IN, Johnston PB, Hogan WJ, et al. Infused autograft lymphocyte to monocyte ratio and survival in diffuse large B cell lymphoma. *Biol Blood Marrow Transplant* (2014) 20(11):1804–12. doi:10.1016/j.bbmt.2014.07.012

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Supplementary Material

Peripheral Blood Stem Cell Mobilization in Healthy Donors by Granulocyte Colony-Stimulating Factor Causes Preferential Mobilization of Lymphocyte Subsets

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1 Legends to Supplementary Figures

1.1 Legend to Supplementary Figure 1

Gating strategy for lymphoid subsets. The selection and identification of subsets is described from left to right for all panels:

Panel (A): Initial gating of all events included the lymphocyte gate followed by the selection of singlets based on FSC-H (forward scatter-Height) and FSC-W (forward scatter-With) plus SSC-A (side scatter-Areal) and SSC-H (side scatter-Height). All lymphocytes were evaluated for viability by use of Near-IR fluorescent reactive dye. Live $CD3^+$ T cells were separated from live $CD3^-$ lymphocytes with CD3 PECy7, except from in initial characterization of NK cell subsets (Panel (C)), CD3 V450 was then used.

Panel (B): With a CD4/CD8 four-quadrant gate on selected $CD3^+$ cells four T cell subsets could be identified: (i) $CD4^+8^-$ T helper cells (ii) $CD4^-8^+$ T cytotoxic cells (i) $CD4^+8^+$ double positive T cells and $CD4^-8^-$ double negative T cells. A four-quadrant gate was also used for identification of naïve and memory T helper cells based on CD45-RA and CCR7 expression: (i) $CD4^+45^-RA^+CCR7^+$ naïve Th cells (ii) $CD4^+45^-RA^-CCR7^-$ effector memory (EM) Th cells (iii) $CD4^+45^-RA^-CCR7^+$ central memory (CM) Th cells (iv) $CD4^+45^-RA^-CCR7^-$ terminally differentiated (TD) Th cells. Identical gating strategy was used for identification of $CD8^+$ naïve and memory cytotoxic T cells. A distinct $CD45RO^+CD26^{hi}$ subpopulation was identified in selected $CD8^+$ cells, and the corresponding phenotype was also detected for $CD4^+$ cells. Finally, the identification of T cell receptor (TCR) divergent αβ T cells and γδ T cells is shown.

Panel (C): $CD56^+16^{++}$ cytolytic and $CD56^{++}16^{dim}$ cytokine producing NK cells were selected from $CD3^-$ lymphocytes and $V\alpha24^+$ iNKT cells from $CD3^+$ cells. T regulatory cells were selected from $CD4^+25^+$ cells and gated into $CD45RA^+$ $FoxP3^+$ naïve and $CD45RA^-$ $FoxP3^+$ effector T regulatory cells.

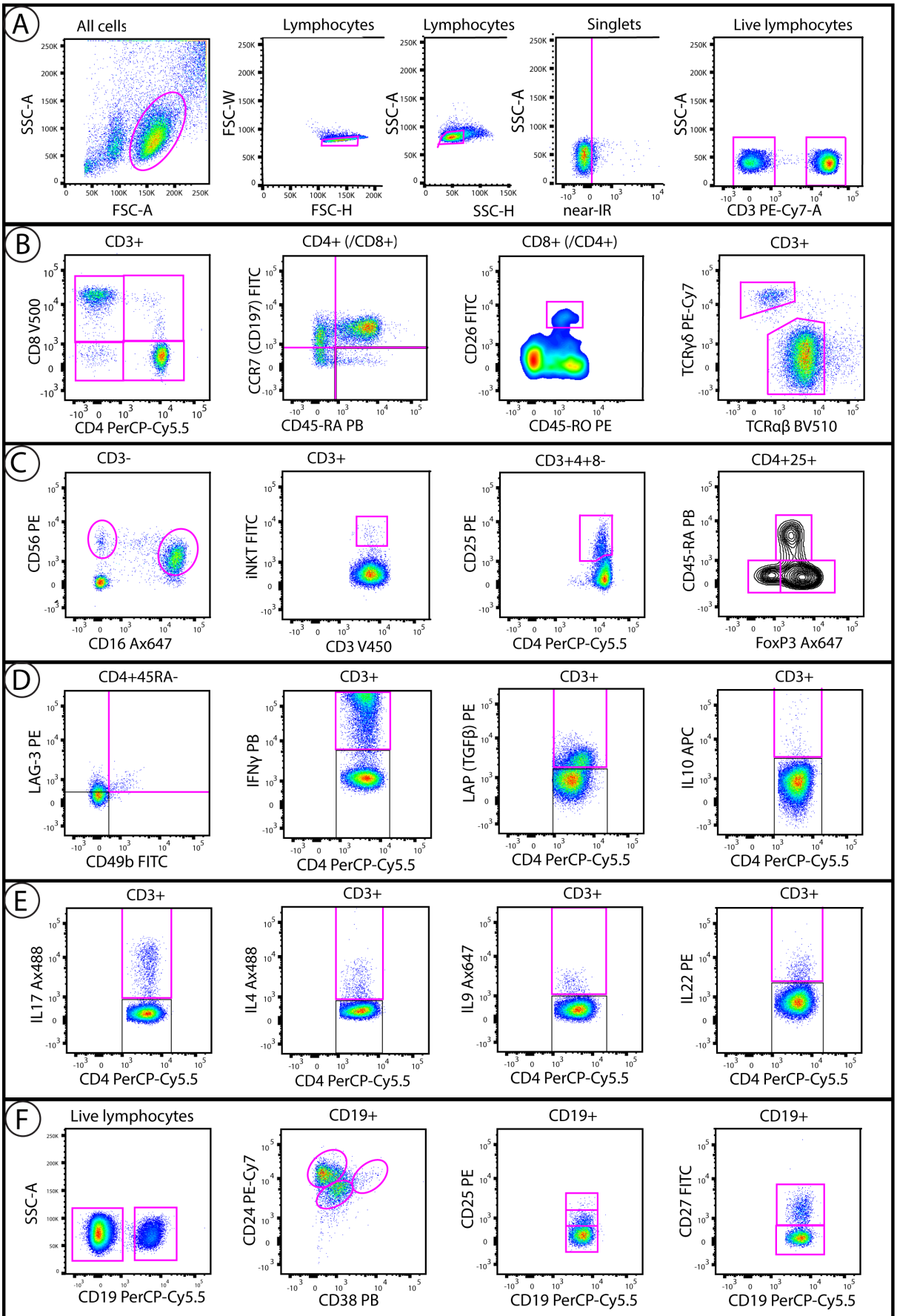
Panel (D): Type 1 regulatory (Tr1) cells were identified as $CD4^+45RA^-49b^+LAG3^+$. Cytokine expressing cells (IFNγ, TGFβ and IL-10) are shown as fractions of $CD4^+$ T cells. Corresponding subset identification was performed for $CD8^+$ T cells, $CD19^+$ B cells, $CD3^-19^-$ lymphocytes (i.e. mainly NK cells and innate lymphoid cells) and $CD4^-8^-$ T cells (i.e. mainly γδ T cells and NKT cells).

Panel (E): Identification of IL17, IL4, IL9 and IL22 expressing cells is like in Panel D shown for $CD4^+$ T cells, but was also identified in $CD8^+$ T cells, $CD19^+$ B cells, $CD3^-19^-$ lymphocytes (i.e. mainly NK cells) and $CD4^-8^-$ T cells (i.e. mainly γδ T cells).

Panel (F): $CD19^+$ B cells was identified and could be separated into $CD24^+38^+$ mature B cells, $CD24^{++}38^-$ memory B cells and $CD24^{++}38^{++}$ transitional B cells. Finally, the expression of IL2-R (CD25) and CD27 on all B-cells was evaluated; the IL2-R expressing cells were classified as $CD25^+$ or $CD25^{dim}$.

1.2 Legend to Supplementary Figure 2

Unsupervised hierarchical cluster analyses based on untreated (A) and G-CSF treated (B) healthy donor T, B and NK cell PB concentrations. All values were median normalized and log-2 transformed before performing the unsupervised hierarchical clustering analyses and complete linkage was used as linkage method. The Pearson correlation was used for distance measure. The heat maps with corresponding dendrograms are presented. Red color indicates concentration higher than the median; whereas blue color indicates concentration lower than the median. The vertical donor clustering into two main clusters is presented to the left of the heat maps, while the rightmost columns present the donor identification numbers of the two clusters marked with different colors based on the donor clustering in (A). With only two exceptions (donor 4 and donor 13), the donors clustered identically into the upper and lower donor cluster during G-CSF (B).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SSC-H
Singlets
SSC-A
near-IR
Live lymphocytes
SSC-A
CD3 PE-Cy7-A
B
CD3+
CD8 V500
CD4 PerCP-Cy5.5
CD4+ (/CD8+)
CCR7 (CD197) FITC
CD45-RA PB
CD8+ (/CD4+)
CD26 FITC
CD45-RO PE
CD3+
TCRγδ PE-Cy7
TCRαβ BV510
C
CD3-
CD56 PE
CD16 Ax647
CD3+
iNKT FITC
CD3 V450
CD3+4+8-
CD25 PE
CD4 PerCP-Cy5.5
CD4+25+
CD45-RA PB
FoxP3 Ax647
D
CD4+45RA-
LAG-3 PE
CD49b FITC
CD3+
IFNγ PB
CD4 PerCP-Cy5.5
CD3+
LAP (TGFβ) PE
CD4 PerCP-Cy5.5
CD3+
IL10 APC
CD4 PerCP-Cy5.5
E
CD3+
IL17 Ax488
CD4 PerCP-Cy5.5
CD3+
IL4 Ax488
CD4 PerCP-Cy5.5
CD3+
IL9 Ax647
CD4 PerCP-Cy5.5
CD3+
IL22 PE
CD4 PerCP-Cy5.5
F
Live lymphocytes
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CD19 PerCP-Cy5.5
CD19+
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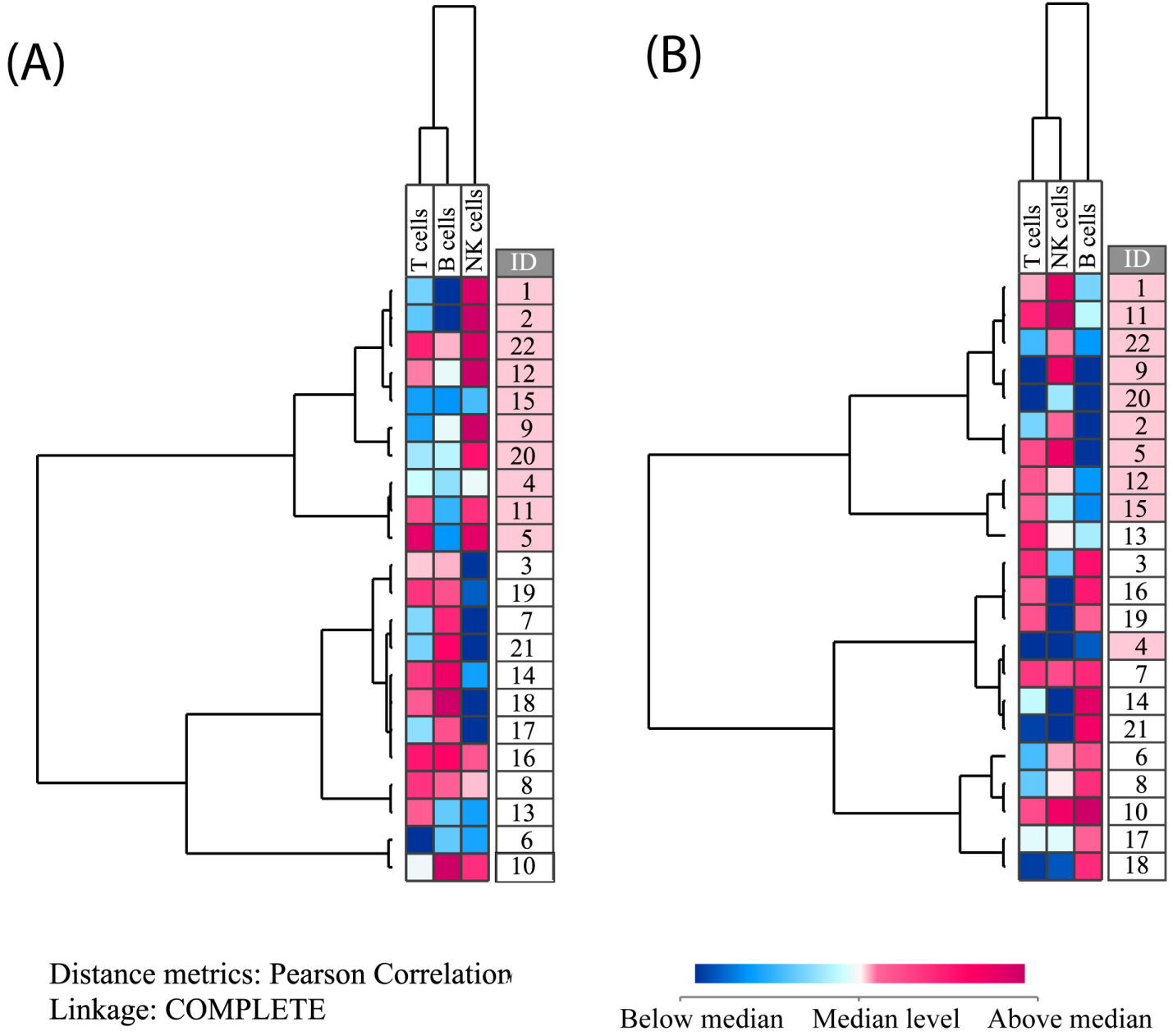
CD38 PB
CD19+
CD25 PE
CD19 PerCP-Cy5.5
CD19+
CD27 FITC
CD19 PerCP-Cy5.5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Distance metrics: Pearson Correlation
Linkage: COMPLETE
Below median
Median level
Above median

Supplementary Tables

Peripheral Blood Stem Cell Mobilization in Healthy Donors by Granulocyte Colony-Stimulating Factor Causes Preferential Mobilization of Lymphocyte Subsets

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1 Supplementary Tables

Supplementary Table 1: Clinical characteristicsof the 20 allogeneic stem cell recipients.

Identity[1]	Gender (M/F)	Gender match[2]	Age (years)	Diagnosis[3]	Complete remission[4]	EBMT risk score[5]	HCT CI[6]	HLA match	ABO incompatibility	Conditioning[7]	Stem cell dose[8]	WBC dose[9]	aGVHD	Relapse
1	M	-	57	MDS[L]	-	6	1-2	10/10	-	RIC	8.2	16.4	+	-
2	F	+	52	AML	CR1	2	≥3	10/10	-	RIC	5.6	18.8	+	-
3	M	+	43	AML	CR1	2	1-2	10/10	-	MAC	5.1	8.7	+	-
4	M	+	43	AML	CR1	2	≥3	10/10	-	RIC	8.0	4.7	-	-
5	M	+	64	MDS[E]	-	3	0	09/10	major	RIC	5.9	24.9	-	+
7	F	+	45	AML	-	5	≥3	10/10	minor	MAC	6.3	24.6	+	-
8	M	+	69	MDS[I]	CR2	4	0	10/10	-	RIC	5.5	27.3	-	+
9	F	+	49	AML	CR1	2	0	10/10	major	MAC	5.5	18.2	-	-
10	F	+	39	MDS[I]	-	2	1-2	10/10	-	MAC	5.9	5.1	+	-
11	M	-	44	AML	CR1	3	0	10/10	-	MAC	6.2	11.5	-	+
12	M	+	64	CMF[E]	-	3	0	10/10	major	RIC	5.0	5.9	-	-

13	F	+	36	ALL	CR2	3	1-2	10/10	-	MAC	5.0	3.8	-	-
14	M	-	44	AML	CR1	3	1-2	10/10	-	MAC	5.0	16.1	-	-
15	M	+	60	CMF[E]	-	4	1-2	09/10	-	RIC	3.9	16.6	-	-
16	F	+	35	AML	CR1	1	0	10/10	-	MAC	5.2	5.4	-	-
17	F	+	41	ALL	CR1	2	0	10/10	-	MAC	5.7	7.1	+	-
18	M	+	63	AML	CR1	2	≥3	10/10	-	RIC	5.5	7.4	-	+
19	M	-	45	AML	CR1	3	0	10/10	-	MAC	5.4	10.4	-	+
21	M	+	66	CLL[L]	-	5	0	10/10	-	RIC	6.2	4.2	-	+
22	M	+	62	AML	CR1	2	≥3	10/10	-	RIC	5.9	12.1	-	-

[1]For the patients ID6 and ID20 the planned transplantations were cancelled due to disease exacerbation.
[2]Male to male, female to female and male to female donations are indicated as +, female to male donation is indicated as -
[3]AML, acute myeloid leukemia; ALL, acute lymphoblastic leukemia; CLL, chronic lymphocytic leukemia; CMF, chronic myelofibrosis; MDS, myelodysplastic syndrome. For patients with other diseases than acute leukemic the disease stage from the EBMT risk score is indicated after the diagnosis (E, early; I, intermediate; L, late).
[4] CR1, first complete remission, CR2, second complete remission.
[5]Gratwohl A, Stern M, Brand R, Apperley J, Baldomero H, de Witte T, et al. Risk score for outcome after allogeneic hematopoietic stem celtransplantation: a retrospective analysis. *Cancer* (2009) 115(20):4715-26. doi: 10.1002/cncr.24531. PubMed PMID: 19642176.
[6]Sorror ML, Maris MB, Storb R, Baron F, Sandmaier BM, Maloney DG, et al. Hematopoietic cell transplantation (HCT)-specific comorbidity index: a new tool for risk assessment before allogeneic HCT. Blood (2005) 106(8):2912-9. doi: 10.1182/blood-2005-05-2004. PubMed PMID: 15994282; PubMed Central PMCID: PMC1895304.
[7]MAC, myeloablative conditioning; RIC, reduced intensity conditioning.
[8]Stem cell dosesare given as x 10^6/kg body weight of the recipient.
[9] White blood cell doses are given as x 10^7/kg body weight of the recipient.

Supplementary Table 2: Definitions ofimmunophenotypes of the main T, B and NK cell subsets identified

Lymphoidcellsubsets	Immunophenotype	Monoclonalantibodies/Fluorochromes	Reference
T cells			
Naïve T_H	$CD4^+45RA^+CCR7^+$	CD3-PE-Cy7 (SK7), CD4-PerCP-Cy5.5 (RPA-T4), CD45-RA-V450 (HI100), CD197/CCR7-Ax647 (150503)	(1)
Central memory (T_{CM})	$CD4^+45RA^-CCR7^+$	CD3-PE-Cy7 (SK7), CD4-PerCP-Cy5.5 (RPA-T4), CD45-RA-V450 (HI100), CD197/CCR7-Ax647 (150503)	(1)
Effectormemory (T_{EM})	$CD4^+45RA^-CCR7^-$	CD3-PE-Cy7 (SK7), CD4-PerCP-Cy5.5 (RPA-T4), CD45-RA-V450 (HI100), CD197/CCR7-Ax647 (150503)	(1)
Terminallydifferentiated (T_{TD})	$CD4^+45RA^+CCR7^-$	CD3-PE-Cy7 (SK7), CD4-PerCP-Cy5.5 (RPA-T4), CD45-RA-V450 (HI100), CD197/CCR7-Ax647 (150503)	(1)
Naïve T_c	$CD8^+45RA^+CCR7^+$	CD3-PE-Cy7 (SK7), CD8-V500 (RPA-T8), CD45-RA-V450 (HI100), CD197/CCR7-Ax647 (150503)	(1)
Central memory	$CD8^+45RA^-CCR7^+$	CD3-PE-Cy7 (SK7), CD8-V500 (RPA-T8), CD45-RA-V450 (HI100), CD197/CCR7-Ax647 (150503)	(1)
Effectormemory	$CD8^+45RA^-CCR7^-$	CD3-PE-Cy7 (SK7), CD8-V500 (RPA-T8), CD45-RA-V450 (HI100), CD197/CCR7-Ax647 (150503)	(1)
Effector (TEMRA)	$CD8^+45RA^+CCR7^-$	CD3-PE-Cy7 (SK7), CD8-V500 (RPA-T8), CD45-RA-V450 (HI100), CD197/CCR7-Ax647 (150503)	(1)
	$CD4^+45RO^+CD26^{++}$	CD3-PE-Cy7 (SK7), CD4-PerCP-Cy5.5 (RPA-T4), CD45-RO-PE (UCHL), CD26-FITC (M-A261)	(2)
	$CD8^+45RO^+CD26^{++}$	CD3-PE-Cy7 (SK7), CD8-V500 (RPA-T8), CD45-RO-PE (UCHL), CD26-FITC (M-A261)	(2)
αβ T cells	$CD3^+TCR\alpha\beta^+$	CD3-V450 (UCHT1),TCRαβ-BV510 (T10B91.A)	(3)
γδ T cells	$CD3^+4^-8^-TCR\gamma\delta^+$	CD3-V450 (UCHT1),TCRγδ-PE-Cy7 (11F2)	(3)
Naïve T regulatorycells	$CD4^+25^+45RA^+FOXP3^+$	CD3-PE-Cy7 (SK7), CD4-PerCP-Cy5.5 (RPA-T4), CD25-PE (M-A251), CD45-RA-V450 (HI100), FoxP3-Ax647 (236A/E7)	(4)
Effector Tregulatorycells	$CD4^+25^+45RA^-FOXP3^+$	CD3-PE-Cy7 (SK7), CD4-PerCP-Cy5.5 (RPA-T4), CD25-PE (M-A251), CD45-RA-V450 (HI100), FoxP3-Ax647 (236A/E7)	(4)
Tr1	$CD4^+45RA^-49b^+LAG3^+$	CD3-PE-Cy7 (SK7), CD4-PerCP-Cy5.5 (RPA-T4), CD45-RA-V450 (HI100), CD49b-FITC (AK7), LAG-3-PE (FAB2319P)	(5)
B cells			
Transitional	$CD19^+24^{++}38^{++}$	CD19-PerCP-Cy5.5 (SJ25C1), CD24- PE-Cy7 (ML5), CD38-PB (HIT2)	(6)
Mature	$CD19^+24^{++}38^-$	CD19-PerCP-Cy5.5 (SJ25C1), CD24- PE-Cy7 (ML5), CD38-PB (HIT2)	(6)
Memory	$CD19^+24^+38^+$	CD19-PerCP-Cy5.5 (SJ25C1), CD24- PE-Cy7 (ML5), CD38-PB (HIT2)	(6)
	$CD19^+27^+$	CD19-PerCP-Cy5.5 (SJ25C1), CD27-FITC (M-T271),	(6)
IL-$2R^+$	$CD19^+25^+$	CD19-PerCP-Cy5.5 (SJ25C1), CD25-PE (M-A251)	(7)
IL-$2R^w$	$CD19^+25^w$	CD19-PerCP-Cy5.5 (SJ25C1), CD25-PE (M-A251)	(7)
NK cells			
Cytolytic	$CD56^+16^{++}$	CD3-V450 (UCHT1), CD56-PE (B159), CD16-Ax647 (3G8)	(8)
Cytokineproducing	$CD56^{++}16^+$	CD3-V450 (UCHT1), CD56-PE (B159), CD16-Ax647 (3G8)	(8)
Invariant NKT (iNKT)	$CD3^+V\alpha24^+$	CD3-V450 (UCHT1),iNKT(Vα24)-FITC (6b11)	(9)

1. Matteucci E, Ghimenti M, Di Beo S, Giampietro O. Altered proportions of naive, central memory and terminally differentiated central memory subsets among CD4+ and CD8 + T cells expressing CD26 in patients with type 1 diabetes. *Journal of clinical immunology* (2011) 31(6):977-84. doi: 10.1007/s10875-011-9573-z. PubMed PMID: 21887518.

2. Hildebrandt M, Dijkstra D, Gollasch H, Daemen K, Stevanovic-Meyer M, Ludwig WD. Apheresis-related enrichment of CD26++ T lymphocytes: phenotypic characterization and correlation with unfavorable outcome in autologous hematopoietic progenitor cell transplantation. *Transfusion* (2012) 52(4):765-76. doi: 10.1111/j.1537-2995.2011.03351.x. PubMed PMID: 21950525.

3. Vantourout P, Hayday A. Six-of-the-best: unique contributions of gammadelta T cells to immunology. *Nature reviews Immunology* (2013) 13(2):88-100. doi: 10.1038/nri3384. PubMed PMID: 23348415; PubMed Central PMCID: PMC3951794.

4. Simonetta F, Bourgeois C. CD4+FOXP3+ Regulatory T-Cell Subsets in Human Immunodeficiency Virus Infection. *Frontiers in immunology* (2013) 4:215. doi: 10.3389/fimmu.2013.00215. PubMed PMID: 23908654; PubMed Central PMCID: PMC3727053.

5. Gagliani N, Magnani CF, Huber S, Gianolini ME, Pala M, Licona-Limon P, et al. Coexpression of CD49b and LAG-3 identifies human and mouse T regulatory type 1 cells. *Nature medicine* (2013) 19(6):739-46. doi: 10.1038/nm.3179. PubMed PMID: 23624599.

6. Carsetti R, Rosado MM, Wardmann H. Peripheral development of B cells in mouse and man. *Immunological reviews* (2004) 197:179-91. Epub 2004/02/14. PubMed PMID: 14962195.

7. Brisslert M, Bokarewa M, Larsson P, Wing K, Collins LV, Tarkowski A. Phenotypic and functional characterization of human CD25+ B cells. *Immunology* (2006) 117(4):548-57. Epub 2006/03/25. doi: IMM2331 [pii]

10.1111/j.1365-2567.2006.02331.x. PubMed PMID: 16556269; PubMed Central PMCID: PMC1782245.

8. Cooper MA, Fehniger TA, Turner SC, Chen KS, Ghaheri BA, Ghayur T, et al. Human natural killer cells: a unique innate immunoregulatory role for the CD56(bright) subset. *Blood* (2001) 97(10):3146-51. PubMed PMID: 11342442.

9. Berzins SP, Smyth MJ, Baxter AG. Presumed guilty: natural killer T cell defects and human disease. *Nature reviews Immunology* (2011) 11(2):131-42. doi: 10.1038/nri2904. PubMed PMID: 21267014.

Supplementary Table 3: The effect of G-CSF on peripheral blood and graft concentrations and percentages of various leukocyte subsets (n = 22) presented as median levels with variation ranges in parentheses. In the left part of the table the reults are presented as PB and graft concentrations, whereas in the right part the same values are presented as percentages of their parent populations acquired by flow cytometry. Neutrophils, monocytes and lymphocytes are presented as percentages of total WBC count, and T-, B- and NK cells as percentages of the total number of lymphocytes. All values are given as medians with variation ranges in parentheses. The Wilcoxon's test for paired samples was used for comparison of pre-treatment and G-CSF treated/pre-apheresis concentrations and percentages and pre-apheresis and graft concentrations and percentages.

Leukocytesubset	Prior to G-CSF (x 10^9/L)	During G-CSF (x 10^9/L)	P	Stem cellgraft (x 10^9/L)	P	Prior to G-CSF (%)	During G-CSF (%)	P	Stem cellgraft (%)	P
Neutrophils	3.4 (2.4-11.0)	36.8 (21.0-65.5)	0.00004	100.6 (29.6-234.0)	0.00005	60.5 (47.3-83.3)	84.9 (77.7-92.2)	0.00004	42.4 (13.8-68.8)	0.00004
Monocytes	0.5 (0.2-0.7)	1.9 (0.9-3.9)	0.00004	35.1 (5.5-75.6)	0.00004	7.1 (3.1-11.4)	4.6 (2.3-8.3)	0.001	16.2 (1.6-38.3)	0.00006
Lymphocytes	1.7 (0.9-2.8)	3.9 (2.4-6.5)	0.00004	78.1 (42.2-182.6)	0.00004	27.7 (8.3-42.1)	9.3 (4.4-13.2)	0.00005	36.6 (17.6-59.9)	0.00004
T-cells	1.25 (0.60-2.26)	2.92 (1.29-4.17)	0.00004	53.92 (23.72-145.71)	0.00009	73.3 (54.8-82.1)	69.4 (44.1-81.9)	NS	62.1 (45.9-85.2)	0.001
B-cells	0.15 (0.03-0.33)	0.50 (0.21-1.77)	0.00004	13.50 (3.12-26.46)	0.0001	8.4 (2.28-17.5)	10.8 (5.9-29.9)	0.0001	14.5 (6.1-36.5)	0.003
NK-cells	0.22 (0.05-0.50)	0.25 (0.07-0.68)	NS	4.46 (1.74-14.47)	0.00009	11.7 (3.0-30.9)	6.4 (1.5-15.8)	0.00006	5.6 (2.3-18.4)	0.023

Supplementary Table 4. The effect of stem cell mobilization of 22 healthy donors with G-CSF on the concentrations and percentages of T, B and NK cell subsets in peripheral blood and in the stem cell graft. In the left part of the table the results are presented as PB and graft concentrations (x 10^9/L), whereas in the right part the same values are presented as percentages of their parent populations acquired by flow cytometry. All values are given as medians with variation ranges in parentheses. The Wilcoxon's test for paired samples was used for comparison of pre-treatment and G-CSF treated/pre-apheresis concentrations and percentages.

Lymphoidcellsubsets	Immunophenotype	Prior to G-CSF	During G-CSF	P	Prior to G-CSF	During G-CSF	P	Stem cellgraft (x 10^9/L)	Stem cellgraft %
T cells									
T helpercells (T_H)	$CD4^+$	0.83 (0.39-1.37)	2.11 (0.92-3.47)	0.00004(↑)	67.3 (49.1-84.8)	74.5 (56.6-89.0)	0.001(↑)	41.10 (17.85-107.76)	73.7 (61.8-89.4)
Cytotoxic T cells (T_c)	$CD8^+$	0.29 (0.09-0.79)	0.58 (0.14-1.08)	0.0003(↑)	24.6 (11.6-46.3)	20.4 (8.9-35.8)	0.001 (↓)	10.85 (3.37-33.15)	22.7 (8.0-35.9)
Naïve T_H	$CD4^+45RA^+CCR7^+$	0.45 (0.13-0.95)	1.21 (0.34-2.05)	0.00004(↑)	48.4 (20.8-74.4)	53.6 (21.3-74.0)	0.0002(↑)	21.82 (7.30-60.24)	54.8 (35.0-73.5)
Central memory (T_{CM})	$CD4^+45RA^-CCR7^+$	0.20 (0.09-0.39)	0.37 (0.13-0.87)	0.00007(↑)	22.2 (15.0-40.7)	20.6 (12.1-36.9)	0.001 (↓)	7.38 (2.79-24.35)	19.4 (11.2-30.0)
Effectormemory (T_{EM})	$CD4^+45RA^-CCR7^-$	0.14 (0.06-0.28)	0.29 (0.08-0.72)	0.00004(↑)	17.4 (4.8-32.8)	14.2 (6.2-36.0)	0.015 (↓)	5.44 (4.01-13.36)	17.8 (7.9-26.2)
Terminallydifferentiated (T_{TD})	$CD4^+45RA^+CCR7^-$	0.05 (0.02-0.18)	0.11 (0.05-0.38)	0.00008(↑)	6.8 (3.0-22.2)	6.8 (2.4-17.8)	NS	3.09 (1.12-8.05)	8.5 (2.9-21.7)
	$CD4^+45RA^+CCR7^-$	0.02 (0.01-0.07)	0.05 (0.02-0.20)	0.00004(↑)	3.0 (0.6-6.6)	2.6 (1.4-5.7)	0.044 (↓)	0.82 (0.31-3.25)	2.0 (1.0-5.6)
Naïve T_c	$CD8^+45RA^+CCR7^+$	0.13 (0.04-0.36)	0.24 (0.06-0.66)	0.0002(↑)	45.2 (24.1-69.3)	52.3 (22.5-76.8)	0.003(↑)	5.77 (1.63-12.46)	47.6 (25.6-67.4)
Central memory	$CD8^+45RA^-CCR7^+$	0.023 (0.003-0.080)	0.030 (0.007-0.137)	0.004(↑)	6.6 (2.7-17.3)	6.8 (2.0-16.5)	NS	0.67 (0.08-3.03)	4.9 (2.0-12.4)
Effectormemory	$CD8^+45RA^-CCR7^-$	0.03 (0.01-0.10)	0.06 (0.01-0.17)	0.0002(↑)	10.8 (5.3-26.5)	11.8 (4.0-29.7)	NS	1.05 (0.52-3.85)	11.2 (4.4-26.4)
Effector (TEMRA)	$CD8^+45RA^+CCR7^-$	0.08 (0.02-0.41)	0.12 (0.02-0.36)	0.036(↑)	33.7 (13.9-53.1)	24.4 (13.1-50.7)	0.002 (↓)	2.93 (0.88-14.35)	26.0 (19.4-57.0)
	$CD8^+45RO^+CD26^{++}$	0.011 (0.001-0.088)	0.016 (0.002-0.101)	NS	4.1 (0.7-16.6)	3.0 (0.7-13.3)	0.006 (↓)	0.24 (0.02-1.83)	2.9 (0.3-8.3)
αβ T cells	$CD3^+TCR\alpha\beta^+$	1.18 (0.58-2.14)	2.76 (1.21-4.04)	0.00005(↑)	94.8 (76.6-98.9)	96.2 (86.0-98.8)	0.012(↑)	52.60 (20.63-140.47)	96.5 (86.2-98.4)
γδ T cells	$CD3^+4^-8^-TCR\gamma\delta^+$	0.048 (0.004-0.118)	0.046 (0.009-0.178)	0.017(↑)	3.0 (0.3-11.0)	1.9 (0.6-6.1)	0.0001 (↓)	1.15 (0.30-4.20)	2.0 (0.9-11.2)
Naïve T regulatorycells	$CD4^+25^+45RA^+FOXP3^+$	0.010 (0.003-0.042)	0.019 (0.007-0.124)	0.00008(↑)	1.1 (0.5-4.9)	1.0 (0.4-5.6)	NS	0.457 (0.165-1.817)	1.0 (0.5-6.5)
Effector Tregulatorycells	$CD4^+25^+45RA^-FOXP3^+$	0.030 (0.016-0.068)	0.071 (0.027-0.178)	0.00004(↑)	3.8 (1.6-7.0)	3.5 (2.2-6.9)	NS	1.268 (0.541-4.207)	3.4 (1.5-5.7)
Tr1	$CD4^+45RA^-49b^+LAG3^+$	0.006 (0.002-0.018)	0.011 (0.004-0.064)	0.003(↑)	1.4 (0.4-3.6)	1.3 (0.4-2.9)	NS	0.217 (<0.001-0.920)	1.2 (0.5-3.6)
B cells									
Transitional	$CD19^+24^{++}38^{++}$	0.005 (0.001-0.021)	0.013 (0.005-0.034)	0.00004(↑)	3.7 (1.2-10.6)	2.6 (1.5-5.6)	0.007 (↓)	0.311 (0.087-1.045)	3.0 (1.5-5.0)
Mature	$CD19^+24^{++}38^-$	0.023 (0.002-0.097)	0.059 (0.016-0.295)	0.00004(↑)	14.4 (6.2-50.0)	13.3 (4.7-35.9)	0.0003 (↓)	1.898 (0.319-9.011)	13.7 (5.2-42.9)

Memory	$CD19^{+}24^{+}38^{+}$	0.094 (0.022-0.274)	0.352 (0.147-1.471)	0.00004(↑)	67.6 (36.6-85.7)	76.3 (51.7-89.4)	0.00006(↑)	7.61 (1.52-19.09)	74.9 (44.6-90.5)
	$CD19^{+}27^{+}$	0.027 (0.003-0.131)	0.067 (0.018-0.459)	0.00004(↑)	20.4 (5.5-63.1)	14.4 (3.9-54.8)	0.00004 (↓)	1.055 (0.535-13.742)	13.0 (3.5-52.0)
$IL\text{-}2R^{+}$	$CD19^{+}25^{+}$	0.002 (<0.001-0.017)	0.002 (0.001-0.043)	NS	1.4 (0.4-7.5)	0.6 (0.1-5.0)	0.0004 (↓)	0.065 (0.012-1.028)	0.6 (0.1-3.9)
$IL\text{-}2R^{w}$	$CD19^{+}25^{w}$	0.017 (0.002-0.070)	0.031 (0.007-0.228)	0.0001(↑)	10.3 (5.5-33.8)	7.4 (2.9-27.2)	0.00005 (↓)	0.671 (0.196-6.422)	6.8 (3.0-24.3)
NK cells									
Cytolytic	$CD56^{+}16^{++}$	0.191 (0.025-0.447)	0.201 (0.025-0.521)	NS	38.9 (5.0-73.3)	17.9 (2.0-53.5)	0.00008 (↓)	3.901 (0.882-11.986)	12.5 (3.2-52.9)
Cytokineproducing	$CD56^{++}16^{+}$	0.018 (0.006-0.038)	0.029 (0.005-0.230)	0.001(↑)	3.4 (1.3-13.9)	2.3 (0.3-12.7)	NS	0.619 (0.261-2.117)	2.2 (0.5-7.2)
Invariant NKT (iNKT)	$CD3^{+}V\alpha24^{+}$	0.003 (0.001-0.022)	0.003 (0.001-0.023)	NS	0.26 (0.05-1.28)	0.16 (0.02-1.03)	0.016 (↓)	0.089 (0.006-2.147)	0.15 (0.02-6.86)

(↑) = significant increased concentration, (↓) = significant decreased concentration, NS = non-significant

Supplementary Table 5: The effect of stem cell mobilization with G-CSF on the intracellular cytokine concentrations in lymphoid cells from healthy donors (n = 22). From left to right, the results for T helper cells (T_H), T cytotoxic cells (T_C), $CD3^+4^-8^-$ T cells, B cells and $CD3^-19^-$ cells are presented. For each cytokine the median untreated concentrations (x 10^9/L) of positive cells are shown together with the concentrations during G-CSF treatment. On the line below the same values are presented as median percentages of their parent populations acquired by flow cytometry.The Wilcoxon's test for paired samples was used for comparison of pre-treatment and G-CSF treated/pre-apheresis concentrations and percentages.

	T_H cells			**T_c cells**			**$CD3^+4^-8^-$ T cells**			**B cells**			**$CD3^-19^-$cells**		
Cytokine	**Prior to G-CSF**	**During G-CSF**	**P**	**Prior to G-CSF**	**During G-CSF**	**P**	**Prior to G-CSF**	**During G-CSF**	**P**	**Prior to G-CSF**	**During G-CSF**	**P**	**Prior to G-CSF**	**During G-CSF**	**P**
IFNɣ	0.157/ 20.6	0.374/ 20.6	0.00004 (↑)/ NS	0.154/ 60.4	0.302/ 56.2	0.001 (↑)/ 0.018 (↓)	0.030/ 69.2	0.040/ 61.3	0.022 (↑)/ 0.001(↓)	0.0013/ 1.9	0.0036/ 1.1	0.002 (↑)/ 0.025(↓)	0.0789/ 41.8	0.0776/ 32.1	NS/ 0.002 (↓)
IL10	0.0042/ 0.5	0.0100/ 0.7	0.00004 (↑)/ 0.006 (↑)	0.0010/ 0.64	0.0019/ 0.8	0.000061 (↑)/ 0.029 (↑)	0.0001/ 0.4	0.0003/ 0.4	0.001 (↑)/ NS	0.0012/ 6.7	0.0041/ 4.4	0.001 (↑)/ NS	0.0005/ 0.3	0.0010/ 0.4	0.005 (↑)/ 0.004 (↑)
TGFβ	0.178/ 24.1	0.367/ 24.2	0.000061 (↑)/ NS	0.112/ 43.94	0.231/ 40.3	0.004 (↑)/ NS	0.0150/ 50.9	0.0297/ 48.9	0.024 (↑)/ NS	0.0130/ 10.5	0.0249/ 6.5	0.000295 (↑)/ 0.001 (↓)	0.0043/ 3.9	0.0033/ 2.4	0.022 (↑)/ 0.003 (↓)
IL4	0.0175/ 2.7	0.0322/ 2.0	0.000367 (↑)/ NS	0.0053/ 2.8	0.0070/ 2.2	NS/ 0.039 (↓)	0.0002/ 1.0	0.0002/ 0.8	NS/ NS	0.0013/ 2.0	0.0049/ 1.8	0.000069 (↑) NS	0.0039/ 2.9	0.0078/ 4.6	0.003 (↑)/ 0.030 (↑)
IL9	0.0048/ 1.1	0.0152/ 1.1	0.001 (↑)/ 0.024 (↑)	0.0112/ 4.8	0.0107/ 4.3	0.011 (↓) / NS	0.0025/ 16.6	0.0055/ 14.3	NS/ NS	0.0011/ 1.0	0.0012/ 0.4	NS/ 0.001(↓)	0.0925/ 50.0	0.1086/ 40.3	NS/ 0.011 (↓)
IL17	0.0091/ 1.2	0.0269/ 1.5	0.000046 (↑)/ NS	0.0008/ 0.3	0.0014/ 0.5	0.005 (↑)/ NS	0.0001/ 0.5	0.0004/ 0.8	0.007 (↑)/ 0.016 (↑)	0.0001/ 0.1	0.0003/ 0.1	0.001 (↑)/ NS	0.0001/ 0.04	0.0004/ 0.04	NS/ NS
IL22	0.0153/ 2.6	0.0211/ 1.6	NS/ NS	0.0033/ 1.1	0.0034/ 0.9	NS/ 0.050 (↓)	0.0001/ 0.4	0.0001/ 0.3	NS/ NS	0.0010/ 1.1	0.0030/ 0.9	0.000187 (↑)/ NS	0.0006/ 0.4	0.0006/ 0.4	NS/ NS

(↑) = significant increased concentration, (↓) = significant decreased concentration, NS = non-significant

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STEM CELL MOBILIZATION

The healthy donor profile of immunoregulatory soluble mediators is altered by stem cell mobilization and apheresis

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Abstract

Background. Peripheral blood stem cells from healthy donors mobilized by granulocyte colony-stimulating factor (G-CSF) and thereafter harvested by leukapheresis are commonly used for allogeneic stem cell transplantation. *Methods.* Plasma levels of 38 soluble mediators (cytokines, soluble adhesion molecules, proteases, protease inhibitors) were analyzed in samples derived from healthy stem cell donors before G-CSF treatment and after 4 days, both immediately before and after leukapheresis. *Results.* Donors could be classified into two main subsets based on their plasma mediator profile before G-CSF treatment. Seventeen of 36 detectable mediators were significantly altered by G-CSF; generally an increase in mediator levels was seen, including pro-inflammatory cytokines, soluble adhesion molecules and proteases. Several leukocyte- and platelet-released mediators were increased during apheresis. Both plasma and graft mediator profiles were thus altered and showed correlations to graft concentrations of leukocytes and platelets; these concentrations were influenced by the apheresis device used. Finally, the mediator profile of the allotransplant recipients was altered by graft infusion, and based on their day +1 post-transplantation plasma profile our recipients could be divided into two major subsets that differed in overall survival. *Discussion.* G-CSF alters the short-term plasma mediator profile of healthy stem cell donors. These effects together with the leukocyte and platelet levels in the graft determine the mediator profile of the stem cell grafts. Graft infusion also alters the systemic mediator profile of the recipients, but further studies are required to clarify whether such graft-induced alterations have a prognostic impact.

Key Words: *allogeneic stem cell transplantation, apheresis, chemokine, granulocyte colony-stimulating factor, hematopoietic stem cell mobilization, interleukin, peripheral blood stem cell grafts, plasma profile, protease, soluble adhesion molecule*

Introduction

Allogeneic stem cell grafts from both bone marrow and peripheral blood allografts contain hematopoietic stem cells as well as large populations of immunocompetent cells and platelets [1]. Previous studies have demonstrated that T-cell graft depletion reduced the risk of severe graft-versus-host disease (GVHD) but increased the risk of leukemia relapse and graft failure [2–5]. Thus, the risk of immune-dependent post-transplantation complications was dependent on the number of graft immunocompetent cells and especially the number of T cells. One would therefore expect an increased frequency and/or severity of GVHD when using peripheral blood stem cells (PBSCs) mobilized by granulocyte colony-stimulating factor (G-CSF) because such grafts contain high T-cell numbers [6]. However, the incidence of acute GVHD after allogeneic peripheral blood stem cell transplantation (PBSCT) has been reported to be lower than expected [7–9], an observation indicating that T cells in blood grafts differ from bone marrow

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grafts. The plasma levels of soluble mediators may then reflect a G-CSF–induced immunomodulation that could involve the graft immunocompetent cells and thereby be important for the risk of post-transplantation immune-mediated complications. It is not known whether such G-CSF effects can influence donor health. A recent study showed that G-CSF therapy in healthy stem cell donors induced changes in the $CD34^+$ cell expression of more than 2000 genes and microRNAs involved in regulation of cell cycle progression, proliferation, angiogenesis and immune responses [10]. These changes increased during the first 30 days after G-CSF treatment, and lasted for at least 1 year. G-CSF also alters the short-term systemic metabolic regulation [11]. We previously described altered cytokine levels in autologous donors after both hematopoietic stem cell mobilization and apheresis [12,13] and an association between the pretransplantation serum cytokine profile of allotransplant recipients and risk of post-transplantation complications [14]. Taken together, these observations suggest that early cytokine-mediated effects are important for outcome after allotransplantation and donor immunoregulation seems to have an additional impact on patient outcome [15–19]. In this context we have examined the effects of G-CSF treatment and stem cell harvesting on plasma levels of immunoregulatory soluble mediators, the levels of these mediators in allogeneic stem cell grafts and the effects of stem cell infusion on the recipient cytokine network.

Materials and methods

Healthy allogeneic stem cell donors and allotransplant recipients

All studies were approved by the local ethics committee (REK III No.126.01, Regional Committee for Medical and Health Research Ethics of Western Norway: 2011/996, 2011/1237, 2011/1241 and 2013/634). The participants were included after signing a written informed consent. The present study includes 25 consecutive healthy HLA-matched related allogeneic stem cell donors, 16 males and 9 females with median age 54 years (range, 25–77 years), and 16 allogeneic stem cell transplant recipients, 7 males and 9 females with median age 47 years (range, 35–63 years).

Stem cell mobilization and harvesting in the healthy donors

The matched related donors received the stem cell mobilizing agent human non-glycosylated G-CSF 10 µg/kg/d for 4 days prior to stem cell harvesting. Stem cell quantification started on day 4 of G-CSF stimulation, and harvesting was performed when the stem cell count exceeded 15–20 x 10^3/mL. The first nine stem cell donors were harvested by large-volume leukapheresis with four times processing of the total blood volume, using the Mononuclear Cell Removal-protocol with the WBC kit for the Cobe Spectra cell separator version 7 (Cobe Laboratories). During the study the apheresis devices of our department were replaced due to timely equipment upgrade; hence the mononuclear cell (MNC) procedure with the Spectra Optia Collection Set on the Spectra Optia cell separator version 9 (Terumo BCT Inc.) was used for the 16 last donors.

Allogeneic stem cell transplantation

Eleven of the 16 allotransplant recipients were diagnosed with acute myeloid leukemia (AML), three with acute B cell lymphoblastic leukemia (B-ALL), one with myelofibrosis and one with myelodysplastic syndrome (MDS). At the time of transplantation, all leukemia patients were in complete hematologic remission; 14 patients received myeloablative conditioning with intravenous busulfan plus cyclophosphamide, and two patients received reduced intensity conditioning with intravenous fludarabine plus busulfan. All allotransplant recipients received G-CSF mobilized peripheral blood stem cell grafts derived from HLA-matched family donors and GVHD prophylaxis with cyclosporine A plus methotrexate. Neutrophil counts exceeding 0.5 x 10^9/L and stable platelet counts exceeding 50 x 10^9/L without platelet transfusions for at least 3 consecutive days were defined as neutrophil and platelet reconstitution, respectively.

Preparation of plasma and stem cell graft supernatant samples

Venous blood samples from the allogeneic stem cell donors were collected (i) at the time of the pretransplantation evaluation prior to G-CSF treatment, median 20.5 days before apheresis, (ii) during G-CSF therapy in the morning immediately before apheresis, (iii) immediately after apheresis and (iv) approximately 24 h after start of apheresis. From the allotransplant recipients venous blood samples were collected (i) between 0700 and 0900 AM the day of transplantation, (ii) between 0700 and 0900 AM the day after stem cell infusion and (iii) between 0700 and 0900 AM approximately 1 week after allogeneic stem cell transplantation (median, 6 days; variation range, 4–13 days). All venous blood samples were collected into Vacuette 9NC tubes with sodium citrate and acid-citrate-dextrose solution A (Greiner Bio-One GmbH). Plastic tubes without additives were used for samples from stem cell allografts. All blood and graft samples were centrifuged at 1310*g* for 10 min at room temperature within 30 min of sampling. The plasma

supernatants were immediately transferred to plastic tubes, frozen and stored at -70°C until analyzed.

Plasma mediator levels

The concentrations of the following 38 mediators were determined using Luminex analyses (R&D Systems): (i) the immunomodulatory cytokines interferon-γ (IFN-γ), CD40 ligand (CD40L) and tumor necrosis factor-α (TNF-α); (ii) the interleukins IL1-β, IL-6, IL-8/CXCL-8, IL-10, IL-12 and interleukin 1 receptor antagonist (IL-1 RA); (iii) the chemokines CCL-2/4/5/11 and CXCL-5/10/11; (iv) the growth factors G-CSF, granulocyte macrophage colony-stimulating factor (GM-CSF), vascular endothelial growth factor (VEGF), thrombopoietin (TPO), hepatocyte growth factor (HGF) and leptin; (v) the soluble adhesion molecules P-selectin, E-selectin, intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1); (vi) the matrix metalloproteases MMP-1, MMP-2, MMP-3, MMP-7, MMP-8, MMP-9, MMP-12 and MMP-13 and (vii) the tissue inhibitors of metalloproteases 1-4; TIMP-1, TIMP-2, TIMP-3 and TIMP-4.

Bioinformatics and statistical analyses

Bioinformatics analyses were performed using J-Express (MolMine AS) [20]. All values were median normalized and log-2 transformed before performing the unsupervised hierarchical clustering analyses. Complete linkage was used as linkage method and the Pearson correlation for distance measure. In unsupervised hierarchical clustering with complete linkage a multivariate dataset is divided into related groups based on similarities between objects without prior information about group similarity patterns. The data are presented as a binary tree, which is shaped as a hierarchy of nested subsets with the most similar patterns situated in closest proximity to each other. The linkage method describes the calculation of the distance between two objects in this hierarchy; in complete linkage the maximum distance possible between the objects is used [21–23].

Additional statistical analyses were performed using the standard computer software package IBM SPSS Statistics 22 (IBM Corporate). The Wilcoxon test for paired samples was applied for analyses of paired observations, and the Independent-Samples Mann-Whitney *U* test and the chi-square test were used for comparison of groups. The Kendall tau-b test was used for analysis of correlations between continuous variables, and the Kaplan-Meier method with two-sided log-rank statistics was used for estimation of survival curves. Variables assumed to have a prognostic impact on survival were further analyzed using the Cox proportional hazards model [24].

Results

The plasma mediator concentrations of healthy donors are altered during stem cell mobilization and apheresis

We compared the plasma concentrations prior to and during G-CSF treatment for each individual mediator (Figure 1; Table I). Plasma levels of IL-12 and GM-CSF showed undetectable levels for all donor samples at all time points and were therefore omitted from all statistical analyses. G-CSF administration caused a significant alteration of the plasma levels of 17 mediators. Fourteen of the 35 detectable mediators showed increased concentrations during mobilization, and the most significant increases were detected for TNFα, IL-1 RA, IL-6, IL-10, CCL4, E-Selectin, VCAM-1, ICAM-1 and MMP-8 (Table I; Figure 1). MMP-3, TIMP-2 and TIMP-4 showed significantly decreased levels during G-CSF treatment (Figure 1; Table I).

Plasma mediator concentrations were thereafter compared before and after leukapheresis; 19 mediators then showed decreased levels post-apheresis, whereas three exceptional mediators (IL-6, CXCL10 and leptin) showed increased levels (Figure 1; Supplementary Table S1). However, it should be emphasized that the differences in median levels were relatively small compared with the G-CSF–induced alterations (Figure 1; Table I) and the wide variation between donors was maintained following apheresis.

Healthy stem cell donors can be subclassified prior to G-CSF treatment based on their plasma mediator profiles

We noticed a substantial variation in plasma mediator levels between donors both prior to and during G-CSF treatment. We first performed a hierarchical clustering analysis including all the donors based on the levels of mediators measured before G-CSF treatment. As shown in Figure 2A, the donors could be divided into an upper main cluster with 12 donors and a lower main cluster with 13 donors, and the 36 detectable mediators also formed two main horizontal clusters (Figure 2A).

The two main donor clusters (Figure 2A, left side of the figure) were compared with regard to the levels of each single mediator. Sixteen mediators showed significant differences between the two donor clusters; CD40L ($P < 0.000005$), MMP-13 ($P < 0.0005$), IL-8 ($P < 0.0005$), HGF ($P < 0.001$), TIMP-3 ($P < 0.001$), MMP-1, MMP-2, TPO, CXCL11, CCL11 and IFN-γ ($P < 0.005$) and CXCL5, CCL4, MMP-12, IL-1 RA and G-CSF ($P < 0.05$). All these mediators belonged to the left mediator cluster (Figure 2A, top of the figure), whereas none of the 16 mediators in the right cluster differed significantly between the two main donor clusters (chi-square test; $P = 0.000002$).

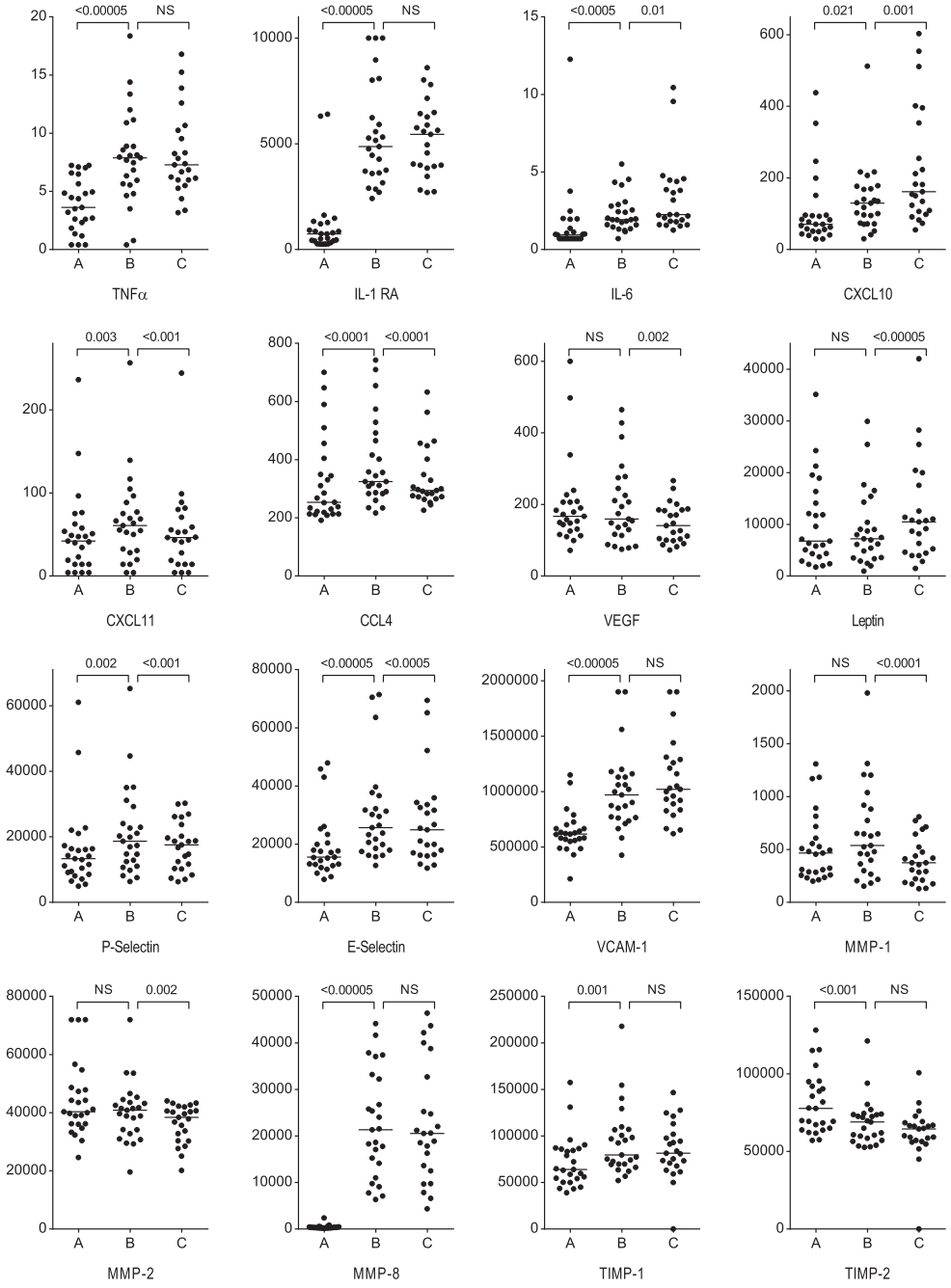

Figure 1. The effect of stem cell mobilization and apheresis on healthy donor plasma levels (pg/mL) of representative soluble mediators from each mediator group (Table I and Supplementary Table S1). Mediator levels were determined (A) prior to treatment with G-CSF, (B) after stem cell mobilization (immediately before apheresis) and (C) immediately after apheresis. The individual values and medians (solid line) for 25 consecutive healthy donors prior to G-CSF and immediately before apheresis and 23 donors immediately after apheresis are presented.

Table I. Median plasma mediator levels for the 25 allogeneic stem cell donors during stem cell mobilization.

Mediator	Prior to G-CSF	During G-CSF	G-CSF effect (*P*)
Immunomodulatory cytokines			
IFNγ	3.7 (<2.2–34.7)	2.8 (<2.2–25.5)	NS
CD40L	1361 (<998–10 1562)	1309 (<998–95 487)	NS
TNFα	3.6 (<0.8–7.2)	7.9 (<0.8–18.4)	0.000016 (↑)
Interleukins			
IL-1 RA	741.1 (248.1–6400)	4867 (2415– > 7528)	0.000012 (↑)
Il-1β	<0.3 (<0.3–1.0)	<0.3 (<0.3–0.9)	NS
IL-6	0.9 (<0.9–12.3)	1.9 (<0.9–5.5)	0.000296 (↑)
IL-8 (CXCL-8)	15.7 (<8.3–146.5)	14.3 (<8.3–149.5)	NS
IL-10	<0.5 (<0.5–7.9)	0.51 (<0.5–12.8)	0.000219 (↑)
Chemokines			
CXCL5 (ENA-78)	170.2 (<61.1–2384)	155.9 (<61.1–3220)	NS
CXCL10 (IP-10)	70.9 (29.3–438.2)	129.9 (30.0–512.3)	0.021 (↑)
CXCL11 (I-TAC)	42.1 (<17.0–236.3)	58.1 (<17.0–256.6)	0.003 (↑)
CCL2 (MCP-1)	267.1 (93.7–437.0)	240.9 (89.4–477.8)	NS
CCL4 (MIP-1β)	253.4 (191.4–700.5)	324.5 (216.6–742.5)	0.00009 (↑)
CCL5 (RANTES)	>6000 (923.5– > 6000)	4120.9 (885.9– > 6000)	NS
CCL11 (Eotaxin)	292.1 (<124.6–1580)	279.2 (<124.6–1977)	NS
Growth factors			
TPO	1324.5 (<378.2– > 109,611)	1394.7 (<378.2– > 109,611)	NS
VEGF	166.8 (71.7–600.0)	159.4 (74.8–464.8)	NS
HGF	202.5 (<41.6–1518)	260.6 (<41.6–1459)	NS
Leptin	6760 (1747–35135)	7216 (966.1–29917)	NS
G-CSF	46.9 (<29.9–241.7)	>7425 (3687–14178)	0.000012 (↑)
Adhesion molecules			
P-Selectin	13 322 (4953–61 068)	18 663 (6358.1–65 218)	0.002 (↑)
E-Selectin	15 575 (7920–47 953)	25 661 (12 691–71 422)	0.000012 (↑)
VCAM-1	617,232 (212,562–1,150,000)	970,313 (425,794– > 1,807,879)	0.000012 (↑)
ICAM-1	184,793 (55,037–1,310,000)	236,484 (119,891–1,470,000)	0.000012 (↑)
Matrix metalloproteases			
MMP-1	465.4 (201.5–1309)	538.1 (153.3–1979)	NS
MMP-2	40 364 (24 626– > 58 201)	40 851 (19 616– > 58 201)	NS
MMP-3	8289 (3417– > 8924)	6508 (2487– > 8924)	0.004 (↓)
MMP-7	3711 (1190–7353)	3468 (630.0–7624)	NS
MMP-8	332.7 (181.6–2407)	21 316 (6354.4–44 166)	0.000012 (↑)
MMP-9	4148.0 (1773–12111)	7386.7 (3276– > 37370)	0.000296 (↑)
MMP-12	<29.8 (<29.8–69.7)	<29.8 (<29.8–109.3)	NS
MMP-13	309.1 (<220.4 -1872)	392.8(<220.4–2031)	NS
Metalloprotease inhibitors			
TIMP-1	64 100 (39 044–157,544)	79 591 (52 049.5–217,884)	0.001 (↑)
TIMP-2	77 727 (57 078–128,221)	69 037 (52 632–121,228)	0.000403 (↓)
TIMP-3	15 308 (<3897–34 409)	16 055 (<3897–37 381)	NS
TIMP-4	1275 (699.2–2097)	1129.3 (574.7–2199)	0.005 (↓)

All mediator concentrations are given in pg/mL and presented as median values with variation ranges given in parentheses. IL-12 and GM-CSF showed undetectable levels and were not included in the table. Untreated mediator levels are compared with concentrations after G-CSF treatment (Wilcoxon test for paired samples).
NS, not significant; (↑), increased level; (↓), decreased level.

There were no significant differences between the two main donor clusters with respect to age, gender, weight, height, HLA-A, B or DR phenotype, processed blood volume, later stem cell mobilization or yield, pretreatment hemoglobin values and peripheral blood leukocyte/platelet counts (data not shown).

The plasma mediator profile of healthy stem cell donors is altered by in vivo G-CSF treatment

We then did a clustering analysis of the mediator levels after G-CSF treatment (Figure 2B). G-CSF mainly altered the levels of mediators that were previously shown to not differ significantly between the two main donor clusters identified before G-CSF therapy (Figure 2A, four of 20 versus 13 of 16; chi-square test, $P < 0.0005$).

Furthermore, 15 of the 35 mediators differed significantly between the two main donor clusters identified during G-CSF treatment (Figure 2B); 12 of these 15 mediators also differed significantly between the two main donor clusters identified before G-CSF (Figure 2A). Thus, differences between donors with regard to their plasma mediator profile are maintained

during G-CSF therapy, and this constancy of relative concentrations of single mediators between donors can probably explain why most of the donors and most of the mediators that cluster in each of the pretherapy groups also cluster close to each other even after G-CSF treatment (Figure 2B).

The mediators that were most significantly altered between the two donor clusters during G-CSF therapy were CD40L ($P < 0.00005$), IL-8 ($P < 0.00005$), MMP-13 ($P < 0.0001$), TPO ($P < 0.001$) and HGF ($P < 0.001$), all belonging to the same cytokine sub cluster (Figure 2B). Finally, in the upper donor cluster, the median stem cell yield was 3.4 x 10^6 $CD34^+$ cells per kg donor-weight (n = 15; range, 0.8–22.4), whereas for the lower cluster it was 5.8 x 10^6 $CD34^+$ cells per kg (n = 10; range, 3.1–15.1; $P = 0.026$; Mann-Whitney *U* test). The other clinical variables mentioned in the section above did not differ between these two donor clusters.

Stem cell harvesting causes a further modulation of the plasma mediator profile–effects of apheresis device

We also performed an unsupervised hierarchical clustering analysis for plasma mediator levels immediately after apheresis (Supplementary Figure S1), and the cluster results differed from those seen during G-CSF treatment immediately before apheresis (Figure 2B). Still, two main donor clusters were observed after clustering, and 20 mediators differed significantly between these two clusters; the most significant differences were observed for TPO ($P < 0.000005$), IL-8 ($P < 0.00005$), CCL11 ($P < 0.00005$) and CD40L ($P < 0.0005$). All but one of the nine donors harvested with Cobe Spectra clustered together in the upper main cluster. Thus, there was a significant association between apheresis device used and plasma mediator profile ($P < 0.0005$; Pearson chi-square).

The soluble mediator profiles in the stem cell graft supernatants are associated with the preharvesting plasma cytokine profile, the graft levels of platelets and leukocytes and the apheresis device

Samples from the graft supernatants were available for 22 donors. The majority of mediators showed increased levels in graft supernatants compared with the plasma concentrations before and/or during G-CSF treatment, especially IL-1 RA, HGF, leptin, MMP-8 and MMP-9 (Supplementary Table S2). Levels were decreased for IL-6, CCL2, ICAM-1 and MMP-3 compared with pre-apheresis plasma concentrations. We also did a hierarchical clustering analysis of the graft supernatant concentrations (Figure 3); the grafts/donors could then be divided into two main clusters with 13 and nine grafts, respectively. For 15 of the 35 mediators, the corresponding graft supernatant mediator concentrations differed significantly between these two graft clusters: CD40L ($P = 0.036$), TNFα ($P = 0.001$), IL-1β ($P = 0.006$), CXCL10 ($P = 0.025$), CCL11 ($P = 0.043$), TPO ($P < 0.0005$), VEGF ($P = 0.036$), HGF ($P < 0.00005$), leptin ($P = 0.043$), P-Selectin ($P = 0.011$), ICAM ($P = 0.007$), MMP-8 ($P = 0.001$), MMP-9 ($P < 0.0005$), TIMP-1 ($P = 0.006$) and TIMP-2 ($P < 0.000005$).

As shown in Figure 3, the nine grafts harvested with Cobe Spectra and all 12 grafts harvested with Spectra Optia clustered together/close to each other ($P = 0.001$, Pearson chi-square). Grafts collected with Cobe Spectra and Spectra Optia also differed significantly with regard to the levels of two immunoregulators (TNFα $P < 0.00001$; IL-1β $P < 0.000005$), several chemokines (CXCL5 $P = 0.003$; CXCL10 $P = 0.004$; CCL4 $P = 0.036$) and growth factors (TPO $P = 0.001$; VEGF $P < 0.0005$; HGF $P < 0.00001$), soluble P-Selectin ($P = 0.001$) as well as several MMPs (MMP-7 $P < 0.0005$; MMP-8 $P = 0.001$; MMP-9 $P = 0.004$) and their inhibitors (TIMP-1 $P < 0.0005$; TIMP-2 $P < 0.00005$).

The concentrations of white blood cells and platelets in the stem cell graft were dependent on the apheresis device (Table II). The graft leukocyte concentration was positively correlated with the graft levels of IL-1β ($r = 0.707/P < 0.00005$), TIMP-2 (0.550/ < 0.0005), HGF (0.541/ < 0.0005), TNFα and TIMP-1 (0.506/0.001), MMP-8 (0.498/0.003) and VEGF (0.472/0.002), and the graft platelet concentration correlated positively to VEGF (0.524/0.001), CXCL5 (0.498/0.001) and P-Selectin (0.455/0.003) for the 22 available graft samples.

Donor mediator levels are not normalized 24 h after finalized G-CSF treatment

The desired stem cell dose was achieved after 1 day of apheresis for 10 donors and therefore G-CSF treatment was ended. This group of good mobilizers was younger than the donors needing two apheresis to achieve the target dose (Supplementary Table S3). Plasma samples were collected from both groups 24 h after start of the first apheresis (ongoing versus ended G-CSF treatment), and the plasma mediator levels did not differ between the groups except for CXCL10 and HGF (Supplementary Table S3). We also did a hierarchical clustering of the mediator levels in 24 h post-apheresis samples derived from the 10 good mobilizers; the clustering showed that the mediator profiles had not normalized compared with the profiles found before G-CSF treatment and a significant association between levels of mediators and the apheresis

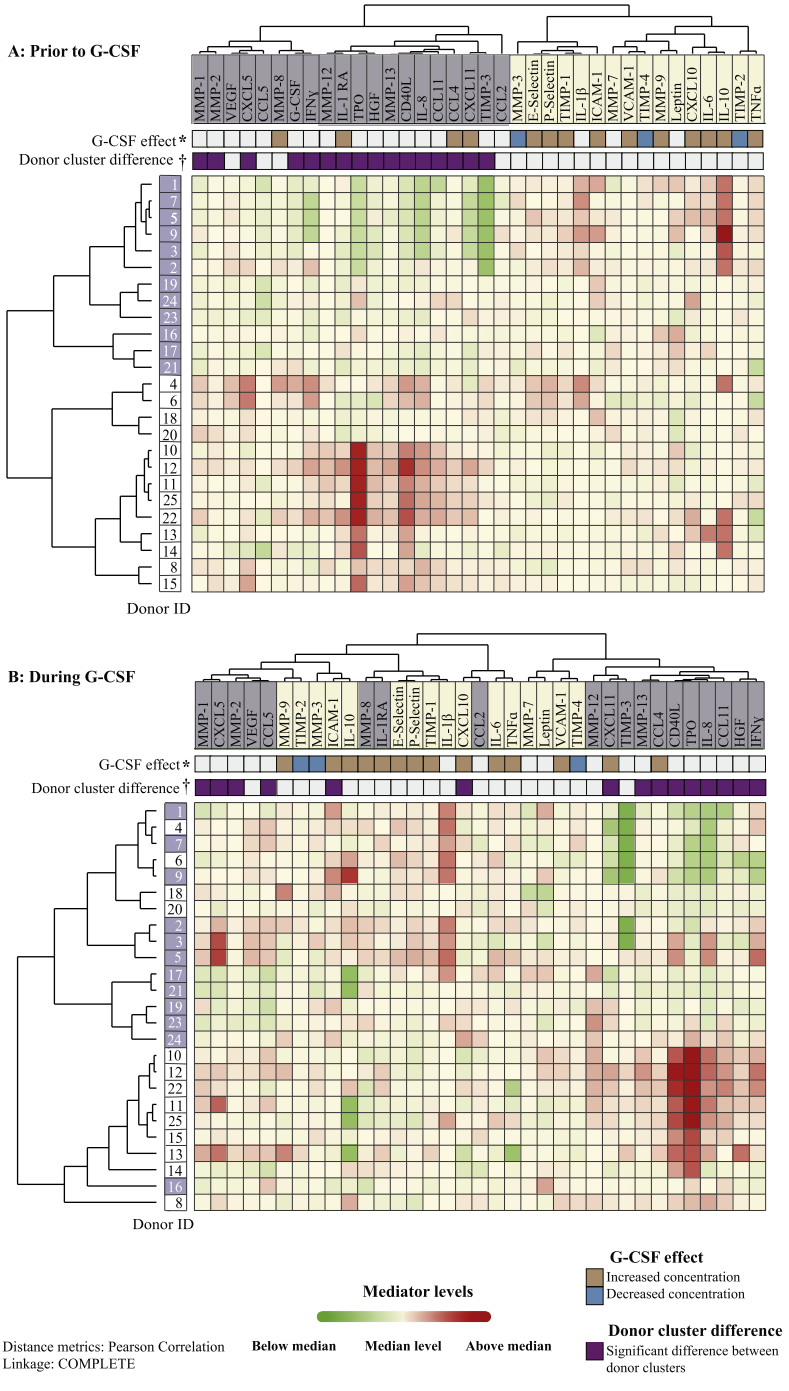
A: Prior to G-CSF
MMP-1
MMP-2
VEGF
CXCL5
CCL5
MMP-8
G-CSF
IFNγ
MMP-12
IL-1 RA
TPO
HGF
MMP-13
CD40L
IL-8
CCL11
CCL4
CXCL11
TIMP-3
CCL2
MMP-3
E-Selectin
P-Selectin
TIMP-1
IL-1β
ICAM-1
MMP-7
VCAM-1
TIMP-4
MMP-9
Leptin
CXCL10
IL-6
IL-10
TIMP-2
TNFα
G-CSF effect *
Donor cluster difference †
1 7 5 9 3 2 19 24 23 16 17 21 4 6 18 20 10 12 11 25 22 13 14 8 15
Donor ID
B: During G-CSF
MMP-1
CXCL5
MMP-2
VEGF
CCL5
MMP-9
TIMP-2
MMP-3
ICAM-1
IL-10
MMP-8
IL-1RA
E-Selectin
P-Selectin
TIMP-1
IL-1β
CXCL10
CCL2
IL-6
TNFα
MMP-7
Leptin
VCAM-1
TIMP-4
MMP-12
CXCL11
TIMP-3
MMP-13
CCL4
CD40L
TPO
IL-8
CCL11
HGF
IFNγ
G-CSF effect *
Donor cluster difference †
1 4 7 6 9 18 20 2 3 5 17 21 19 23 24 10 12 22 11 25 15 13 14 16 8
Donor ID
G-CSF effect
Increased concentration
Decreased concentration
Mediator levels
Below median
Median level
Above median
Donor cluster difference
Significant difference between donor clusters
Distance metrics: Pearson Correlation
Linkage: COMPLETE

Figure 2. Unsupervised hierarchical clustering analysis based on the pretreatment plasma levels of 36 detectable mediators in 25 allogeneic stem cell donors (A). All color schemes are explained in the lower part of the figure. Each figure shows the heat map with corresponding dendrograms. Red color in the donor/mediator columns of the heat maps indicates levels higher than the corresponding median level for each mediator, whereas green color indicates low levels compared with the median. G-CSF concentrations were included in the analysis prior to but not after injections of recombinant human G-CSF. (A/Upper) This part of the figure shows the clustering analysis of plasma mediator levels before G-CSF therapy. The horizontal mediator clustering into two main clusters marked with different colors (dark grey and yellow) is presented at the top of the figure, whereas the column to the left of the heat map presents the two main donor clusters marked with different colors (blue and white) and with the donor identification/number within each square. (B/Lower) This figure presents the unsupervised hierarchical clustering analysis of soluble mediator levels after treatment with G-CSF. The clustering of both mediators (horizontal, upper part) and donors (vertical, left column) was altered by G-CSF. The dark grey and yellow coloring of the mediators at the top of figure B indicates the mediator clustering from Figure 2A (pretreatment samples), whereas the column to the left of the heat map presents the two main donor clusters identified in Figure 2A marked with different colors (pigeon grey and white) and with the donor identification/number within each square. *G-CSF effect. The upper horizontal row in Figures A and B between the heat map and the mediator clustering indicate the G-CSF effect on the plasma concentration of each individual mediator. Significant increased (Wilcoxon test for paired samples, beige color) and decreased mediator levels (turquoise color) during G-CSF therapy are indicated. †Donor cluster difference. The lower horizontal rows of Figures A and B between the heat map and the mediator clustering indicates single mediators that differed significantly between the two donor clusters; they are marked with purple color. Mediators that differed significantly between the donor clusters identified both prior to and after G-CSF are marked with an X in Figure B. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

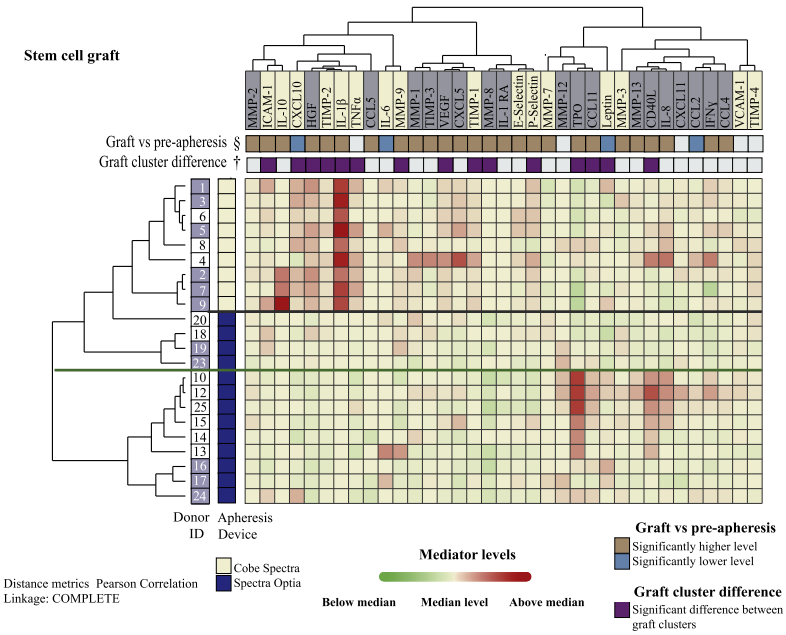

Figure 3. Unsupervised hierarchical cluster analyses based on mediator concentrations in the graft supernatant. All color schemes are explained in the lower part of the figure. The dendrogram showing the horizontal mediator clustering is presented at the top of the heat map, and the vertical donor clustering is shown to the left of the dendrogram. Red color in the patient/mediator columns indicates levels higher than the corresponding median level for each mediator, whereas green color indicates low levels compared with the median. The green horizontal line indicates the border between the two donor clusters, and the black line marks the border between the donor groups harvested with Cobe Spectra and Spectra Optia. Two columns to the left are marked with Donor ID and Apheresis Device, respectively. These two columns indicate (i) the two main donor clusters from the analysis of pretreatment samples (Figure 2A/upper; grey and white color), and (ii) the apheresis device used for each donor (Cobe Spectra in yellow, and Spectra Optia in blue). §Graft versus pre-apheresis. This upper horizontal row between the heat map and the mediator clustering indicates the mediator levels in the graft supernatant compared with pre-apheresis plasma levels; beige color indicates significantly higher levels and turquoise color lower levels in the graft (Wilcoxon test for paired samples; Supplementary Table S2). †In this lower horizontal row single mediators differing significantly between the two graft clusters are marked with purple color. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table II. Comparison of apheresis procedure characteristics, graft cellular concentrations and decrements of peripheral blood cell concentrations for Cobe Spectra (n = 9) and Spectra Optia (n = 13).

	Cobe Spectra (n = 9)	Spectra Optia (n = 13)	*P*
Apheresis variables			
Number of TBV processed	4.7 (3.0–7.0)	3.1 (2.0–4.0)	<0.00001
Graft volume (mL)	378 (294–463)	327 (146–536)	NS
Apheresis time (min)	300 (231–360)	322 (221–377)	NS
Graft components			
$CD34^+$ stem cells (10^9/L)	1.2 (0.6–5.4)	1.0 (0.3–2.4)	NS
Total leukocytes (10^9/L)	304 (226–418)	189 (130–253)	<0.000005
Neutrophils (10^9/L)	131 (53–234)	70 (30–120)	<0.001
Monocytes (10^9/L)	39 (6–112)	28 (3–46)	NS
Lymphocytes (10^9/L)	126 (56–183)	64 (37–113)	<0.0005
Platelets (10^9/L)	2509 (1200–3753)	1444 (689–2237)	0.003
Red blood cell volume (mL)	9.7 (3.5–28.6)	3.0 (1.5–16.0)	<0.001
Apheresis-induced decrements (PB)			
Total leukocytes (10^9/L)	12.1 (1.5–20.0)	3.8 (-7.2–10.4)	0.002
Neutrophils (10^9/L)	7.4 (-1.6–13.8)	2.2 (-9.7–6.2)	0.002
Lymphocytes (10^9/L)	1.7 (1.0–2.8)	1.5 (-0.5–2.9)	NS
Monocytes (10^9/L)	0.9 (0.2–2.0)	0.6 (-1.0–2.8)	NS
Platelets (10^9/L)	152 (92–238)	95 (34–154)	0.003
Hgb (g/dL)	1.3 (0.6–2.4)	1.0 (-0.5–1.4)	NS

The TBV of the donor is calculated by the apheresis device based on donor gender, height and weight in accordance with Nadler's equation [25]. The decrements of peripheral blood cells during apheresis are calculated as the difference between post-apheresis and pre-apheresis concentrations. Increments are presented as figures with negative signs. All values are given as medians with variation ranges in parentheses.
Hgb, hemoglobin; PB, peripheral blood; TBV, total peripheral blood volume.

device (Cobe Spectra versus Spectra Optia) could still be detected (Supplementary Figure S2).

The plasma mediator levels in allotransplant recipients are altered after graft infusion

The pretransplantation mediator concentrations in the allotransplant recipients were generally significantly higher compared with the untreated levels of the healthy stem cell donors, in particular, for TNFα, IL-1β, IL-6, IL-10, CXCL11, CCL2, VCAM-1, MMP-1, MMP-2, TIMP-1 and TIMP-4 (Supplementary Table S4). Two main patient clusters were identified in the hierarchical clustering analysis of pretransplantation plasma samples (Figure 4A), and these two clusters differed significantly with respect to the concentrations of MMP-8 ($P < 0.0005$), MMP-9 ($P < 0.0005$), IL-1β ($P < 0.0005$), CXCL11 ($P < 0.0005$), CCL4 ($P < 0.0005$), IL-8 ($P < 0.001$), VEGF ($P = 0.007$), CCL5 ($P = 0.007$) and P-Selectin ($P = 0.007$). As shown in Figure 4A, the patient mediator profile identified using hierarchical clustering analysis correlated significantly to the Hematopoietic Cell Transplantation Comorbidity Index [26] ($P = 0.008$, Pearson chi-square).

The plasma mediator profiles were also examined 1 and median 6 days post-transplantation. The immediate response of allogeneic stem cell infusion on mediator levels was rather individual and variable (Figure 4B; Supplementary Figure S3). However, decreased TPO level and slightly increased concentrations of P-Selectin and MMP-8 were observed (Supplementary Table S4; Supplementary Figure S3). Finally, samples 4–13 days post-transplantation showed increased concentrations of IL-6, IL-8 and G-CSF (Supplementary Table S4; Supplementary Figure S3).

The post-transplantation patient mediator profile is associated with disease-free and overall survival

After allotransplantation the patients were observed until death (n = 8) or the end of the study (n = 8). Median time of observation was 1125 days (range, 8–1715 days). The median time until peripheral blood neutrophil counts $>0.5 \times 10^9$/L was 17 days (range, 13–28 days), whereas the median time until the first of 3 consecutive days with stable platelet counts $>50 \times 10^9$/L was 15 days (range, 11–39 days). Two of the 16 patients were diagnosed with acute GVHD grade II–IV, nine with chronic GVHD and four with leukemia relapse. Early death before day +100 occurred in four patients. The time until neutrophil and platelet reconstitution and the incidence of acute or chronic GVHD, early death or relapse did not differ between the major patient subsets identified by

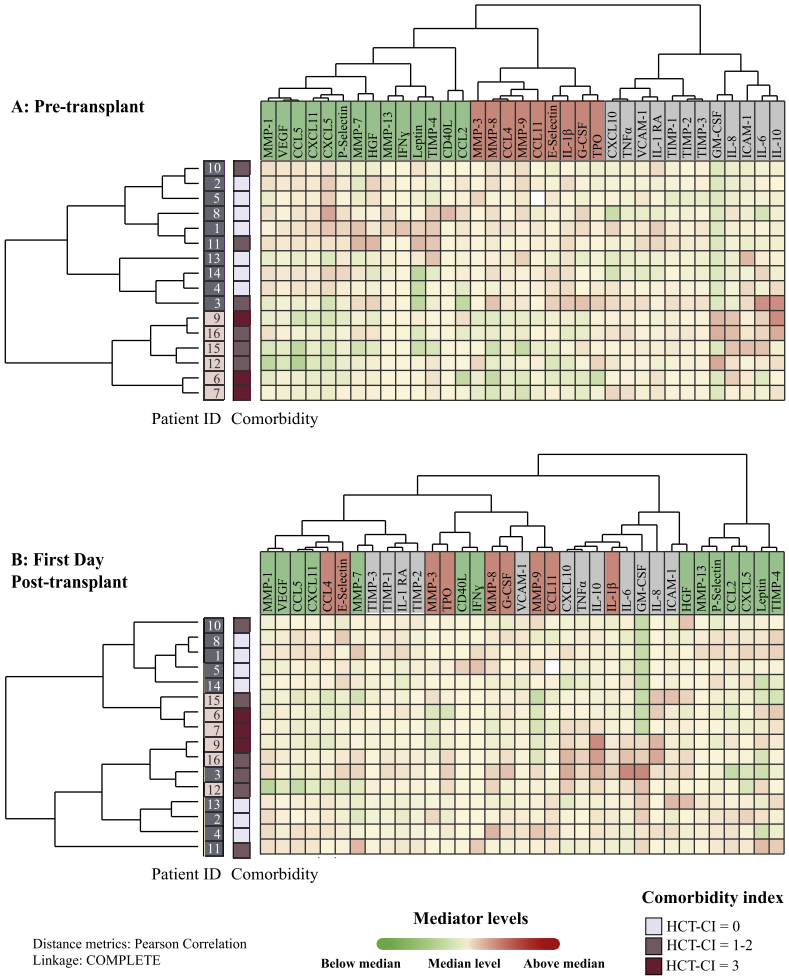

Figure 4. Unsupervised hierarchical cluster analysis based on plasma mediator levels in 16 allogeneic stem cell recipients (Part A/Upper) in the morning on the day of stem cell transplantation (pretransplantation) and (Part B/Lower) the first day after stem cell infusion (post-transplantation). All color schemes are presented in the lower part of the figure. Red color in the patient/mediator columns indicates levels higher than the corresponding median level for each mediator, whereas green color indicates low levels compared with the median. The heat maps with corresponding dendrograms are shown with horizontal mediator clustering at the top of the figures and the vertical patient clustering to the left. (A/Top) The pretransplantation cluster shows three main mediator subsets indicated by green, red and grey, respectively, at the top of the figure. Two main patient clusters marked with either blue or pink color were identified as indicated in the Patient ID column to the left in the figure. The Comorbidity column to the left in the figure indicates the scoring of each individual patient according to the Hematopoietic Stem Cell Transplantation Comorbidity Index (HCT-CI) [26]. (B/Lower) This figure presents the clustering analysis of patient mediator levels on the first day post-transplantation (day +1). The mediator clustering is shown at the top of the figure, and the colors indicate the mediator clustering from the pretransplantation analysis (Figure 4A). Two main patient clusters marked with either blue or pink color were identified as indicated to the left in the figure, and the Patient ID column shows how the patients clustered in the pretransplantation analysis. The Comorbidity column to the left in the figure indicates the scoring of each individual patient as explained in the legend above for Figure 4A. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

hierarchical clustering of pretransplantation samples, immediately after or 6–8 days after transplantation.

Median disease-free survival of the 16 allotransplant recipients (see above) was estimated to be 573 days (95% confidence interval [CI], 0–1184 days) and median overall survival to be 1179 days. There was no significant difference in survival based on pretransplantation patient clustering (Figure 4A) or

Table III. Results from Cox-regression of time from allogeneic stem cell transplantation to death in 16 patients from Haukeland University Hospital, Bergen, Norway included from 2012–2014 and followed up till 2017.

	Unadjusted models			Fully adjusted model		
Predictor	HR	95% CI	*P*	HR	95% CI	*P*
Post-transplantation mediator profile			0.003			0.003
Upper donor cluster (Figure 4B)	1.00	Reference		1.00	Reference	
Lower donor cluster (Figure 4B)	12.20	(1.48, 100.6)		17.73	(1.52, 207.3)	
Age (per 10 y)	1.18	(0.71, 1.96)	0.526	1.17	(0.31, 4.39)	0.810
Gender (female/male)			0.559	-	Not included[a]	-
Female	1.00	Reference				
Male	1.33	(0.51, 3.46)				
HCT-CI			0.522			0.276
0	1.00	Reference		1.00	Reference	
1–2	1.94	(0.41, 9.16)		2.23	(0.36, 13.83)	
3	2.33	(0.48, 11.25)		6.09	(0.66, 56.25)	

HR, hazard ratio; CI, confidence interval; *P*, *P* value from the likelihood ratio test.
[a]Gender could not be included in the fully adjusted model due to nonconvergence of the estimation procedure. In reduced models, however, gender was not significant and including it did not change the results substantially.

graft clustering (Figure 3). However, when the patients were divided into two main clusters/subsets based on hierarchical clustering of plasma levels on the first day post-transplantation (Figure 4B), these groups differed significantly with respect to both disease-free ($P = 0.011$) and overall survival ($P = 0.003$). In the Cox regression (Table III) the unadjusted effect of the mediator profile on overall survival showed a hazard ratio (HR) of 12.20 (95% CI, 1.48–100.6; $P = 0.003$). When adjusting for age and Hematopoietic Cell Transplantation Comorbidity Index (HCT-CI) the mediator profile still showed a significant effect on overall survival with an increased HR of 17.73 (95% CI, 1.52–207.3; $P = 0.003$). Gender showed no effect on survival.

Discussion

G-CSF–mobilized PBSC grafts derived from healthy donors are commonly used for reconstitution of hematopoiesis but also have additional immunomodulatory effects [11]. These last effects may be mediated through or reflected by altered systemic levels of cytokines, soluble adhesion molecules, proteases and protease inhibitors. These mediators form an interacting network for intercellular communication, but there is also additional crosstalk at the receptor level (i.e., trans-signaling) and between downstream intracellular signaling pathways [27,28]. In this context we have investigated the effects of G-CSF mobilization and stem cell harvesting on the systemic concentrations of a large number of soluble mediators, and we have focused on the altered profiles of immunoregulatory mediators rather than variations in single mediator concentrations. The effects of G-CSF and leukapheresis on donor and graft mediator profiles may have impacts both on donor health and on the post-transplantation function of graft immunocompetent cells. Previous studies of systemic mediator levels in healthy stem cell donors are few and have focused on a limited number of single mediators that are mainly relevant for stem cell mobilization and engraftment [29–34].

The most significant increases in mediator levels during G-CSF treatment were observed for MMP-8, the soluble adhesion molecules E-Selectin, VCAM and ICAM, the pro-inflammatory cytokines TNFα and IL-6 and the anti-inflammatory cytokines IL-10 and IL-1 RA. An increase of MMP-8 concentrations is probably caused by enhanced release by neutrophils, while increased levels of soluble adhesion molecules are most likely due to down-regulation of their ligands, which is an expected effect of G-CSF [35,36].

Previous studies have also described altered systemic cytokine levels during G-CSF mobilization, including increased levels of anti-inflammatory cytokines as a part of the tolerogenic or immunosuppressive effect of G-CSF [37,38]. However, two previous studies investigating a limited number of soluble mediators (10 and 4 mediators, respectively) described increased levels of certain pro-inflammatory mediators during G-CSF treatment [29,39]. The stem cell donors in these two studies were younger (median age, 39 and 28 years, respectively) than the donors included in our present study (median age, 54 years), but our present study investigating an extended mediator profile also showed that G-CSF increased the systemic levels of a large number of mediators usually regarded to mediate pro-inflammatory effects.

A recent study compared cytokine levels in allogeneic PBSC grafts versus bone marrow grafts [40]. IL-10 was then the only cytokine (10 mediators

examined) that was significantly increased during G-CSF treatment, and they observed increased levels of IFNγ as well as other pro-inflammatory mediators in the graft supernatants compared with bone marrow grafts. However, animal models suggest that pro-inflammatory cytokines may also have immunosuppressive effects through induction of donor T-cell apoptosis resulting in a reduced risk of acute graft-versus-host disease (aGVHD) development [41–43]. Thus, the final effect of pro-inflammatory cytokines seems to depend on the biological context. It is not known whether "paradoxical" immunosuppressive effects of pro-inflammatory cytokines on donor T cells in the stem cell grafts contribute to the tolerogenic effects of G-CSF therapy in humans.

Platelets express several mediators that are released during activation, including immunomodulators (TNFα, CD40L), IL1β, chemokines (e.g., CXCL5, CCL2 and CCL5), VEGF and P-selectin as well as MMPs and their inhibitors (MMP1, MMP2 and TIMP1-3) [44–46]. Ten of these 12 mediators showed increased plasma concentrations after *in vivo* G-CSF administration (Table I), even though the peripheral blood platelet counts were not significantly altered during G-CSF treatment (data not shown). G-CSF leads to more than a five-fold increment of white blood cell counts, and a corresponding increase of the leukocyte mediator release is a possible explanation for the generally increased plasma mediator level. However, *in vivo* G-CSF treatment also induces platelet activation [47]; an observation suggesting that G-CSF induced platelet activation may contribute to the increased levels of these mediators.

Our study clearly demonstrates that G-CSF treatment leads to substantial alterations of the plasma mediator levels of healthy stem cell donors, but with a high degree of individual variations, and the donor heterogeneity in mediator profiles prior to mobilization is largely maintained during G-CSF treatment (Figure 2A and 2B). Furthermore, we found a weak but significant association between the systemic mediator profile during G-CSF administration and the stem cell yield, indicating that the overall systemic mediator profile influences the complex process of stem cell mobilization.

We found associations between the apheresis device used and the mediator profiles of the stem cell grafts as well as donor plasma profiles after apheresis. The leukocyte and platelet counts were higher in the grafts prepared with Cobe Spectra (Table II), and the positive correlations between graft leukocyte/platelet levels and the graft concentration of various mediators further substantiate different leukocyte and platelet concentrations (see discussion above) as the most important and most likely explanation for the device-dependent differences in mediator levels in the grafts and in the donor plasma after apheresis. However, one cannot exclude the possibility that differences in separation and isolation of cells by themselves also contribute to differences in mediator profiles in the stem cell grafts. Cobe Spectra and Spectra Optia apply different mechanisms for separation and collection of mobilized stem cells [48], the major differences being (i) continuous automatic versus intermittent manual interface position control during centrifugation, (ii) intermediate versus high centrifugation force, (iii) intermediate versus low extracorporeal blood volume and (iv) continuous collection of buffy coat versus intermittent deposition of platelet-rich buffy coat into a collection chamber [48,49]. These differences may lead to additional variations in processing- and product-volumes as well as apheresis time in addition to the differences in graft composition [49,50]. A higher number of total blood volumes was also processed with Cobe Spectra compared with Spectra Optia (Table II); this may contribute to a stronger activation of cells in the grafts and thereby increased mediator release (Figure 3).

Even though G-CSF treatment leads to increased plasma concentrations of several mediators, and the levels of most mediators are further increased in the graft, the infused graft volume is relatively small and the pretransplantation level of many mediators is even higher in the recipient. Thus, a post-transplantation effect in the recipient caused by the infused cytokines is probably small; in our opinion, potential modification of graft immunocompetent cells more likely influences the patient.

We observed an association between patient comorbidity and pretransplantation mediator profile (Figure 4A). However, the change in recipient mediator profile early after stem cell infusion was not predicted by comorbidity and the patients with increased comorbidity index were evenly distributed between the two main donor clusters identified in Figure 4B.

The immediate post-transplantation mediator profile of the patients was significantly associated to both overall and disease-free survival, and this correlation was not weakened by adjustment for HCT-CI and age. On the contrary, when we expanded our analysis with the Cox proportional hazards model to calculate the effect size for each factor, the HR associated with inclusion in the lower patient cluster in Figure 4B after adjustment for comorbidity and age increased from 12.20 to 17.73. Even though the sample size in our study is small and the results, therefore, should be interpreted with greatest caution and regarded as an exploratory survey, these results suggest that larger prospective studies should be done to clarify whether the post-transplantation mediator profile represents a prognostic marker for patient outcome.

To conclude, both G-CSF treatment and apheresis procedures alter the systemic mediator levels in stem cell donors and in graft supernatants. Our results suggest that infusion of allogeneic stem cell grafts from healthy family donors will alter the short-term systemic mediator levels in the recipients/patients. Altered donor mediator levels caused by stem cell mobilization and apheresis may also lead to potentially more sustained changes of the properties of graft immunocompetent cells. Whether these effects have any impact on patient outcomes needs to be investigated in future clinical studies.

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References

[1] Melve GK, Ersvaer E, Kittang AO, Bruserud O. The chemokine system in allogeneic stem-cell transplantation: a possible therapeutic target? Expert Rev Hematol 2011;4:563–76. http://www.ncbi.nlm.nih.gov/pubmed/21939423.

[2] Kernan NA, Collins NH, Juliano L, Cartagena T, Dupont B, O'Reilly RJ. Clonable T lymphocytes in T cell-depleted bone marrow transplants correlate with development of graft-v-host disease. Blood 1986;68:770–3. http://www.ncbi.nlm.nih.gov/pubmed/3527302.

[3] Muller S, Schulz A, Reiss U, Schwarz K, Schreiner T, Wiesneth M, et al. Definition of a critical T cell threshold for prevention of GVHD after HLA non-identical PBPC transplantation in children. Bone Marrow Transplant 1999;24:575–81. http://www.ncbi.nlm.nih.gov/pubmed/10490721.

[4] Walshe J, Bishop MR. Factors affecting engraftment of allogeneic hematopoietic stem cells after reduced-intensity conditioning. Cytotherapy 2004;6:589–602. http://www.ncbi.nlm.nih.gov/pubmed/15773022.

[5] Goldman JM, Gale RP, Horowitz MM, Biggs JC, Champlin RE, Gluckman E, et al. Bone marrow transplantation for chronic myelogenous leukemia in chronic phase. Increased risk for relapse associated with T-cell depletion. Ann Intern Med 1988;108:806–14. http://www.ncbi.nlm.nih.gov/pubmed/3285744.

[6] Korbling M, Huh YO, Durett A, Mirza N, Miller P, Engel H, et al. Allogeneic blood stem cell transplantation: peripheralization and yield of donor-derived primitive hematopoietic progenitor cells (CD34+ Thy-1dim) and lymphoid subsets, and possible predictors of engraftment and graft-versus-host disease. Blood 1995;86:2842–8. http://www.ncbi.nlm.nih.gov/pubmed/7545476.

[7] Korbling M, Przepiorka D, Huh YO, Engel H, van Besien K, Giralt S, et al. Allogeneic blood stem cell transplantation for refractory leukemia and lymphoma: potential advantage of blood over marrow allografts. Blood 1995;85:1659–65. http://www.ncbi.nlm.nih.gov/pubmed/7888684.

[8] Flowers ME, Inamoto Y, Carpenter PA, Lee SJ, Kiem HP, Petersdorf EW, et al. Comparative analysis of risk factors for acute graft-versus-host disease and for chronic graft-versus-host disease according to National Institutes of Health consensus criteria. Blood 2011;117:3214–19. http://www.ncbi.nlm.nih.gov/pubmed/21263156.

[9] Anasetti C, Logan BR, Lee SJ, Waller EK, Weisdorf DJ, Wingard JR, et al. Peripheral-blood stem cells versus bone marrow from unrelated donors. N Engl J Med 2012;367:1487–96. http://www.ncbi.nlm.nih.gov/pubmed/23075175.

[10] Baez A, Martin-Antonio B, Piruat JI, Prats C, Alvarez-Laderas I, Barbado MV, et al. Granulocyte colony-stimulating factor produces long-term changes in gene and microRNA expression profiles in CD34+ cells from healthy donors. Haematologica 2014;99:243–51. http://www.ncbi.nlm.nih.gov/pubmed/24056818.

[11] Hatfield KJ, Melve GK, Bruserud O. Granulocyte colony-stimulating factor alters the systemic metabolomic profile in healthy donors. Metabolomics 2017;13:2. http://www.ncbi.nlm.nih.gov/pubmed/27980502.

[12] Akkok CA, Hervig T, Stamnesfet S, Nesthus I, Melve GK, Lassalle P, et al. Effects of peripheral blood stem cell apheresis on systemic cytokine levels in patients with multiple myeloma. Cytotherapy 2011;13:1259–68. http://www.ncbi.nlm.nih.gov/pubmed/21905955.

[13] Mosevoll KA, Akkok CA, Hervig T, Melve GK, Bruserud O, Reikvam H. Stem cell mobilization and harvesting by leukapheresis alters systemic cytokine levels in patients with multiple myeloma. Cytotherapy 2013;15:850–60. http://www.ncbi.nlm.nih.gov/pubmed/23623276.

[14] Reikvam H, Mosevoll KA, Melve GK, Gunther CC, Sjo M, Bentsen PT, et al. The pretransplantation serum cytokine profile in allogeneic stem cell recipients differs from healthy individuals, and various profiles are associated with different risks of posttransplantation complications. Biol Blood Marrow Transplant 2012;18:190–9. http://www.ncbi.nlm.nih.gov/pubmed/22019725.

[15] Ljungman P, Brand R, Einsele H, Frassoni F, Niederwieser D, Cordonnier C. Donor CMV serologic status and outcome of CMV-seropositive recipients after unrelated donor stem cell transplantation: an EBMT megafile analysis. Blood 2003;102:4255–60. http://www.ncbi.nlm.nih.gov/pubmed/12933590.

[16] Blaser BW, Roychowdhury S, Kim DJ, Schwind NR, Bhatt D, Yuan W, et al. Donor-derived IL-15 is critical for acute allogeneic graft-versus-host disease. Blood 2005;105:894–901. http://www.ncbi.nlm.nih.gov/pubmed/15374888.

[17] Socie G, Loiseau P, Tamouza R, Janin A, Busson M, Gluckman E, et al. Both genetic and clinical factors predict the development of graft-versus-host disease after allogeneic hematopoietic stem cell transplantation. Transplantation 2001;72:699–706. http://www.ncbi.nlm.nih.gov/pubmed/11544434.

[18] Keen LJ, DeFor TE, Bidwell JL, Davies SM, Bradley BA, Hows JM. Interleukin-10 and tumor necrosis factor alpha region haplotypes predict transplant-related mortality after unrelated donor stem cell transplantation. Blood 2004;103:3599–602. http://www.ncbi.nlm.nih.gov/pubmed/14701704.

[19] Karabon L, Wysoczanska B, Bogunia-Kubik K, Suchnicki K, Lange A. IL-6 and IL-10 promoter gene polymorphisms of patients and donors of allogeneic sibling hematopoietic stem cell transplants associate with the risk of acute graft-versus-host disease. Hum Immunol 2005;66:700–10. http://www.ncbi.nlm.nih.gov/pubmed/15993715.

[20] Stavrum AK, Petersen K, Jonassen I, Dysvik B. Analysis of gene-expression data using J-Express, Current protocols in bioinformatics / editorial board, Andreas D. Baxevanis … [et al.] Chapter 7, 2008, Unit 7 3. http://www.ncbi.nlm.nih.gov/pubmed/18428687.

[21] Do JH, Choi DK. Clustering approaches to identifying gene expression patterns from DNA microarray data. Mol Cells 2008;25:279–88. http://www.ncbi.nlm.nih.gov/pubmed/18414008.

[22] Bergkvist A, Rusnakova V, Sindelka R, Garda JM, Sjogreen B, Lindh D, et al. Gene expression profiling—Clusters of possibilities. Methods 2010;50:323–35. http://www.ncbi.nlm.nih.gov/pubmed/20079843.

[23] Herrero J, Valencia A, Dopazo J. A hierarchical unsupervised growing neural network for clustering gene expression patterns. Bioinformatics 2001;17:126–36. http://www.ncbi.nlm.nih.gov/pubmed/11238068.

[24] Cox DR. Regression models and life tables [with discussion]. J Royal Statist Soc B 1972;34:187–220. http://www.jstor.org/stable/2985181.

[25] Neyrinck MM, Vrielink H, E. Joint Task Force for, Certification. Calculations in apheresis. J Clin Apher 2015;30:38–42. http://www.ncbi.nlm.nih.gov/pubmed/25041907.

[26] Sorror ML, Maris MB, Storb R, Baron F, Sandmaier BM, Maloney DG, et al. Hematopoietic cell transplantation (HCT)-specific comorbidity index: a new tool for risk assessment before allogeneic HCT. Blood 2005;106:2912–19. http://www.ncbi.nlm.nih.gov/pubmed/15994282.

[27] Ozaki K, Leonard WJ. Cytokine and cytokine receptor pleiotropy and redundancy. J Biol Chem 2002;277:29355–8. http://www.ncbi.nlm.nih.gov/pubmed/12072446.

[28] Cameron MJ, Kelvin DJ. Cytokines and chemokines—their receptors and their genes: an overview. Adv Exp Med Biol 2003;520:8–32. http://www.ncbi.nlm.nih.gov/pubmed/12613570.

[29] Lysak D, Hrabetova M, Vrzalova J, Koza V, Navratilova J, Svoboda T, et al. Changes of cytokine levels during granulocyte-colony-stimulating factor stem cell mobilization in healthy donors: association with mobilization efficiency and potential predictive significance. Transfusion 2011;51:319–27. http://www.ncbi.nlm.nih.gov/pubmed/20804529.

[30] Okano A, Ashihara E, Shimazaki C, Uchiyama H, Inaba T, Taniguchi K, et al. Predictive parameters for granulocyte colony-stimulating factor-induced peripheral blood stem cell mobilization. J Clin Apher 2008;23:171–7. http://www.ncbi.nlm.nih.gov/pubmed/18988229.

[31] Rodriguez-Cortes O, Vela-Ojeda J, Lopez-Santiago R, Montiel-Cervantes L, Reyes-Maldonado E, Estrada-Garcia I, et al. Granulocyte colony-stimulating factor produces a decrease in IFNgamma and increase in IL-4 when administrated to healthy donors. J Clin Apher 2010;25:181–7. http://www.ncbi.nlm.nih.gov/pubmed/20818712.

[32] Sharp JG, McGuire TR, Mann SL, Murphy B, Kessinger A. Plasma from poorly mobilizing human subjects inhibits cytokine-induced murine blood stem-cell mobilization. Cytotherapy 2002;4:365–73. http://www.ncbi.nlm.nih.gov/pubmed/12396837.

[33] McGuire TR, Kessinger A, Hock L, Sharp JG. Elevated transforming growth factor beta levels in the plasma of cytokine-treated cancer patients and normal allogeneic stem cell donors. Cytotherapy 2001;3:361–4. http://www.ncbi.nlm.nih.gov/pubmed/11953015.

[34] Young JC, Lin K, Travis M, Hansteen G, Abitorabi A, Sirenko O, et al. Investigation into an engraftment defect induced by culturing primitive hematopoietic cells with cytokines. Cytotherapy 2001;3:307–20. http://www.ncbi.nlm.nih.gov/pubmed/12171719.

[35] Papayannopoulou T. Current mechanistic scenarios in hematopoietic stem/progenitor cell mobilization. Blood 2004;103:1580–5. http://www.ncbi.nlm.nih.gov/pubmed/14604975.

[36] Kronenwett R, Martin S, Haas R. The role of cytokines and adhesion molecules for mobilization of peripheral blood stem cells. Stem Cells 2000;18:320–30. http://www.ncbi.nlm.nih.gov/pubmed/11007916.

[37] Franzke A. The role of G-CSF in adaptive immunity. Cytokine Growth Factor Rev 2006;17:235–44. http://www.ncbi.nlm.nih.gov/pubmed/16807060.

[38] Rutella S, Zavala F, Danese S, Kared H, Leone G. Granulocyte colony-stimulating factor: a novel mediator of T cell tolerance. J Immunol 2005;175:7085–91. http://www.ncbi.nlm.nih.gov/pubmed/16301609.

[39] Pollmacher T, Korth C, Mullington J, Schreiber W, Sauer J, Vedder H, et al. Effects of granulocyte colony-stimulating factor on plasma cytokine and cytokine receptor levels and on the *in vivo* host response to endotoxin in healthy men. Blood 1996;87:900–5. http://www.ncbi.nlm.nih.gov/pubmed/8562960.

[40] Fidyk W, Mitrus I, Ciomber A, Smagur A, Chwieduk A, Glowala-Kosinska M, et al. Evaluation of proinflammatory and immunosuppressive cytokines in blood and bone marrow of healthy hematopoietic stem cell donors. Cytokine 2017;102:181–6. http://www.ncbi.nlm.nih.gov/pubmed/28927758.

[41] Sykes M, Szot GL, Nguyen PL, Pearson DA. Interleukin-12 inhibits murine graft-versus-host disease. Blood 1995;86:2429–38. http://www.ncbi.nlm.nih.gov/pubmed/7662991.

[42] Yang YG, Sergio JJ, Pearson DA, Szot GL, Shimizu A, Sykes M. Interleukin-12 preserves the graft-versus-leukemia effect of allogeneic CD8 T cells while inhibiting CD4-dependent graft-versus-host disease in mice. Blood 1997;90:4651–60. http://www.ncbi.nlm.nih.gov/pubmed/9373279.

[43] Reddy P, Teshima T, Kukuruga M, Ordemann R, Liu C, Lowler K, et al. Interleukin-18 regulates acute graft-versus-host disease by enhancing Fas-mediated donor T cell apoptosis. J Exp Med 2001;194:1433–40. http://www.ncbi.nlm.nih.gov/pubmed/11714750.

[44] Semple JW, Italiano JE Jr, Freedman J. Platelets and the immune continuum. Nat Rev Immunol 2011;11:264–74. http://www.ncbi.nlm.nih.gov/pubmed/21436837.

[45] Bruserud O. Bidirectional crosstalk between platelets and monocytes initiated by Toll-like receptor: an important step in the early defense against fungal infections? Platelets 2013;24:85–97. http://www.ncbi.nlm.nih.gov/pubmed/22646762.

[46] Seizer P, May AE. Platelets and matrix metalloproteinases. Thromb Haemost 2013;110:903–9. http://www.ncbi.nlm.nih.gov/pubmed/23864155.

[47] Spiel AO, Bartko J, Schwameis M, Firbas C, Siller-Matula J, Schuetz M, et al. Increased platelet aggregation and *in vivo* platelet activation after granulocyte colony-stimulating factor administration. A randomised controlled trial. Thromb Haemost 2011;105:655–62. http://www.ncbi.nlm.nih.gov/pubmed/21301783.

[48] Reinhardt P, Brauninger S, Bialleck H, Thorausch K, Smith R, Schrezenmeier H, et al. Automatic interface-controlled apheresis collection of stem/progenitor cells: results from an autologous donor validation trial of a novel stem cell apheresis device. Transfusion 2011;51:1321–30. http://www.ncbi.nlm.nih.gov/pubmed/21155834.

[49] Brauninger S, Bialleck H, Thorausch K, Seifried E, Bonig H. Mobilized allogeneic peripheral stem/progenitor cell apheresis with Spectra Optia v.5.0, a novel, automatic interface-controlled apheresis system: results from the first feasibility trial. Vox Sang 2011;101:237–46. http://www.ncbi.nlm.nih.gov/pubmed/21517897.

[50] Brauninger S, Bialleck H, Thorausch K, Felt T, Seifried E, Bonig H. Allogeneic donor peripheral blood "stem cell" apheresis: prospective comparison of two apheresis systems. Transfusion 2012;52:1137–45. http://www.ncbi.nlm.nih.gov/pubmed/22044384.

Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.jcyt.2018.02.366.

Supplementary Material

THE HEALTHY DONOR PROFILE OF IMMUNOREGULATORY SOLUBLE MEDIATORS IS ALTERED BY STEM CELL MOBILIZATION AND APHERESIS

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Supplementary Table 1: Median plasma mediator levels for the 25 allogeneic stem cell donors during stem cell apheresis. All mediator concentrations are given in pg/ml and presented as median values with variation ranges given in parentheses. IL-12 and GM-CSF showed undetectable levels and were not included in the table. From left to right, unstimulated mediator levels are compared to concentrations after G-CSF treatment, and these pre-apheresis levels are then compared to the mediator concentrations immediately after apheresis (Wilcoxon's test for paired samples).

Mediator	Pre-apheresis	Post-apheresis	Apheresis effect (p)
Immunomodulatory cytokines			
IFNɣ	2.8 (<2.2 - 25.5)	<2.2 (<2.2 - 21.2)	0.004 (↓)
CD40L	1309 (<998 - 95487)	<998 (<998 - 88418)	0.000291 (↓)
TNFα	7.9 (<0.8 - 18.4)	7.3 (3.2 - 16.8)	ns
Interleukins			
IL-1 RA	4867 (2415 - > 7528)	5443 (2701 - >7528)	ns
Il-1β	<0.3 (<0.3 - 0.9)	<0.3 (<0.3 - 1.3)	ns
IL-6	1.9 (<0.9 - 5.5)	2.3 (1.3 - 10.5)	0.01 (↑)
IL-8 (CXCL-8)	14.3 (<8.3 - 149.5)	<8.3 (<8.3 - 135.8)	0.004 (↓)
IL-10	0.51 (<0.5 - 12.8)	<0.5 (<0.5 - 14.5)	ns
Chemokines			
CXCL5 (ENA-78)	155.9 (<61.1 - 3220)	90.4 (<61.1 - 1330)	0.000099 (↓)
CXCL10 (IP-10)	129.9 (30.0 - 512.3)	161.7 (54.9 - 603.2)	0.001 (↑)
CXCL11 (I-TAC)	58.1 (<17.0 - 256.6)	46.2 (<17.0 - 244.2)	0.000103 (↓)
CCL2 (MCP-1)	240.9 (89.4 - 477.8)	251.9 (89.5 - 473.1)	ns
CCL4 (MIP-1β)	324.5 (216.6 - 742.5)	294.4 (226.0 - 632.2)	0.000068 (↓)
CCL5 (RANTES)	4120.9 (885.9 - >6000)	2789 (912.2 - >6000)	0.016 (↓)
CCL11 (Eotaxin)	279.2 (<124.6 - 1977)	221.7 (<124.6 - 1379)	0.000162 (↓)
Growth factors			
TPO	1394.7 (<378.2 - >109611)	1267.6 (<378.2 - >109611)	0.000438 (↓)
VEGF	159.4 (74.8 - 464.8)	141.0 (72.8 - 266.6)	0.002 (↓)
HGF	260.6 (<41.6 - 1459)	535.2 (<41.6 - 7641)	ns
Leptin	7216 (966.1 - 29917)	10469.5 (1488 - 42033)	0.000027 (↑)
G-CSF	>7425 (3687 - 14178)	5899 (1437 - >7425)	0.001 (↓)
Adhesion molecules			
P-Selectin	18663 (6358.1 - 65218)	17531 (6293 - 30248)	0.000262 (↓)
E-Selectin	25661 (12691 - 71422)	24976 (11759 - 69414)	0.000233 (↓)
VCAM-1	970313 (425794 - > 1807879)	1020000 (619125 - >1807879)	ns
ICAM-1	236484 (119891 - 1470000)	212718 (100571 - 1300000)	0.000087 (↓)
Matrix metalloproteases			
MMP-1	538.1 (153.3 - 1979)	373.9 (129.4 - 807.9)	0.000068 (↓)
MMP-2	40851 (19616 - >58201)	38415 (20145 - 44084)	0.002 (↓)
MMP-3	6508 (2487 - >8924)	4477 (2519 - >8924)	0.00006 (↓)
MMP-7	3468 (630.0 - 7624)	3212 (905.4 - 8078)	ns
MMP-8	21316 (6354.4 - 44166)	20531 (4362 - 46387)	ns
MMP-9	7386.7 (3276 - >37370)	7476.3 (3743 - 21406)	ns
MMP-12	<29.8 (<29.8 - 109.3)	<29.8 (<29.8 - 94.9)	0.008 (↓)
MMP-13	392.8(<220.4 - 2031)	325.0 (<220.4 - 1863)	0.000398 (↓)
Metalloprotease inhibitors			
TIMP-1	79591 (52049.5 - 217884)	81399 (<12.7 - 146644)	ns
TIMP-2	69037 (52632 - 121228)	64526 (<42.1 - 100684)	ns
TIMP-3	16055 (<3897 - 37381)	14425 (<3897 - 44804)	ns
TIMP-4	1129.3 (574.7 - 2199)	1241.1 (<6.8 - 1994)	ns

Abbreviations: Ns; not significant, (↑): increased level, (↓): decreased level.

Supplementary Table 2: Mediator levels in the stem cell graft supernatants of 22 allogeneic stem cell donors. All mediator concentrations are given in pg/ml and presented as median values with variation ranges given in parentheses. IL-12 and GM-CSF showed undetectable levels, and were not included in the table. Graft mediator levels are compared to plasma concentrations prior to G-CSF and during G-CSF treatment (Wilcoxon's test for paired samples, see Table 1). *Abbreviations:* PB: peripheral blood plasma; Ns: not significant; (↑): increased level; (↓): decreased level.

Mediator	**Stem cell graft**	**PB, (prior to G-CSF)**	**Graft vs PB, untreated p-value**	**PB, (during G-CSF)**	**Graft vs PB, during G-CSF p-value**
Immunomodulatory cytokines					
IFNγ	4.2 (<2.2 - 53.2)	3.7 (<2.2 -34.7)	ns	2.8 (<2.2 - 25.5)	ns
CD40L	2414 (1275 - 106709)	1361 (<998 - 101562)	0.020 (↑)	1309 (<998 - 95487)	ns
TNFα	6.6 (1.8 - 40.9)	3.6 (<0.8 - 7.2)	0.000260 (↑)	7.9 (<0.8 - 18.4)	ns
Interleukins					
IL-1 RA	>7528 (3024 - > 7528)	741.1 (248.1 - 6400)	0.000040 (↑)	4867 (2415 - > 7528)	0.000245 (↑)
Il-1β	<0.3 (<0.3 - 50.2)	<0.3 (<0.3 - 1.0)	0.008 (↑)	<0.3 (<0.3 - 0.9)	0.010 (↑)
IL-6	<0.9 (<0.9 - 7.35)	0.9 (<0.9 - 12.3)	ns	1.9 (<0.9 - 5.5)	0.001 (↓)
IL-8 (CXCL-8)	16.7 (<8.3 - 272.2)	15.7 (<8.3 - 146.5)	ns	14.3 (<8.3 - 149.5)	ns
IL-10	<0.5 (<0.5 - 10.35)	<0.5 (<0.5 - 7.9)	ns	0.51 (<0.5 - 12.8)	ns
Chemokines					
CXCL5 (ENA-78)	294.0 (116.4 - 12816)	170.2 (<61.1 - 2384)	ns	155.9 (<61.1 - 3220)	ns
CXCL10 (IP-10)	80.6 (22.2 - 371.9)	70.9 (29.3 - 438.2)	ns	129.9 (30.0 - 512.3)	ns
CXCL11 (I-TAC)	55.6 (14.0 - 256.0)	42.1 (<17.0 - 236.3)	0.016 (↑)	58.1 (<17.0 - 256.6)	ns
CCL2 (MCP-1)	65.8 (21.8 - 199.4)	267.1 (93.7 - 437.0)	0.000040 (↓)	240.9 (89.4 - 477.8)	0.000040 (↓)
CCL4 (MIP-1β)	376.2 (245.1 - 779.3)	253.4 (191.4 - 700.5)	0.000483 (↑)	324.5 (216.6 - 742.5)	ns
CCL5 (RANTES)	>6000 (2630 - >6000)	>6000 (923.5 - >6000)	0.007 (↑)	4120.9 (885.9 - >6000)	0.002 (↑)
CCL11 (Eotaxin)	301.2 (165.3 - 1502)	292.1 (<124.6 - 1580)	ns	279.2 (<124.6 - 1977)	ns
Growth factors					
TPO	1804.0 (<378.2 - >109611)	1324.5 (<378.2 - >109611)	ns	1394.7 (<378.2 - >109611)	ns
VEGF	231.0 (129.3 - 1887)	166.8 (71.7 - 600.0)	0.000260 (↑)	159.4 (74.8 - 464.8)	0.002 (↑)
HGF	554.3 (165.3 - 13685)	202.5 (<41.6 - 1518)	0.002 (↑)	260.6 (<41.6 - 1459)	0.000245 (↑)
Leptin	8215.3 (1265 - 34603)	6760 (1747 - 35135)	ns	7216 (966.1 - 29917)	0.000427 (↑)
G-CSF	>7425 (1704 - >7425)	46.9 (<29.9 - 241.7)	0.000040 (↑)	>7425 (3687 - 14178)	0.001 (↑)
Adhesion molecules					
P-Selectin	25281 (7337 - 110510)	13322 (4953 - 61068)	0.000040 (↑)	18663 (6358.1 - 65218)	0.001 (↑)
E Selectin	27750 (12107 - 71067)	15575 (7920 - 47953)	0.000040 (↑)	25661 (12691 - 71422)	0.004 (↑)
VCAM-1	1095000.0 (551763 - > 1807879)	617232 (212562 - 1150000)	0.000040 (↑)	970313 (425794 - > 1807879)	0.001 (↑)
ICAM-1	241441 (107728 - 1230000)	184793 (55037 - 1310000)	0.004 (↑)	236484 (119891 - 1470000)	0.000201 (↓)
Matrix metalloproteases					
MMP-1	790.9 (294.5 - 7045)	465.4 (201.5 - 1309)	0.000091 (↑)	538.1 (153.3 - 1979)	0.006 (↑)
MMP-2	40678 (23630 - >58201)	40364 (24626 - >58201)	ns	40851 (19616 - >58201)	ns
MMP-3	5062 (2650 - 12087)	8289 (3417 - >8924)	0.000089 (↓)	6508 (2487 - >8924)	0.000089 (↓)
MMP-7	4238 (1289 - 7440)	3711 (1190 - 7353)	ns	3468 (630.0 - 7624)	0.012 (↑)
MMP-8	>48433.0 (10810 - >48433)	332.7 (181.6 - 2407)	0.000040 (↑)	21316 (6354 - 44166)	0.000105 (↑)
MMP-9	33651 (10491 - >37370)	4148.0 (1773 - 12111)	0.000060 (↑)	7387 (3276 - >37370)	0.000089 (↑)
MMP-12	<29.8 (<29.8 - 70.4)	<29.8 (<29.8 - 69.7)	ns	<29.8 (<29.8 - 109.3)	ns
MMP-13	445.9 (233.9 - 2030)	309.1 (<220.4 -1872)	0.006 (↑)	392.8(<220.4 - 2031)	ns
Metalloprotease inhibitors					
TIMP-1	109200 (48013 - 530498)	64100 (39044 - 157544)	0.000040 (↑)	79591 (52050 - 217884)	0.001 (↑)
TIMP-2	88727 (48959 - 192928)	77727 (57078 - 128221)	ns	69037 (52632 - 121228)	0.001 (↑)
TIMP-3	19797 (12402 - 210872)	15308 (<3897 - 34409)	0.003 (↑)	16055 (<3897 - 37381)	0.001 (↑)
TIMP-4	1200 (610.4 - 2046)	1275 (699.2 - 2097)	ns	1129 (574.7 - 2199)	0.005 (↑)

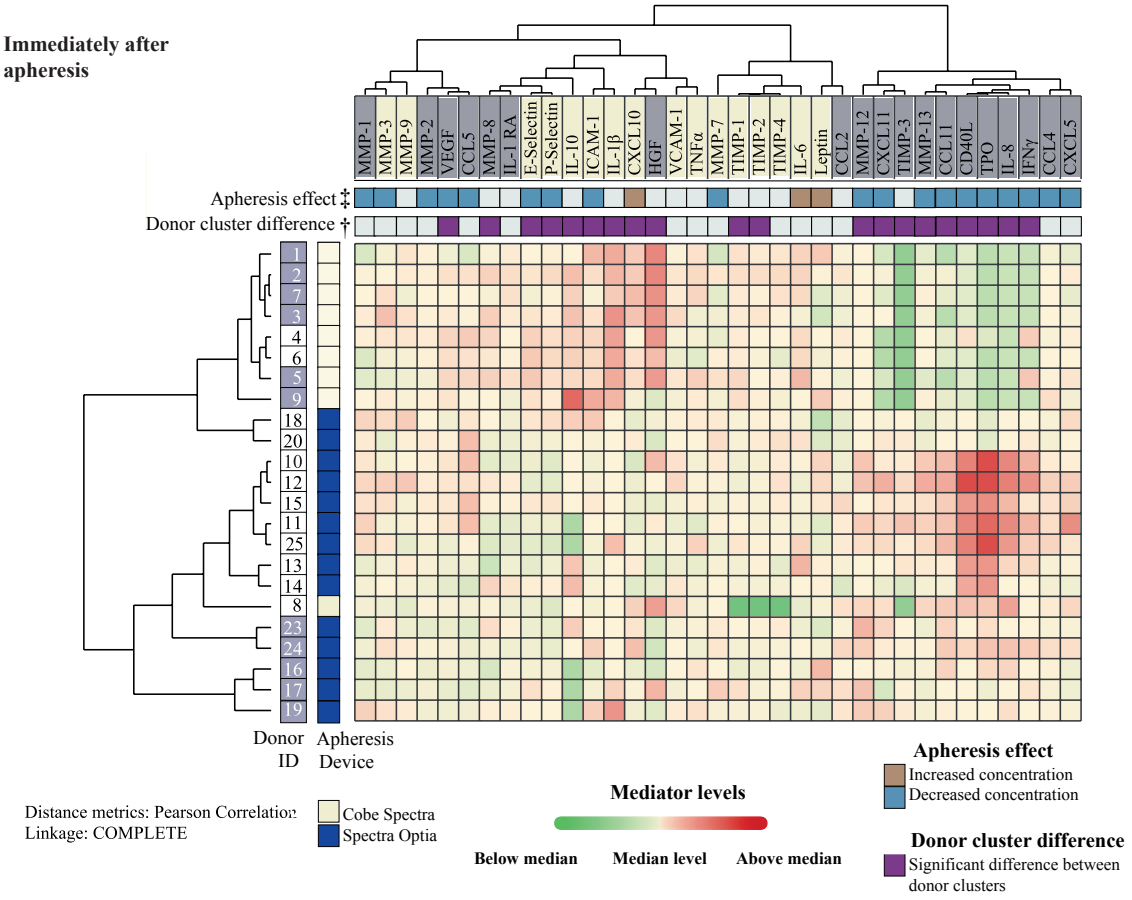
Supplementary Table 3: Comparison of clinical parameters and plasma levels of single mediators 24 hours after start of apheresis in the donor group achieving the target dose of CD34+ stem cells within one day of stem cell collection and the donor group in need of several aphereses to achieve the target dose. In the first group (I) the last dose of G-CSF treatment was given before start of apheresis. In the second group (II) G-CSF therapy was continued during sampling until the last day of apheresis. All values are given as medians with variation ranges in parenthesis. *Stem cell yield achieved per kg donor weight during the first day of apheresis.

Clinical variable/ Mediator	**Single stem cell apheresis (I) (n = 10)**	**2 - 3 stem cell aphereses (II) (n = 15)**	**p-value**
Clinical variables			
Age (years)	43 (25 - 77)	60 (45 - 73)	0.007
Weight (kg)	84 (63 - 91)	86 (51 - 160)	ns
Apheresis device (CS/SO)	(4/6)	(5/10)	ns
Apheresis duration (minutes)	305 (231 - 363)	313 (221 - 377)	ns
No. of TBV processed	3.8 (3.0 -7.0)	3.4 (2.0 - 6.0)	ns
PB [CD34+] x 10^3/mL	62.2 (30.4 - 147.8)	22.1 (16.7 - 58.8)	0.004
$CD34^+$ yield x 10^6/kg*	7.2 (4.3 - 22.4)	3.2 (0.8 - 5.6)	0.000007
Immunomodulatory cytokines			
IFNγ	<2.2 (<2.2 - 22.0)	<2.2 (<2.2 - 8.5)	ns
CD40L	1442 (<998 - 95723)	<998 (<998 - 18947)	ns
TNFα	6.1 (3.3 - 13.4)	9.0 (6.0 - 16.8)	ns
Interleukins			
IL-1 RA	3562 (1935 - 6711)	4921 (3078 - >7528)	ns
Il-1β	<0.3 (<0.3 - 1.3)	<0.3 (<0.3 - 1.1)	ns
IL-6	1.9 (1.2 - 8.1)	2.9 (1.5 - 7.1)	ns
IL-8 (CXCL-8)	<8.3 (<8.3 - 140.2)	<8.3 (<8.3 - 116.1)	ns
IL-10	0.5 (<0.5 - 1.5)	0.6 (<0.5 - 15.6)	ns
Chemokines			
CXCL5 (ENA-78)	93.3 (<61.1 - 1619)	101.4 (<61.1 - 215.5)	ns
CXCL10 (IP-10)	118.1 (66.6 - 164.2)	171.2 (50.4 - 503.7)	0.004
CXCL11 (I-TAC)	47.8 (<17.0 - 251.2)	52.5 (<17.0 - 91.1)	ns
CCL2 (MCP-1)	198.2 (69.6 - 282.5)	215.7 (129.1 - 448.5)	ns
CCL4 (MIP-1β)	291.1 (230.4 - 647.5)	329.7 (237.6 - 613.9)	ns
CCL5 (RANTES)	2678 (738.4 - >6000)	2633 (1293 - >6000)	ns
CCL11 (Eotaxin)	225.6 (<124.6 - 1456)	230.9 (<124.6 - 941.8)	ns
Growth factors			
TPO	1827 (<378.2 - >109611)	987.7 (<378.2 - >109611)	ns
VEGF	154.0 (63.1 - 342.2)	129.2 (80.1 - 238.7)	ns
HGF	143.8 (<41.6 - 531.1)	284.3 (131.8 - 762.5)	0.012
Leptin	8367 (2170 - 37720)	8105 (1061 - 24254)	ns
G-CSF	232.6 (116.7 - >7425)	>7425 (5557 - >7425)	0.001
Adhesion molecules			
P-Selectin	17750 (9137 - 32103)	19049 (6501 - 27253)	ns
E Selectin	28131 (13860 - 73710)	24425 (12569 - 71748)	ns
VCAM-1	1025780 (425136 - >1807879)	1040000 (810567 - >1807879)	ns
ICAM-1	257593 (114756 - 546034)	237033 (176383 - 1430000)	ns
Matrix metalloproteases			ns
MMP-1	341.6 (179.7 - 959.4)	379.2 (169.8 - 711.7)	ns
MMP-2	39420 (28144 - 52346)	38831 (21012 - 50582)	ns
MMP-3	6792 (5146 - >8924)	7112 (4109 - >8924)	ns
MMP-7	3568 (2467 - 6635)	4009 81128 - 8901)	ns
MMP-8	27550 (3749 - >48433)	28889 (15907 - 72608)	ns
MMP-9	6334 (5761 - 11583)	7914 (5164 - 26402)	ns
MMP-12	<29.8 (<29.8 - 67.9)	<29.8 (<29.8 - 94.3)	ns
MMP-13	326.9 (<220.4 - 1906)	266.6 (<220.4 - 905.2)	ns
Metalloprotease inhibitors			
TIMP-1	80738 (51985 - 125305)	85570 (66786 - 194546)	ns
TIMP-2	64060 (52849 - 94557)	72271 (56042 - 147914)	ns
TIMP-3	13313 (<3897 - 27096)	15308 (<3897 - 22237)	ns
TIMP-4	996.4 (707.2 - 1502)	1296 (957.4 - 3251)	ns

Abbreviations: CS: Cobe Spectra; SO: Spectra Optia; PB: Peripheral blood

Supplementary Table 4: Median recipient plasma mediator levels during infusion of allogeneic stem cell grafts given in pg/ml with variation ranges given in parentheses. The pre-transplant mediator concentration was measured at 9 am before transplantation and compared to mediator levels in healthy untreated stem cell donors (Wilcoxon's test for paired samples, N =16). The mediator levels were measured at 9 am the first post-transplant day and compared (Wilcoxon's test for paired samples) to pre-transplant concentrations (N = 16) and to late post-transplant levels (median 6 days post-transplant, range 4-13 days, N=8).

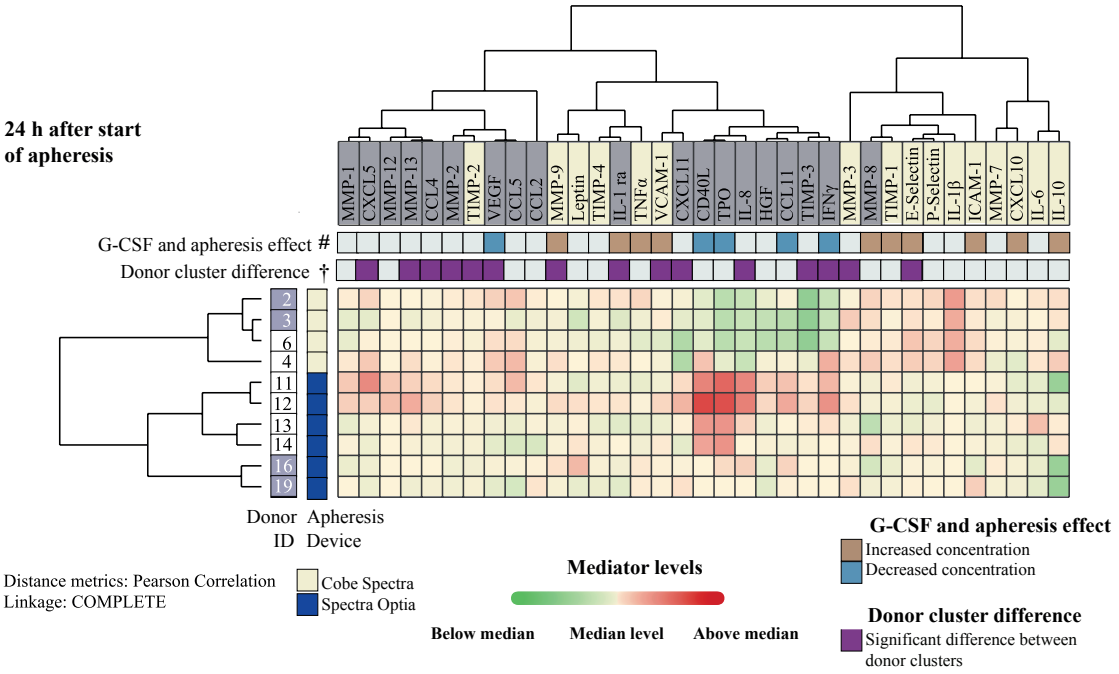
Mediator	Pre-transplant level (pg/ml)	Pre-transplant vs healthy (p, N = 16)	Early post-transplant level (pg/ml)	Early post-transplant vs. pre-transplant (p, N =16)	Late post-transplant level (pg/ml)	Early vs late post-transplant (p, N = 8)
Immunomodulatory cytokines						
IFNγ	<2.2 (<2.2 - 4.9)	0.011 (↓)	(<2.2 (<2.2 - 5.3)	ns	<2.2 (<2.2 - 4.7)	ns
CD40L	<998 (<998 - 2618)	0.049 (↓)	<998 (<998 - 1468)	ns	<998 (<998 - 1919)	ns
TNFα	7.3 (3.8 - 11.6)	0.000073 (↑)	6.4 (3.9 - 13.3)	ns	6.2 (3.4 - 8.7)	ns
Interleukins						
IL-1 RA	838 (403 - 2000)	ns	721 (347 - 1253)	0.034 (↓)	550 (133 - 910)	0.036 (↓)
IL-1 β	0.58 (<0.37 - 1.52)	0.000048 (↑)	0.65 (0.42 - 1.36)	ns	0.68 <0.37 - 1.58)	ns
IL-6	4.5 (6.0 - 32.4)	0.00000043 (↑)	4.5 (2.3 - 61.9)	ns	30.2 (3.3 - 157)	0.025 (↑)
IL-8 (CXCL-8)	9.7 (<8.3 - 147)	ns	<8.3 (<8.3 - 32.4)	0.013 (↓)	87.8 (11.1- 273)	0.012 (↑)
IL-10	0.53 (<0.50 - 5.5)	0.00000018 (↑)	0.48 (<0.50 - 5.3)	ns	0.85 (<0.50 - 5.8)	0.043 (↑)
Chemokines						
CXCL5 (ENA-78)	205 (<61.1 - 1254)	ns	176.5 <61.1 - 357)	ns	89.2 (<61.1 - 231)	0.050 (↓)
CXCL10 (IP-10)	137(19.3 - 267)	0.030 (↑)	89.9 (28.3 - 465)	ns	83.6 (29.7 - 135)	ns
CXCL11 (I-TAC)	358 (117 - 579)	0,00000000058 (↑)	329 (141 - 493)	ns	183 (84 - 309)	0.012 (↓)
CCL2 (MCP-1)	869 (<44.0 - 1479)	0.000017 (↑)	812.5 (<44.0 - 1259)	ns	1014 (483 - 2729)	ns
CCL4 (MIP-1β)	370 (191 - 701)	ns	325 (217 - 743)	ns	294 (226 - 632)	ns
CCL5 (RANTES)	>6000 (476 - >6000)	ns	>6000 (827 - >6000)	ns	2746 (198 - >6000)	0.017 (↓)
CCL11 (Eotaxin)	191 (<125 - 245)	0.000343 (↓)	181 (<125 - 315)	ns	186 (<125 - 328)	ns
Growth factors						
TPO	3998 (614 - 8966)	ns	3100 (781 - 6593)	0.003 (↓)	4737 (3670 - 5748)	0.017 (↑)
VEGF	198 (58.7 - 267)	ns	178 (60.7 - 229)	ns	111 (60.4 - 191)	0.050 (↓)
HGF	516.6 (210 - 1890)	0.002 (↑)	461.1 (262 - 1536)	ns	274.6 (<41.6 - 745)	ns
Leptin	22218 (1978 - 81310)	0.024 (↑)	18820 (3108 - 64331)	ns	23491 (2249 - 53739)	ns
GM-CSF	<0.37(<0.37 - 0.66)	0.007 (↑)	<0.37 (<0.37- 3.7)	0.008 (↑)	0.87 (<0.37 - 6.3)	0.018 (↑)
G-CSF	84.5 (32.3 - 253)	0.007 (↑)	83.3 (51.1 - 259)	ns	1516 (140 - >743)	0.012 (↑)
Adhesion molecules						
P-Selectin	22744 (10059 - 43881)	0.005 (↑)	26707 (14084 - 46942)	0.001 (↑)	16284 (6686 - 27812)	0.012 (↓)
E Selectin	23315 (7581 - 47928)	ns	23772 (7175 - 57410)	ns	17175 (4211 - 36079)	0.012 (↓)
VCAM-1	>1807879 (838307 - >1807879)	0.00000000037 (↑)	>1807879 (425794 - >1807879)	ns	1020000 (619125 - >1807879)	ns
ICAM-1	341980 (209065 - 1310000)	ns	360966 (221834 - 1340000)	ns	343005 (210369 - 519830)	ns
Matrix metalloproteases						
MMP-1	9890 (922 - > 7832)	0.00000000014 (↑)	9326 (741 - > 7832)	ns	9756 (2576 - > 7832)	ns
MMP-2	>58201 (>58201 - >58201)	0.000000018 (↑)	>58201 (>58201 - >58201)	ns	>58201 (>58201 - >58201)	ns
MMP-3	>8924 (4277 - >8924)	0.026	>8924 (3012 - >8924)	ns	>8924 (4306 - >8924)	ns
MMP-7	5031 (1020 - 20143)	ns	5130 (1126 - 19500)	ns	6995 (4769 - 18526)	ns
MMP-8	1291 (<222- 3731)	0.000304 (↑)	2479 (1365 - 10127)	0.001 (↑)	601 (216- 1845)	0.012 (↓)
MMP-9	3633 (458 - 7879)	ns	4029 (521 - 10981)	ns	989 (387 - 1714)	0.012 (↓)
MMP-12	<29.8 (<29.8 - 382)	ns	<29.8 (<29.8 - 428)	ns	<29.8 (<29.8 - 475)	ns
MMP-13	<220 (<220 - 381)	0.000489 (↓)	<220 (<220 - 315)	ns	<220 (<220 - 216)	ns
Metalloprotease inhibitors						
TIMP-1	127345 (61886 - 191929)	0,000001 (↑)	115104.9 (93523 - 193347)	ns	119504 (91655 - 1466560)	ns
TIMP-2	101000 (59798 - 136151)	0.003 (↑)	104648 (87344 - 134799)	ns	100457 (76774 - 144122)	ns
TIMP-3	25027 (12267 - 36426)	0.000162 (↑)	26965 (20228 - 29454)	ns	26324 (20228 - 31852)	ns
TIMP-4	2631 (739 - 7606)	0.000041 (↑)	2056 (498- 4448)	0.011 (↓)	2605 (1079 - 5121)	ns



Supplementary Figure 1
Unsupervised hierarchical cluster analyses based on plasma mediator levels immediately after apheresis. All color schemes are presented in the lower part of the figure. The figure shows the heat map with corresponding dendrograms. Red color in the donor/mediator columns of the heat maps indicates levels higher than the corresponding median level for each mediator, whereas green color indicates low levels compared to the median. The dendrogram showing the horizontal mediator clustering is presented at the top of the heat map, and each mediator is marked with its original yellow or dark grey color from the pre-treatment clustering analysis (see Figure 2A. Two columns to the left are marked with Donor ID and Apheresis Device, respectively. These two columns indicate (i) the two main donor clusters from the analysis of pre-treatment samples (see Figure 2A/upper; pigeon grey and white color), and (ii) the apheresis device used for each donor (Cobe Spectra in yellow, and Spectra Optia in blue).

‡Apheresis effect. The upper horizontal row between the heat map and the mediator clustering indicates the apheresis effect on the plasma concentration of each individual mediator. Significant increased (Wilcoxon's test for paired samples, beige color) and decreased mediator levels (turquoise color) during G-CSF therapy are indicated.

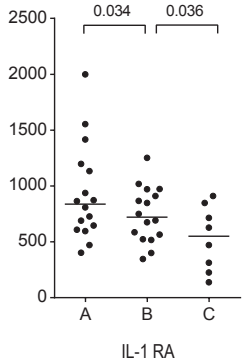
†Donor cluster difference. The lower horizontal row between the heat map and the mediator clustering indicates single mediators that differed significantly between the two donor clusters and they are marked with purple color.



Supplementary Figure 2
Unsupervised hierarchical cluster analyses based on plasma mediator levels 24 hours after start of apheresis. All color schemes are presented in the lower part of the figure. The figure shows the heat map with corresponding dendrograms. Red color in the donor/mediator columns of the heat maps indicates levels higher than the corresponding median level for each mediator, whereas green color indicates low levels compared to the median. The dendrogram showing the horizontal mediator clustering is presented at the top of the heat map, and each mediator is marked with its original yellow or dark grey color from the pre-treatment clustering analysis (see Figure 2A. Two columns to the left are marked with Donor ID and Apheresis Device, respectively. These two columns indicate (i) the two main donor clusters from the analysis of pre-treatment samples (see Figure 2A/upper; pigeon grey and white color), and (ii) the apheresis device used for each donor (Cobe Spectra in yellow, and Spectra Optia in blue).

#G-CSF and apheresis effect. The upper horizontal row between the heat map and the mediator clustering indicates the overall effect of G-CSF therapy and apheresis on single mediator levels. Beige color indicates G-CSF induced concentration rise not significantly or modestly modified by apheresis and still present 24 hours after termination of G-CSF therapy (Wilcoxon's test for paired samples). Turquoise color indicates significant increasing effect of G-CSF followed by concentration decrease during apheresis.

†Donor cluster difference. The lower horizontal row between the heat map and the mediator clustering indicates single mediators that differed significantly between the two donor clusters and they are marked with purple color



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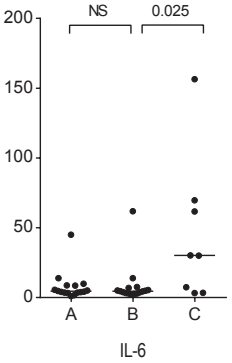

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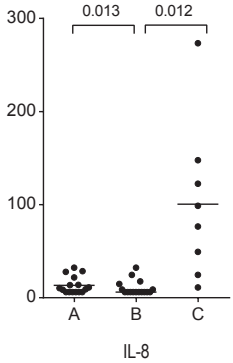

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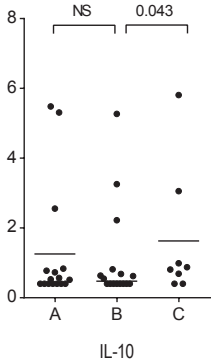

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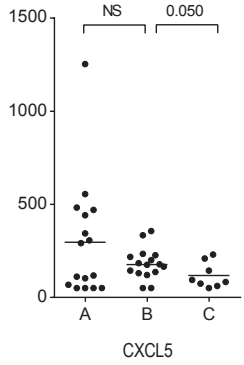

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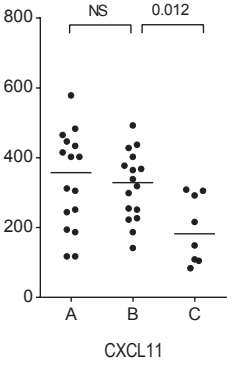

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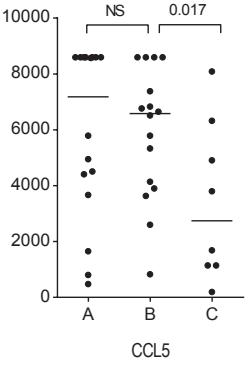
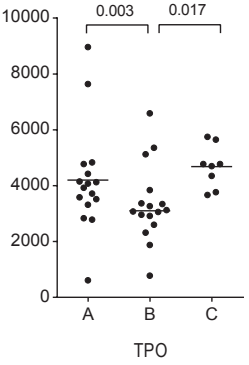
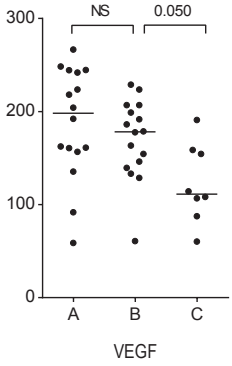


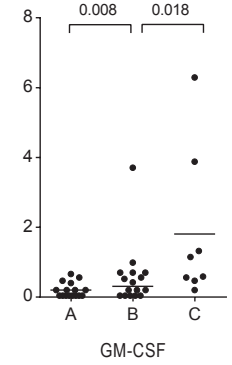
CCL5



TPO



VEGF



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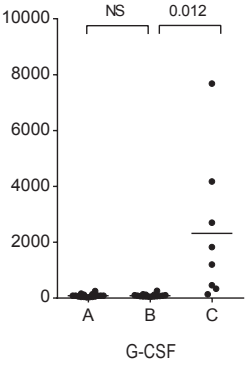
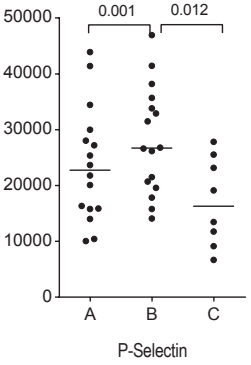
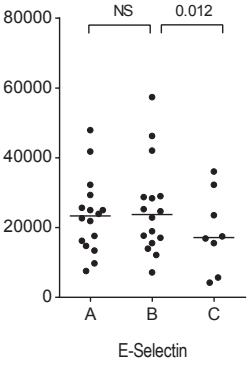


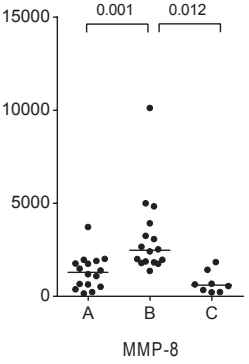
G-CSF



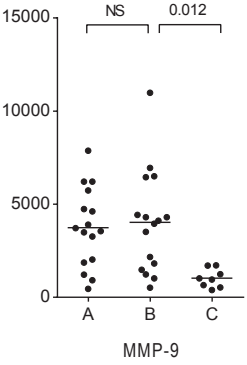
P-Selectin



E-Selectin



MMP-8



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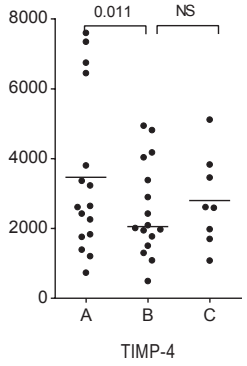

TIMP-4

Supplementary Figure 3
The effect of stem cell infusion on allogeneic stem cell recipient plasma mediator levels. The mediators showing the most significant changes are presented (see Table 4). Pre-transplant mediator levels were determined at 9 am on the day of transplantation (A), early post-transplant levels at 9 am the first day after transplantation (B) and late post-transplant level median day 6 post-transplant (range: day 4–13) (C). The individual values (pg/ml) and medians for 16 allogeneic stem cell recipients are presented, with the p-values shown at the top.

III

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MDPI

Article

Immunomodulation Induced by Stem Cell Mobilization and Harvesting in Healthy Donors: Increased Systemic Osteopontin Levels after Treatment with Granulocyte Colony-Stimulating Factor

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Abstract: Peripheral blood stem cells from healthy donors mobilized by granulocyte colony-stimulating factor (G-CSF) and harvested by leukapheresis are commonly used for allogeneic stem cell transplantation. The frequency of severe graft versus host disease is similar for patients receiving peripheral blood and bone marrow allografts, even though the blood grafts contain more T cells, indicating mobilization-related immunoregulatory effects. The regulatory phosphoprotein osteopontin was quantified in plasma samples from healthy donors before G-CSF treatment, after four days of treatment immediately before and after leukapheresis, and 18–24 h after apheresis. Myeloma patients received chemotherapy, combined with G-CSF, for stem cell mobilization and plasma samples were prepared immediately before, immediately after, and 18–24 h after leukapheresis. G-CSF treatment of healthy stem cell donors increased plasma osteopontin levels, and a further increase was seen immediately after leukapheresis. The pre-apheresis levels were also increased in myeloma patients compared to healthy individuals. Finally, in vivo G-CSF exposure did not alter T cell expression of osteopontin ligand CD44, and in vitro osteopontin exposure induced only small increases in anti-CD3- and anti-CD28-stimulated T cell proliferation. G-CSF treatment, followed by leukapheresis, can increase systemic osteopontin levels, and this effect may contribute to the immunomodulatory effects of G-CSF treatment.

Keywords: allogeneic transplantation; hematopoietic stem cell mobilization; granulocyte colony-stimulating factor; osteopontin; apheresis

1. Introduction

Osteopontin is a glycosylated phosphoprotein synthesized and secreted by various cells [1]. The ability to interact with several cell surface receptors, including certain integrins and CD44, makes

osteopontin a functional regulator of cell adhesion, migration, and survival for a wide range of cells [1]. Binding of osteopontin to the intracellular part of CD44 is important for cytoskeletal functions [2,3], transcriptional regulation, and anti-apoptotic signaling in normal and malignant cells [1,4–6]. Finally, osteopontin is important for normal hematopoiesis and is a component of the hematopoietic stem cell niche, where it regulates the location and cycling of normal stem cells [7,8].

Osteopontin is widely expressed by immunocompetent cells and upregulated both during inflammation and in various tumors [1,9–15]. It has pro-inflammatory effects by stimulating chemotaxis of various immunocompetent cells and by increasing pro-inflammatory cytokine release from macrophages [9] and expression of antigen-presenting and costimulatory molecules by dendritic cells [16]. It is also important for B cell proliferation and immunoglobulin production and is released by activated B cells and T cells as a Th1-associated cytokine [17–19]. However, osteopontin may also have anti-inflammatory effects [1], as observed both in animal models [19,20] and human disease [20,21].

Osteopontin is also important for growth regulation of acute lymphoblastic, and probably also acute myeloid leukemia, cells located at the endosteal stem cell niche [22,23]. Studies in humans have demonstrated that plasma osteopontin levels can reflect local inflammation [24] as well as tumor hypoxia and, thereby, chemo-sensitivity [25].

Systemic administration of granulocyte colony-stimulating factor (G-CSF) is commonly applied to mobilize hematopoietic stem cells for collection by leukapheresis [26–28]. Several apheresis systems have been developed for efficient harvesting of mononuclear cells [29–31]. Peripheral blood stem cell grafts are widely used for allogeneic and autologous hematopoietic stem cell transplantation (allo- and auto-HSCT) in hematological diseases, solid tumors and immune disorders [26,32–36], and increasingly in autoimmune and non-malignant gastrointestinal diseases [37–39]. Additionally, G-CSF mobilized progenitor cells are applicable in regenerative medicine and immunotherapy, and have, e.g., been tried in coronary and limb ischemia, as a possible source for differentiation of dendritic cells and for isolation of mesenchymal stromal cells [40–44].

One important complication associated with allo-HSCT is acute graft versus host disease (acute GVHD). The risk of acute GVHD seems to be similar for peripheral blood and bone marrow allografts [45], suggesting that the potentially adverse effect of the larger number of donor T cells in peripheral blood allografts is counteracted by immunomodulation of graft T cells during mobilization or harvesting.

Animal models suggest that osteopontin stimulates $CD8^+$ T cell-mediated GVHD [46]. This effect may be caused either by pre-transplant modulation of immunocompetent cells in the allogeneic stem cell grafts, or by post-transplant modulation caused by osteopontin in the graft supernatant or osteopontin released in the recipient. Osteopontin has several immunomodulatory effects, and in this context we investigated the levels of osteopontin in autologous and allogeneic stem cell donors and stem cell grafts during mobilization/harvesting and in allogeneic stem cell recipients following graft infusion.

2. Results

2.1. Plasma Osteopontin Levels of Healthy Stem Cell Donors Increase during Granulocyte Colony-Stimulating Factor (G-CSF) Treatment and Reach a Maximal Level Immediately Following Stem Cell Harvesting by Leukapheresis

The median plasma osteopontin levels in healthy allogeneic stem cell donors prior to G-CSF therapy was 45 ng/mL (variation range: 27–62 ng/mL), see Table 1 and Figure 1. During G-CSF treatment, and immediately prior to leukapheresis, the osteopontin concentration in the stem cell donors was increased to a median level of 50 ng/mL (range: 19–75 ng/mL, $p = 0.008$). The healthy allogeneic stem cell donors were compared to a group of 15 healthy platelet donors who did not receive any kind of treatment prior to the apheresis. These healthy platelet donors showed no significant differences compared to the healthy stem cell donors with respect to age, gender distribution, or baseline white blood cell counts (Table 2). The pre-apheresis osteopontin concentrations of the platelet

donors (median 44 ng/mL; range: 28–60 ng/mL) did not differ from the pre-treatment levels of the allogeneic stem cell donors either (Table 1).

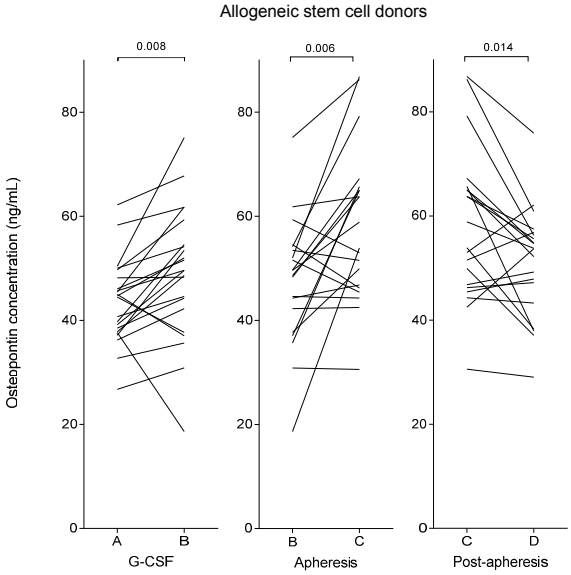

Figure 1. Plasma osteopontin levels in healthy allogeneic stem cell donors during stem cell mobilization and harvesting. Peripheral blood plasma osteopontin concentrations were determined prior to stimulation with granulocyte colony-stimulating factor (G-CSF) (A), after stem cell mobilization and immediately prior to apheresis (B), immediately after apheresis (C) and approximately 24 h after start of apheresis (D).

Table 1. The effect of granulocyte colony-stimulating factor (G-CSF) treatment, apheresis procedures and allogeneic stem cell transplantation on plasma osteopontin (OPN; **Upper part**) and G-CSF (**Lower part**) concentration. (**Upper part**) From the top, the plasma OPN levels are presented for the four study groups: (i) prior to and after G-CSF treatment of allogeneic stem cell donors; (ii) immediately before and after apheresis and in the apheresis product for each study group undergoing apheresis; and (iii) in allotransplanted patients 8–12 h prior to start of stem cell infusion and 12–16 h after infusion; (**Lower part**) Plasma G-CSF concentrations are given for allogeneic stem cell donors prior to and after G-CSF treatment and for autologous stem cell donors only after the G-CSF therapy. All concentrations are given as medians with variation ranges in parentheses.

Patients/Donors	Procedure	Pre-Procedure OPN (ng/mL)	Post-Procedure OPN (ng/mL)	*p* Value	Apheresis Product OPN (ng/mL)
Allogeneic stem cell donors	G-CSF stimulation	45 (27–62)	50 (19–75)	0.008	-
	Stem cell apheresis	50 (19–75)	56 (31–87)	0.006	53 (29–73)
Autologous stem cell donors	Stem cell apheresis	89 (41–356)	109 (55–473)	0.008	86 (7–328)
Healthy platelet donors	Platelet apheresis	44 (28–60)	46 (33–56)	NS	48 (25–75) [1]
Allogeneic HSC recipients	Allogeneic stem cell transplantation	126 (80–438)	103 (72–260)	NS	Not applicable
Patients/Donors	**Procedure**	**Pre-Procedure G-CSF (pg/mL)**	**Post-Procedure G-CSF (pg/mL)**	***p* Value**	**Apheresis Product G-CSF (pg/mL)**
Allogeneic stem cell donors	G-CSF stimulation	50 (22–241)	10,780 (3687–31,947)	0.0003	6673 (1704–21,152)
Autologous stem cell donors	G-CSF stimulation	Not determined	18,366 (9861–46,314)	Not determined	12,906 (8863–41,139)

[1] The osteopontin values measured in platelet concentrate supernatants were adjusted for dilution of the products with platelet additive solution (37% plasma, 63% T-sol). NS, not significant.

Table 2. Clinical and biological characteristics of healthy stem cell donors, autotransplanted myeloma patients, healthy platelet donors, and allotransplant recipients. Number of individuals, age, and gender (M: male, F: female) are presented for each study group. Median basal white blood cell counts (WBC $\times$ 10^9/L) are given for the study groups undergoing apheresis. White blood cell counts and peripheral blood (PB) concentrations of $CD34^+$ stem cells before start of apheresis and yield of $CD34^+$ stem cells are given for G-CSF stimulated allogeneic and autologous donors (multiple myeloma patients). All values are presented as medians with the variation ranges given in parentheses.

Group	Age	Gender (M/F)	Total White Blood Cell Count in the Grafts		$CD34^+$ Cells after G-CSF Treatment	
			Baseline Level ($\times 10^9$/L)	After G-CSF ($\times 10^9$/L)	PB Level ($\times 10^5$/mL)	Yield ($\times 10^6$/kg)
Allogeneic stem cell donors (n = 22)	51 (25–77)	14/8	5.9 (3.1–13.4)	46.0 (30.1–76.3)	44.1 (16.7–147.8)	5.4 (0.8–22.4)
Autologous stem cell donors (n = 15)	57 (44–67)	9/6	5.4 (2.5–9.0)	10.8 (2.7–43.7)	39.9 (9.7–175.0)	5.3 (1.1–27.9)
Platelet donors (n = 15)	47 (26–62)	8/7	6.0 (4.7–13.5)	-	-	-
Allogeneic HSCT recipients (n = 16)	47 (35–63)	7/9	-	-	-	-

HSCT, hematopoietic stem cell transplantation.

The G-CSF-treated allogeneic stem cell donors showed a further increase of the median osteopontin concentration to 56 ng/mL (range: 31–87 ng/mL, $p = 0.008$, Table 1) immediately after leukapheresis, but 18–24 h after start of apheresis the median level had declined to 54 ng/mL (range: 29–76 ng/mL, $p = 0.014$, Figure 1). In contrast, the control group of healthy platelet donors showed stable osteopontin levels throughout the observation period without significant altered concentrations immediately after apheresis or 18–24 h after start of apheresis (Table 1).

Plasma G-CSF concentrations in allogeneic stem cell donors prior to and after mobilization were also investigated. The median pre-treatment G-CSF level was 50 pg/mL (range: 22–241 pg/mL) and after four days of G-CSF it was 10,780 pg/mL (range: 3687–31,947 pg/mL); see lower part of Table 1. G-CSF and osteopontin levels then showed no significant correlation.

There were no significant associations between osteopontin plasma levels and apheresis time (median: 305 min; range: 231–377 min) the absolute number of total blood volumes processed during apheresis (median: 3.6; range: 1.6–6.6), or apheresis device applied.

2.2. Plasma Osteopontin Levels Show an Inverse Correlation with Peripheral Blood Neutrophil Levels during G-CSF Therapy but No Association with Peripheral Blood Levels or Yields of $CD34^+$ Cells

We used simple linear regression analyses with one way analysis of variance (ANOVA) to study the correlation between healthy stem cell donor osteopontin levels (all donors included in the analysis) and the corresponding peripheral blood levels of total leukocytes (Table 2) and leukocyte subsets. Plasma osteopontin levels immediately prior to leukapheresis showed significant inverse correlations with the corresponding peripheral blood neutrophil counts (median: 38.5 $\times$ 10^9/L; range: 24.3–66.4 $\times$ 10^9/L; $R^2 = 0.381$; $p = 0.002$) and total peripheral blood leukocyte counts (median: 46.0 $\times$ 10^9/L; range: 30.1−76.3 $\times$ 10^9/L; $R^2 = 0.366$; $p = 0.003$). With this exception, there were no significant associations between osteopontin levels and the total leukocyte counts or the levels of neutrophils, monocytes, total lymphocytes, $CD3^+$ lymphocytes, or $CD34^+$ cells in peripheral blood or in the stem cell graft at any other time point.

2.3. Myeloma Patients (Autologous Stem Cell Donors) Show Increased Plasma Osteopontin Levels after G-CSF Therapy Compared with Healthy Allogeneic Stem Cell Donors

Plasma samples from myeloma patients receiving G-CSF therapy for mobilization of autologous stem cells were available only immediately before leukapheresis (after five days of G-CSF treatment); the plasma osteopontin levels then showed a wide variation and were significantly increased for

the myeloma patients (median 89 ng/mL; range 41–356 ng/mL) compared with the pre-apheresis levels of the healthy stem cell donors (Mann-Whitney U test, $p = 0.001$). As presented in Table 1 (lower part), the pre-harvesting G-CSF levels were also significantly higher for myeloma patients (median 18,366 pg/mL; range 9861–46,314 pg/mL) than for the healthy stem cell donors (median: 10,780 pg/mL; range: 3687–31,947 pg/mL; $p = 0.005$). There was no significant correlation between pre-harvesting G-CSF and osteopontin plasma levels in the myeloma patients. As shown in Table 1 and Figure 2, myeloma patients had a significant increase in plasma osteopontin level during apheresis, but the increase in median osteopontin level 24 h after apheresis did not reach statistical significance.

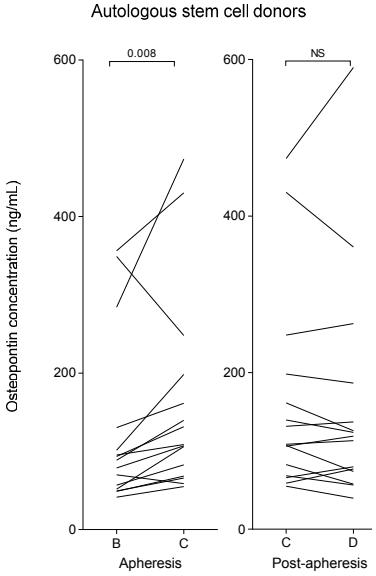

Figure 2. Plasma osteopontin levels in autologous stem cell donors (myeloma patients) after stem cell mobilization and immediately prior to apheresis (B), immediately after apheresis (C) and approximately 24 h after start of apheresis (D).

2.4. Osteopontin Levels Are Higher in Autografts from Myeloma Patients than in Allografts from Healthy Stem Cell Donors

We then compared osteopontin concentrations in the apheresis products from autologous and allogeneic stem cell donors and healthy platelet donors. Autologous stem cell grafts from myeloma patients showed significantly higher supernatant osteopontin levels than the allografts ($p = 0.002$) and the platelet concentrates ($p = 0.005$); the results are summarized in Table 1 and presented in detail in Figure 3. The osteopontin levels in auto- and allografts were higher than unstimulated plasma levels in autologous and allogenic donors, but did not differ significantly from the corresponding plasma levels during G-CSF therapy. Due to dilution with platelet additive solution as described in the experimental section, the osteopontin levels in platelet concentrates were lower than the corresponding plasma levels in the platelet donors, and low compared to allogeneic and autologous stem cell grafts (median: 18 ng/mL; range: 10–28 ng/mL). The patients treated with the platelet concentrates thus received relatively low amounts of osteopontin during platelet infusion. However, after correction for the dilution factor, there was no significant difference between osteopontin levels in platelet concentrates and stem cell grafts from healthy donors or between platelet concentrates and peripheral blood samples from the platelet donors (Table 1, Figure 3).

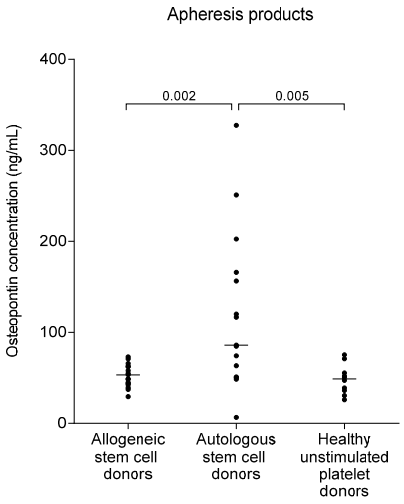

Figure 3. Osteopontin levels in apheresis products, i.e., peripheral blood stem cell grafts and platelet concentrates. The osteopontin levels were determined in allogeneic stem cell products from G-CSF-mobilized healthy stem cell donors (n = 22), autologous stem cell products derived from myeloma patients mobilized by chemotherapy plus G-CSF (n = 15), and platelet concentrates from unstimulated healthy platelet donors (n = 15). The osteopontin levels measured in platelet concentrate supernatants were adjusted for dilution of the products with platelet additive solution (37% plasma, 63% solution).

2.5. Pretransplant Osteopontin Levels of Allotransplant Recipients Are Increased and the High Levels Are Not Altered Following the Infusion of Osteopontin-Containing Stem Cell Grafts

The pre-transplant osteopontin levels in allotransplant recipients were high (median: 126 ng/mL; range: 80–438 ng/mL) and were significantly higher than the levels in healthy individuals ($p < 0.001$; see Table 1), and even higher than for the myeloma patients ($p = 0.02$). The infusion of the osteopontin-containing allograft did not alter the plasma levels significantly; the levels remained high in the allotransplant recipients both when tested one day post-transplant and for eight patients also tested later after the transplantation (median: six days after infusion; range: 4–13 days).

Additional analyses showed no association between recipient osteopontin plasma levels (Table 1) and (i) patient age and gender; (Table 2) (ii) allograft content of leukocytes, $CD34^+$ stem cells, $CD3^+$ T cells, neutrophils, monocytes, lymphocytes or platelets measured as absolute numbers or as the number of cells per kg patient body weight (Table 3).

As presented in Table 3, the median time until neutrophil reconstitution with peripheral blood neutrophil counts above 0.5×10^9/L on the first of three consecutive days was day +17 (range: day +13 to +28). Furthermore, the median time of platelet counts above 50×10^9/L for the first of three consecutive days was day +15 (range: day +11 to +39). There was no significant association between osteopontin levels and time until hematopoietic reconstitution. Finally, for the 16 patients investigated acute GVHD grade II–IV was seen in two patients, early death before day +100 in four patients, chronic GVHD in nine patients, and leukemia relapse in four patients. These observations suggest that our 16 patients are representative for allotransplanted patients.

Table 3. Allogeneic stem cell grafts derived from healthy donors; the levels of various cells in the grafts and the post-transplant clinical course of the allotransplant recipients. The cell content of the stem cell grafts infused to 16 allotransplant recipients is presented as the absolute numbers in the graft (graft content) and as the infused cell doses per kg (infused cells).

Cell Type	Graft Content ($\times 10^8$)	Infused Cells ($\times 10^6$/kg)	Post-Transplant Course [1]	
Total WBC	791 (342–2495)	109 (376–3054)	Neutrophil reconstitution	17 (13–28)
$CD34^+$ stem cells	4.6 (2.4–6.7)	5.5 (3.3–6.8)	Platelet reconstitution	15 (11–39)
$CD3^+$ T cells	278 (71–490)	39 (10–61)	aGVHD	2/16
Neutrophils	285 (112–1048)	45 (15–133)	cGVHD	9/16
Monocytes	127 (18–563)	16 (3–69)	Early death	4/16
Lymphocytes	346 (105–759)	50 (14–96)	Relapse	4/16
Platelets	7068 (3176–11,449)	9607 (3655–14,260)	-	-

[1] Neutrophil and platelet reconstitution is given as the first of three consecutive days after the transplantation with neutrophil counts above 0.5×10^9/L and platelet transfusion independence with platelet counts above 50×10^9/L. aGVHD: acute graft versus host disease grade II–IV, cGVHD: chronic graft versus host disease, early death: defined as death before day +100 after transplantation, WBC: white blood cell count. All values are presented as medians with the variation ranges given in parentheses or as fractions of the total number of 16 patients.

2.6. T and B Lymphocytes Show High Expression of the CD44 Osteopontin Receptor and these High Levels Are Maintained during Stem Cell Mobilization and Harvesting

Interaction between osteopontin and the CD44 receptor mediates chemotaxis of lymphocytes and macrophages [47]. We investigated the expression of CD44 by viable donor lymphocytes during stem cell mobilization and harvesting; the receptor was generally highly expressed and all comparisons are therefore based on the mean fluorescence intensity (MFI), see Figure 4. In $CD19^+$ B cells MFI was reduced from 31,869 to 25,519 (mean values, n = 15) during G-CSF stimulation (p = 0.022). No significant G-CSF induced change in CD44 expression was detected in $CD3^+$ T cell populations; neither was there any significant effect of apheresis on CD44 expression in T and B cells. T cell and B cell CD44-APC MFI did not show any significant correlation to plasma levels of osteopontin or G-CSF at any sampling point.

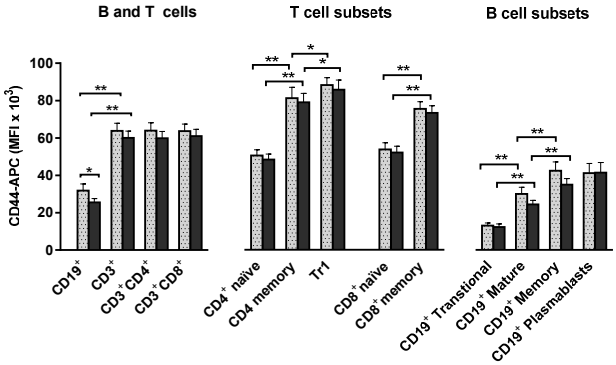

Figure 4. Expression of CD44 in unstimulated (grey-colored bars) and in vivo G-CSF stimulated (black-colored bars) peripheral blood leukocytes from healthy allogeneic stem cell donors. The results are presented as the mean fluorescence intensity (MFI) given as mean values ± standard error of the mean (SEM). (**Left**): The results for $CD19^+$ B cells and $CD3^+$ T cells with $CD4^+$ and $CD8^+$ main subsets are shown; (**Middle**): $CD4^+$ and $CD8^+$ naïve ($CD45RA^+$) T cell subsets are compared with the corresponding T cell memory ($CD45RA^-$) subsets and with T regulatory type 1 (Tr1) cells ($CD4^+$ $CD45RA^-CD49b^+$ $LAG\text{-}3^+$); (**Right**): Transitional B cells ($CD19^+CD24^{hi}CD38^{hi}$) together with mature ($CD19^+CD24^+CD38^+$) and memory ($CD19^+CD24^{hi}38^-$) B-cells and plasmablasts ($CD19^+CD24^{low}CD38^{hi}$) are presented. Statistically significant differences are indicated (** p = 0.001, * p = 0.05).

CD44 expression was consistently higher for $CD3^+$ T cells than for $CD19^+$ B cells; as expected, both $CD4^+$ and $CD8^+$ $CD45RA^-$ memory T cells showed significantly higher CD44 expression than $CD45RA^+$ naïve T cells (Figure 4). Particularly high CD44 expression was found in the subset of $CD49b^+$ LAG-3^+ Tr1 cells (lymphocyte activation gene-3 positive T regulatory type 1 cells) [48].

We also compared CD44 expression in the main $CD19^+$ B cell subsets [49], in unstimulated and G-CSF stimulated peripheral blood mononuclear cells (PBMC) samples. Compared to the $CD24^+CD38^+$ mature subset, transitional $CD24^{hi}CD38^{hi}$ cells showed significantly lower and $CD24^{hi}38^-$ memory B cells significantly higher CD44 expression. $CD19^+CD24^{low}CD38^{hi}$ plasmablasts showed high CD44 expression similar to B memory cells [50].

To summarize, in vivo G-CSF therapy resulted in a modest reduction in CD44 expression in B cells exclusively, and apheresis procedures did not alter T and B cell CD44 expression significantly.

2.7. Osteopontin Causes a Minor Increase of in Vitro Proliferative T Cell Responses

The effect of exogenous osteopontin on T cell proliferative responses was investigated for eight healthy individuals (Figure 5). PBMC were cultured in vitro in the presence of anti-CD3 and anti-CD28. We compared the proliferative responses for cultures prepared in medium alone and cultures with osteopontin 50 ng/mL, i.e., the osteopontin level corresponding to the plasma level in healthy stem cell donors (see Table 1). Osteopontin increased T cell proliferation, but this increase usually corresponded to less than 20% of the corresponding control cultures both when osteopontin was tested in culture medium without G-CSF and medium supplemented with G-CSF.

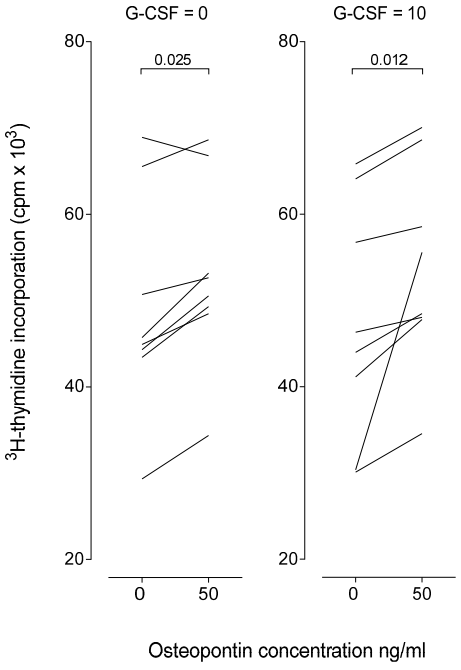

Figure 5. Peripheral blood mononuclear cells (PBMC) from eight healthy unstimulated donors were cultured in serum-free medium and stimulated with anti-CD3 and anti-CD28. The effect of osteopontin 50 ng/mL without G-CSF (**left**) and with G-CSF 10 pg/mL (**right**) on in vitro T cell proliferation was assayed as ^{3}H-thymidine incorporation expressed as median counts per minute (cpm). The proliferation of normal PBMC in control cultures containing isotypic control antibodies instead of anti-CD3/anti-CD28 antibodies corresponded to <1000 cpm.

3. Discussion

Osteopontin can mediate both pro- and anti-inflammatory effects through its binding to specific receptors expressed by various immunocompetent cells [20,21]. In the present study we describe that systemic osteopontin levels are altered during stem cell mobilization and harvesting. Elevated osteopontin levels are detected in the stem cell grafts, and we hypothesize that osteopontin may thereby affect the immunocompetent cells in the grafts.

Some of the statistically significant differences in osteopontin plasma levels described in our present study were relatively small. However, the biological day-to-day variation, time of day variation, and week-to-week variation in osteopontin level in healthy blood donors has been shown to be low [51]. Furthermore, several previous studies have demonstrated that differences corresponding to 15%–25% of control levels reflect differences of biological and clinical significance, e.g., in cancer patients and cardiovascular disease patients [52–54]. These observations suggest that even relatively small variations in plasma osteopontin levels may have a clinical/biological relevance. Our own observations are also in agreement with these previous observations, e.g., we had similar results in base-line samples for our two independent groups of healthy individuals.

Our present study compared plasma osteopontin levels in two independent groups of healthy individuals (G-CSF treated stem cell donors, untreated platelet donors) undergoing apheresis with or without G-CSF stimulation. Osteopontin concentrations increased during G-CSF treatment, and the levels showed a further increase after leukapheresis/stem cell harvesting. This was a transient effect and osteopontin levels decreased during the 24 h period post harvesting. On the other hand, the control group of healthy untreated platelet donors showed stable osteopontin levels with no detectable effect of the apheresis.

We also compared the healthy allogeneic stem cell donors with a group of myeloma patients receiving G-CSF treatment for mobilization of autologous stem cells; the myeloma patients then showed higher pre-harvesting osteopontin levels and a similar increase as the healthy donors following leukapheresis. The higher pre-harvesting osteopontin concentrations in myeloma patients may be due to the combination of G-CSF and chemotherapy for autologous stem cell mobilization in these patients and five days of treatment with G-CSF in contrast to four days of treatment in the allogeneic donors. Alternatively, the difference could be disease dependent; increased levels in myeloma patients are associated with disease burden and decrease when patients respond to anti-myeloma treatment [55,56]. It should be emphasized that only a minority of our patients achieved a complete response prior to the autologous stem cell harvesting.

Samples drawn prior to G-CSF therapy were not available from our myeloma patients. In a recent study of myeloma patients mobilized for stem cell harvest, no significant effect of G-CSF on osteopontin levels could be detected [57]. However, as the regulation of the osteopontin concentration during stem cell mobilizing in these patients is complex and influenced by both disease stage and chemotherapy [55], possible effects of G-CSF might be difficult to detect.

Thus, the effect of apheresis (and possibly the effect of G-CSF treatment) on osteopontin levels is not only seen in healthy donors, but also in myeloma patients. However, the levels were not altered in healthy blood donors undergoing unstimulated thrombapheresis, which suggests that this is probably an effect induced by the G-CSF therapy and not a general effect of all kinds of apheresis procedures. This is further supported by reports of a relatively high degree of product manipulation and activation in the apheresis device used for platelet collection [58,59]. In contrast to our findings, an eventual effect of apheresis procedures on osteopontin levels would, therefore, be expected to be stronger during platelet collection compared to stem cell apheresis. However, it is not possible to exclude that differences in apheresis techniques between stem cell harvesting and platelet collection (e.g., processed blood volume, separation techniques, anti-coagulation) contributed to the different effects of apheresis on osteopontin levels.

G-CSF treatment both in healthy individuals and myeloma patients caused increased levels of circulating neutrophils that express the osteopontin receptor CD44 [60]. One would, therefore, expect

increased binding of osteopontin to neutrophils during G-CSF treatment, but despite this increased binding we could still detect increased osteopontin plasma levels during the treatment.

A recent study of patients with hematological malignancies described an association between genetic CD44 polymorphisms and the efficiency of $CD34^+$ cell mobilization [61], suggesting that CD44-osteopontin are important regulators of stem cell retention to the bone marrow during G-CSF mobilization, at least in myeloma patients. However, we did not observe any association between osteopontin levels and $CD34^+$ cell mobilization/yield, neither in the myeloma patients, nor in the healthy stem cell donors.

We investigated the osteopontin levels in the graft supernatants. The high pre-harvesting plasma levels and the difference between healthy stem cell donors, myeloma patients, and platelet donors were also reflected in the osteopontin levels in the supernatants. The stem cell transplantation thereby also includes an infusion of osteopontin.

The osteopontin receptor CD44 is widely expressed by immunocompetent cells; the T cell expression was not altered by in vivo G-CSF exposure whereas B cell expression was moderately decreased. Exposure of T cells to osteopontin during in vitro activation caused a slight increase in anti-CD3 + anti-CD28 initiated T cell proliferation. These experiments show that osteopontin can alter T cell responses when tested at concentrations corresponding to the in vivo levels. However, additional studies are required to clarify whether this is a direct stimulatory effect on the proliferating cells, a reduced effect of T regulatory cells or an indirect effect mediated by the accessory cells.

The highest levels of osteopontin were found in allogeneic stem cell transplant recipients at the time of transplantation. The levels were high even compared to myeloma patients who had received both induction therapy and stem cell mobilization, and they were not significantly changed by stem cell transplantation. This observation indicates that high osteopontin concentrations is one of the characteristics of the pro-inflammatory state induced by conditioning therapy and underlying disease in allogeneic stem cell transplant recipients. This pro-inflammatory cytokine balance is considered as an important basis for development of GVHD [45], and osteopontin blockade is shown to reduce $CD8^+$ T-cell mediated GVHD in mice [46]. Our findings suggest greater importance of the osteopontin level in the patient compared to the donor and stem cell graft. The osteopontin levels during conditioning therapy and allogeneic stem cell transplantation in humans and the possible importance for development of GVHD should be studied in further detail in order to evaluate osteopontin as a possible therapeutic target in graft versus host disease.

Previous studies have demonstrated that G-CSF has immunomodulatory effects and can suppress T lymphocytes [62].Such effects are probably important in allotransplant recipients receiving peripheral blood stem cell grafts because the frequency of GVHD is similar for bone marrow and mobilized peripheral blood stem cell grafts even though a higher frequency would be expected for the blood grafts due to their larger number of T cells in these grafts [62]. The molecular mechanisms behind this are not known, but our present study suggests that effects of osteopontin on immunocompetent cells may be a part of the G-CSF-induced immunomodulation in healthy stem cell donors. A better understanding of the mechanisms behind the G-CSF associated immunomodulation will be important for the future development of therapeutic strategies to target graft T cells and thereby reduce the risk of severe GVHD without reducing the graft versus leukemia reactivity.

4. Materials and Methods

4.1. Stem Cell Donors and Allotransplant Recipients

All studies were conducted in accordance with the Declaration of Helsinki and approved by the local ethics committee (REK III No. 126.01, Regional Committee for Medical and Health Research Ethics of Western Norway: 2008/1580, 2011/996, 2011/1237, 2011/1241, and 2013/634) and donors and patients were included after signing a written informed consent. The present studies included (i) 22 consecutive healthy human leukocyte antigen matched (HLA-matched), related, allogeneic

stem cell donors; (ii) 15 consecutive autologous stem cell donors, all patients with newly-diagnosed symptomatic multiple myeloma; (iii) 16 allogeneic stem cell transplant recipients; and (iv) 15 healthy platelet donors (Table 2). The allogeneic stem cell donors did not differ from myeloma patients and healthy platelet donors with regard to age, gender distribution, or initial peripheral blood leukocyte count.

4.2. Stem Cell Mobilization in Healthy Donors and Myeloma Patients

The matched related donors received stem cell mobilizing with human non-glycosylated G-CSF 10 μg/kg per day for four days before stem cell harvesting. Initial induction therapy for the myeloma patients was two cycles of either intravenous cyclophosphamide 1 g/m^2 on day 1 at four weeks intervals (14 patients) or bortezomib 1.3 mg/m^2 on days 1, 4, 8, and 11 at a three-week interval (one patient); both regimens were combined with dexamethasone 40 mg orally on days 1–4 and 9–12. All myeloma patients either responded to the treatment or had stable disease, and stem cells were, thereafter, mobilized with intravenous cyclophosphamide 2 g/m^2 followed by G-CSF 5 μg/kg/day. Peripheral blood leukocyte counts were significantly higher in healthy stem cell donors compared to myeloma patients immediately before stem cell harvesting ($p < 0.001$, Table 2), but the peripheral blood concentration of CD34$^+$ cells did not differ significantly between groups.

4.3. Apheresis Procedures

Stem cell quantification was started on day 4 or 5 of G-CSF stimulation for stem cell donors and myeloma patients, respectively. For the myeloma patients this corresponded to day 10 after the start of cyclophosphamide. Stem cell harvest was performed when the stem cell count exceeded 15–20 $\times$ 10^3/mL. Large-volume leukapheresis with four times processing of the total blood volume on a Cobe Spectra cell separator, version 7 (Cobe Laboratories, Gloucester, UK) was used for nine of the healthy stem cell donors and all the myeloma patients; the other 13 healthy stem cell donors were harvested with a Spectra Optia cell separator, version 9 (Terumo BCT Inc., Lakewood, CO, USA). The automated mononuclear cells (MNC) procedure was used in accordance with the instructions from the manufacturer. The yield of CD34$^+$ cells per kg bodyweight obtained by apheresis and the white blood cell count in the apheresis product did not differ significantly between groups. Finally, single-donor platelet concentrates from unstimulated healthy volunteer donors were prepared with a Fenwal Amicus cell separator (Baxter Healthcare Corp., Deerfield, IL, USA) and leukocyte-reduction provided by elutriation. The platelets were suspended in 37% plasma and 63% platelet additive solution (T-sol, Baxter Healthcare Corp.) as described in detail previously [63,64].

4.4. Allogeneic Stem Cell Transplantation

Eleven of the 16 allotransplant recipients were diagnosed with acute myeloid leukemia (AML), three with acute B cell lymphoblastic leukemia (B-ALL), one with myelofibrosis and one with myelodysplastic syndrome (MDS). All leukemia patients were in complete hematological remission at the time of transplantation. The patients received (i) myeloablative conditioning with intravenous busulfan plus cyclophosphamide and mesna (14 patients); or (ii) reduced intensity conditioning with intravenous fludarabine plus busulfan (two patients). All patients were transplanted with G-CSF mobilized peripheral blood stem cell grafts derived from HLA-matched family donors and received graft versus host disease (GVHD) prophylaxis with cyclosporine A, plus methotrexate. Neutrophil reconstitution was defined as neutrophil counts exceeding 0.2/0.5 $\times$ 10^9/L for at least three consecutive days, and platelet reconstitution as at least three consecutive days with stable platelet counts exceeding 20/50 $\times$ 10^9/L.

4.5. Preparation of Plasma and Peripheral Blood Mononuclear Cells (PBMC)

4.5.1. Blood Sampling

Venous blood samples from the allogeneic stem cell donors were collected (A) prior to G-CSF stimulation at the time of the pre-transplant evaluation (median 20.5 days before apheresis). For the three study groups undergoing apheresis, blood samples were also drawn (B) in the morning immediately before apheresis, (C) immediately after apheresis, and (D) approximately 24 h after start of apheresis. All venous blood samples from allotransplant recipients were collected between 07:00 and 09:00. Samples for plasma preparation were collected into Vacuette 9NC tubes and samples for cell preparation into acid-citrate-dextrose solution A (ACD-A) tubes with sodium citrate and acid-citrate-dextrose solution A as anticoagulants (Greiner Bio-One GmbH, Kremsmünster, Austria). Samples from stem cell allo- and autografts and platelet concentrates were transferred to plastic tubes without additives.

4.5.2. Preparation of Plasma Samples

The blood samples were centrifuged at 2000× *g* (myeloma patients and platelet donors) or 1310× *g* (allotransplant recipients) for ten minutes at room temperature within 30 min of sampling. The supernatants were immediately transferred to plastic tubes, frozen, and stored at −70 °C until analyzed.

4.5.3. Preparation of PBMC Samples

After isolation by density gradient separation (Lymphoprep, AXIS-SHIELD PoC AS, Oslo, Norway; specific density: 1.077 g/mL), PBMC were dissolved in RPMI 1640 medium supplemented with 2 mmol/L L-glutamine, penicillin 100 IE/mL, streptomycin 0.1 mg/mL (Sigma-Aldrich, St. Louis, MO, USA), and 20% fetal bovine serum (FBS, Biowest, Nuaillé, France). 10% dimethyl sulfoxide (DMSO, Sigma-Aldrich, St. Louis, MO, USA) was used as cryoprotectant, and the vials were stored in liquid nitrogen at −150 °C after gradual cooling to −80 °C in Mr. Frosty Freezing Container (Thermo Fisher Scientific, Waltham, MA, USA).

4.6. Analysis of Plasma Osteopontin and G-CSF Concentrations

Plasma osteopontin levels were determined by enzyme-linked immuno-sorbent assays (ELISA) (Quantikine ELISA Human Osteopontin (OPN) Immunoassay from R&D Systems, Minneapolis, MN, USA). Plasma G-CSF concentrations were determined by Luminex analyses (R&D Systems, Minneapolis, MN, USA). All samples were analyzed in duplicates, strictly according to the manufacturer's instructions.

4.7. Flow Cytometry Analyses

PBMC were thawed in a 37 °C water bath, dissolved in supplemented RPMI 1640 medium, and incubated for one hour (37 °C, a humidified atmosphere of 5% CO_2) before incubation with near-IR fluorescent reactive dye (LIVE/DEAD Fixable Dead Cell Stain Kits, Molecular Probes, Eugene, OR, USA) for 30 min to determine cell viability. After washing in phosphate-buffered saline (PBS) with 1% bovine serum albumin fraction V (BSA, Roche Diagnostics GmbH, Mannheim, Germany) the cells were incubated for 20 min with the following mouse anti-human monoclonal antibodies: CD3-PE-Cy7 (SK7), CD4-PerCP-Cy5.5 (RPA-T4), CD8-V500 (RPA-T8), CD19-PerCP-Cy5.5 (SJ25C1), CD45-RA-V450 (HI100), and CD24-PE-Cy7 (ML5) (all from Becton Dickinson Biosciences-BD Pharmingen, San Diego, CA, USA), rat CD44-Ax 488 (IM7) and mouse CD49b-FITC (P1E6-C5) (both from BioLegend, San Diego, CA, USA), mouse CD38-PB (HIT2; EXBIO, Prague, Czech Republic) and goat LAG-3-PE (FAB2319P; R&D Systems, Minneapolis, MN, USA). Eight-color flow cytometry analysis was performed using a FACS Canto II flow cytometer (Becton Dickinson Biosciences-Immunocytometry Systems; San Jose,

CA, USA). Acquisition of 30,000 $CD3^+$ T cells or 10,000 $CD19^+$ B cells per sample was endeavored, and cytometer performance was monitored daily with Cytometer Setup and Tracking Beads (Becton Dickinson Biosciences-BD Pharmingen, San Diego, CA, USA). The data were analyzed with FlowJo software version X (FlowJo LLC, Ashland, OR, USA).

4.8. Analysis of T-Cell Proliferation by ^{3}H-Thymidine Incorporation

PBMC were cultured in 96-well microtiter plates (5×10^4 cells per well, 190 μL medium per well), the culture medium being X-vivo10® with 100 μg/mL gentamycin (BioWhittaker, Walkersville, MA, USA). The T cells were activated by anti-CD3 (clone CLB-T3/4.E, 1XE, PeliCluster, Sanquin, Amsterdam, The Netherlands; final concentration 316 ng/mL) and anti-CD28 (clone: CLB-CD28/1, 15E8 PeliCluster; final concentration 842 ng/mL). The corresponding control antibodies were purchased from R&D Systems (Abingdon, UK). The medium was supplemented with recombinant human osteopontin 50 ng/mL (R&D Systems, Minneapolis, MN, USA) and eventually recombinant human G-CSF 10 pg/mL (PeproTech EC Ltd., Rocky Hill, NJ, USA). After three days of culture ^{3}H-thymidine (280 kBq per well added in 20 μL of saline; TRA 310, Amersham International, Amersham, UK) was added and cultures harvested 18 h later. The median count per minute (cpm) of nuclear radioactivity for triplicate cultures was used for all calculations.

4.9. Statistical Analyses

The statistical analyses were performed by the standard computer software package IBM SPSS Statistics 22 (IBM Corporate, Armonk, NY, USA). The Wilcoxon's test for paired samples was applied for analyses of paired observations, and the independent samples Mann-Whitney U test for comparison of groups. The covariance between different continuous variables was studied with simple linear regression analyses with one way analysis of variance (ANOVA).

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Author Contributions: Øystein Bruserud, Guro Kristin Melve and Elisabeth Ersvaer conceived and designed the experiments; Guro Kristin Melve and Elisabeth Ersvaer performed the experiments; Guro Kristin Melve analyzed the data; Øystein Bruserud, Tor Hervig, Çiğdem Akalın Akkök, Einar K. Kristoffersen and Guro Kristin Melve contributed reagents/materials/analysis tools; Guro Kristin Melve, Øystein Bruserud, Çiğdem Akalın Akkök, Aymen Bushra Ahmed, Elisabeth Ersvaer, Einar K. Kristoffersen and Tor Hervig wrote the paper.

Conflicts of Interest: The authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

Abbreviations

G-CSF	Granulocyte colony-stimulating factor
CD	Cluster of differentiation
HLA	Human leukocyte antigen
GVHD	Graft versus host disease
OPN	Osteopontin
HSC	Hematopoietic stem cell
HSCT	Hematopoietic stem cell transplantation
MFI	Mean fluorescence intensity
PBMC	Peripheral blood mononuclear cells
LAG-3	Lymphocyte activation gene 3
Tr1 cells	T regulatory type 1 cells
^{3}H	Tritiated hydrogen
MNC	Mononuclear cells

AML	Acute myeloid leukemia
B-ALL	B cell lymphoblastic leukemia
MDS	Myelodysplastic syndrome
ACD-A	Acid-citrate-dextrose solution A
FBS	Fetal Bovine Serum
DMSO	Dimethyl sulfoxide
Near-IR	Near-infrared
PBS	Phosphate-buffered saline
BSA	Bovine Serum Albumin

References

1. Wang, K.X.; Denhardt, D.T. Osteopontin: Role in immune regulation and stress responses. *Cytokine Growth Factor Rev.* **2008**, *19*, 333–345. [CrossRef] [PubMed]
2. Zohar, R.; Zhu, B.; Liu, P.; Sodek, J.; McCulloch, C.A. Increased cell death in osteopontin-deficient cardiac Fibroblasts Occurs by a Caspase-3-Independent Pathway. *Am. J. Physiol. Heart Circ. Physiol.* **2004**, *287*, H1730–H1739. [CrossRef] [PubMed]
3. Suzuki, K.; Zhu, B.; Rittling, S.R.; Denhardt, D.T.; Goldberg, H.A.; McCulloch, C.A.; Sodek, J. Colocalization of intracellular osteopontin with CD44 is associated with migration, cell fusion, and resorption in osteoclasts. *J. Bone Miner. Res.* **2002**, *17*, 1486–1497. [CrossRef] [PubMed]
4. Shinohara, M.L.; Lu, L.; Bu, J.; Werneck, M.B.; Kobayashi, K.S.; Glimcher, L.H.; Cantor, H. Osteopontin expression is essential for interferon-α production by plasmacytoid dendritic cells. *Nat. Immunol.* **2006**, *7*, 498–506. [CrossRef] [PubMed]
5. Hsieh, Y.H.; Juliana, M.M.; Hicks, P.H.; Feng, G.; Elmets, C.; Liaw, L.; Chang, P.L. Papilloma development is delayed in osteopontin-null mice: Implicating an antiapoptosis role for osteopontin. *Cancer Res.* **2006**, *66*, 7119–7127. [CrossRef] [PubMed]
6. Inoue, M.; Shinohara, M.L. Intracellular osteopontin (iOPN) and immunity. *Immunol. Res.* **2011**, *49*, 160–172. [CrossRef] [PubMed]
7. Nilsson, S.K.; Johnston, H.M.; Whitty, G.A.; Williams, B.; Webb, R.J.; Denhardt, D.T.; Bertoncello, I.; Bendall, L.J.; Simmons, P.J.; Haylock, D.N. Osteopontin, a key component of the hematopoietic stem cell niche and regulator of primitive hematopoietic progenitor cells. *Blood* **2005**, *106*, 1232–1239. [CrossRef] [PubMed]
8. Li, S.; Zhai, Q.; Zou, D.; Meng, H.; Xie, Z.; Li, C.; Wang, Y.; Qi, J.; Cheng, T.; Qiu, L. A pivotal role of bone remodeling in granulocyte colony stimulating factor induced hematopoietic stem/progenitor cells mobilization. *J. Cell. Physiol.* **2013**, *228*, 1002–1009. [CrossRef] [PubMed]
9. Sodek, J.; Batista Da Silva, A.P.; Zohar, R. Osteopontin and mucosal protection. *J. Dent. Res.* **2006**, *85*, 404–415. [CrossRef] [PubMed]
10. Gomez-Ambrosi, J.; Catalan, V.; Ramirez, B.; Rodriguez, A.; Colina, I.; Silva, C.; Rotellar, F.; Mugueta, C.; Gil, M.J.; Cienfuegos, J.A.; et al. Plasma osteopontin levels and expression in adipose tissue are increased in obesity. *J. Clin. Endocrinol. Metab.* **2007**, *92*, 3719–3727. [CrossRef] [PubMed]
11. Rangaswami, H.; Bulbule, A.; Kundu, G.C. Osteopontin: Role in cell signaling and cancer progression. *Trends Cell Biol.* **2006**, *16*, 79–87. [CrossRef] [PubMed]
12. Ramaiah, S.K.; Rittling, S. Role of osteopontin in regulating hepatic inflammatory responses and toxic liver injury. *Expert Opin. Drug Metab. Toxicol.* **2007**, *3*, 519–526. [CrossRef] [PubMed]
13. Sim, S.H.; Messenger, M.P.; Gregory, W.M.; Wind, T.C.; Vasudev, N.S.; Cartledge, J.; Thompson, D.; Selby, P.J.; Banks, R.E. Prognostic utility of pre-operative circulating osteopontin, carbonic anhydrase IX and CRP in renal cell carcinoma. *Br. J. Cancer* **2012**, *107*, 1131–1137. [CrossRef] [PubMed]
14. Kothari, A.N.; Arffa, M.L.; Chang, V.; Blackwell, R.H.; Syn, W.K.; Zhang, J.; Mi, Z.; Kuo, P.C. Osteopontin—A master regulator of epithelial-mesenchymal transition. *J. Clin. Med.* **2016**, *5*. [CrossRef] [PubMed]
15. Rittling, S.R.; Singh, R. Osteopontin in immune-mediated diseases. *J. Dent. Res.* **2015**, *94*, 1638–1645. [CrossRef] [PubMed]

16. Kawamura, K.; Iyonaga, K.; Ichiyasu, H.; Nagano, J.; Suga, M.; Sasaki, Y. Differentiation, maturation, and survival of dendritic cells by osteopontin regulation. *Clin. Diagn. Lab. Immunol.* **2005**, *12*, 206–212. [CrossRef] [PubMed]
17. Guo, B.; Tumang, J.R.; Rothstein, T.L. B cell receptor crosstalk: B cells express osteopontin through the combined action of the alternate and classical BCR signaling pathways. *Mol. Immunol.* **2009**, *46*, 587–591. [CrossRef] [PubMed]
18. Nagai, S.; Hashimoto, S.; Yamashita, T.; Toyoda, N.; Satoh, T.; Suzuki, T.; Matsushima, K. Comprehensive gene expression profile of human activated T_h1- and T_h2-polarized cells. *Int. Immunol.* **2001**, *13*, 367–376. [CrossRef] [PubMed]
19. Weber, G.F.; Cantor, H. Differential roles of osteopontin/Eta-1 in early and late lpr disease. *Clin. Exp. Immunol.* **2001**, *126*, 578–583. [CrossRef] [PubMed]
20. Heilmann, K.; Hoffmann, U.; Witte, E.; Loddenkemper, C.; Sina, C.; Schreiber, S.; Hayford, C.; Holzlohner, P.; Wolk, K.; Tchatchou, E.; et al. Osteopontin as two-sided mediator of intestinal inflammation. *J. Cell. Mol. Med.* **2009**, *13*, 1162–1174. [CrossRef] [PubMed]
21. Sato, T.; Nakai, T.; Tamura, N.; Okamoto, S.; Matsuoka, K.; Sakuraba, A.; Fukushima, T.; Uede, T.; Hibi, T. Osteopontin/Eta-1 upregulated in Crohn's disease regulates the Th1 immune response. *Gut* **2005**, *54*, 1254–1262. [CrossRef] [PubMed]
22. Boyerinas, B.; Zafrir, M.; Yesilkanal, A.E.; Price, T.T.; Hyjek, E.M.; Sipkins, D.A. Adhesion to osteopontin in the bone marrow niche regulates lymphoblastic leukemia cell dormancy. *Blood* **2013**, *121*, 4821–4831. [CrossRef] [PubMed]
23. Liersch, R.; Gerss, J.; Schliemann, C.; Bayer, M.; Schwoppe, C.; Biermann, C.; Appelmann, I.; Kessler, T.; Lowenberg, B.; Buchner, T.; et al. Osteopontin is a prognostic factor for survival of acute myeloid leukemia patients. *Blood* **2012**, *119*, 5215–5220. [CrossRef] [PubMed]
24. Wong, C.K.; Lit, L.C.; Tam, L.S.; Li, E.K.; Lam, C.W. Elevation of plasma osteopontin concentration is correlated with disease activity in patients with systemic lupus erythematosus. *Rheumatology* **2005**, *44*, 602–606. [CrossRef] [PubMed]
25. Ostheimer, C.; Bache, M.; Guttler, A.; Kotzsch, M.; Vordermark, D. A pilot study on potential plasma hypoxia markers in the radiotherapy of non-small cell lung cancer. Osteopontin, carbonic anhydrase IX and vascular endothelial growth factor. *Strahlenther. Onkol.* **2014**, *190*, 276–282. [CrossRef] [PubMed]
26. Korbling, M.; Freireich, E.J. Twenty-five years of peripheral blood stem cell transplantation. *Blood* **2011**, *117*, 6411–6416. [CrossRef] [PubMed]
27. Devine, H.; Tierney, D.K.; Schmit-Pokorny, K.; McDermott, K. Mobilization of hematopoietic stem cells for use in autologous transplantation. *Clin. J. Oncol. Nurs.* **2010**, *14*, 212–222. [CrossRef] [PubMed]
28. Kindwall-Keller, T. Peripheral stem cell collection: From leukocyte growth factor to removal of catheter. *J. Clin. Apheresis* **2014**, *29*, 199–205. [CrossRef] [PubMed]
29. Flommersfeld, S.; Bakchoul, T.; Bein, G.; Wachtel, A.; Loechelt, C.; Sachs, U.J. A single center comparison between three different apheresis systems for autologous and allogeneic stem cell collections. *Transfus. Apheresis Sci.* **2013**, *49*, 428–433. [CrossRef] [PubMed]
30. Brauninger, S.; Bialleck, H.; Thorausch, K.; Felt, T.; Seifried, E.; Bonig, H. Allogeneic donor peripheral blood "stem cell" apheresis: Prospective comparison of two apheresis systems. *Transfusion* **2012**, *52*, 1137–1145. [CrossRef] [PubMed]
31. Reinhardt, P.; Brauninger, S.; Bialleck, H.; Thorausch, K.; Smith, R.; Schrezenmeier, H.; Seifried, E.; Wiesneth, M.; Bonig, H. Automatic interface-controlled apheresis collection of stem/progenitor cells: Results from an autologous donor validation trial of a novel stem cell apheresis device. *Transfusion* **2011**, *51*, 1321–1330. [CrossRef] [PubMed]
32. Gennery, A.R.; Slatter, M.A.; Grandin, L.; Taupin, P.; Cant, A.J.; Veys, P.; Amrolia, P.J.; Gaspar, H.B.; Davies, E.G.; Friedrich, W.; et al. Transplantation of hematopoietic stem cells and long-term survival for primary immunodeficiencies in Europe: Entering a new century, do we do better? *J. Allergy Clin. Immunol.* **2010**, *126*, 602–610. [CrossRef] [PubMed]
33. Naumann-Winter, F.; Greb, A.; Borchmann, P.; Bohlius, J.; Engert, A.; Schnell, R. First-line tandem high-dose chemotherapy and autologous stem cell transplantation versus single high-dose chemotherapy and autologous stem cell transplantation in multiple myeloma, a systematic review of controlled studies. *Cochrane Database Syst. Rev.* **2012**, *10*. [CrossRef]

34. Jantunen, E.; Sureda, A. The evolving role of stem cell transplants in lymphomas. *Biol. Blood Marrow Transplant. J. Am. Soc. Blood Marrow Transplant.* **2012**, *18*, 660–673. [CrossRef] [PubMed]
35. Passweg, J.R.; Baldomero, H.; Bader, P.; Bonini, C.; Cesaro, S.; Dreger, P.; Duarte, R.F.; Dufour, C.; Kuball, J.; Farge-Bancel, D.; et al. Hematopoietic stem cell transplantation in Europe 2014: More than 40,000 transplants annually. *Bone Marrow Transplant.* **2016**, *51*, 786–792. [CrossRef] [PubMed]
36. Sureda, A.; Bader, P.; Cesaro, S.; Dreger, P.; Duarte, R.F.; Dufour, C.; Falkenburg, J.H.; Farge-Bancel, D.; Gennery, A.; Kroger, N.; et al. Indications for allo- and auto-sct for haematological diseases, solid tumours and immune disorders: Current practice in Europe, 2015. *Bone Marrow Transplant.* **2015**, *50*, 1037–1056. [CrossRef] [PubMed]
37. Farge, D.; Labopin, M.; Tyndall, A.; Fassas, A.; Mancardi, G.L.; van Laar, J.; Ouyang, J.; Kozak, T.; Moore, J.; Kotter, I.; et al. Autologous hematopoietic stem cell transplantation for autoimmune diseases: An observational study on 12 years' experience from the European group for blood and marrow transplantation working party on autoimmune diseases. *Haematologica* **2010**, *95*, 284–292. [CrossRef] [PubMed]
38. D'Addio, F.; Vasquez, A.V.; Nasr, M.B.; Franek, E.; Zhu, D.; Li, L.; Ning, G.; Snarski, E.; Fiorina, P. Autologous nonmyeloablative hematopoietic stem cell transplantation in new-onset type 1 diabetes: A multicenter analysis. *Diabetes* **2014**, *63*, 3041–3046. [CrossRef] [PubMed]
39. Al-toma, A.; Nijeboer, P.; Bouma, G.; Visser, O.; Mulder, C.J. Hematopoietic stem cell transplantation for non-malignant gastrointestinal diseases. *World J. Gastroenterol.* **2014**, *20*, 17368–17375. [CrossRef] [PubMed]
40. Pavo, N.; Charwat, S.; Nyolczas, N.; Jakab, A.; Murlasits, Z.; Bergler-Klein, J.; Nikfardjam, M.; Benedek, I.; Benedek, T.; Pavo, I.J.; et al. Cell therapy for human ischemic heart diseases: Critical review and summary of the clinical experiences. *J. Mol. Cell. Cardiol.* **2014**, *75*, 12–24. [CrossRef] [PubMed]
41. Burt, R.K.; Testori, A.; Oyama, Y.; Rodriguez, H.E.; Yaung, K.; Villa, M.; Bucha, J.M.; Milanetti, F.; Sheehan, J.; Rajamannan, N.; et al. Autologous peripheral blood CD133$^+$ cell implantation for limb salvage in patients with critical limb ischemia. *Bone Marrow Transplant.* **2010**, *45*, 111–116. [CrossRef] [PubMed]
42. Nair, S.K.; Driscoll, T.; Boczkowski, D.; Schmittling, R.; Reynolds, R.; Johnson, L.A.; Grant, G.; Fuchs, H.; Bigner, D.D.; Sampson, J.H.; et al. Ex vivo generation of dendritic cells from cryopreserved, post-induction chemotherapy, mobilized leukapheresis from pediatric patients with medulloblastoma. *J. Neuro-Oncol.* **2015**, *125*, 65–74. [CrossRef] [PubMed]
43. Kassis, I.; Zangi, L.; Rivkin, R.; Levdansky, L.; Samuel, S.; Marx, G.; Gorodetsky, R. Isolation of mesenchymal stem cells from G-CSF-mobilized human peripheral blood using fibrin microbeads. *Bone Marrow Transplant.* **2006**, *37*, 967–976. [CrossRef] [PubMed]
44. Fu, W.L.; Zhou, C.Y.; Yu, J.K. A new source of mesenchymal stem cells for articular cartilage repair: MSCs derived from mobilized peripheral blood share similar biological characteristics in vitro and chondrogenesisin vivo as MSCs from bone marrow in a rabbit model. *Am. J. Sports Med.* **2014**, *42*, 592–601. [CrossRef] [PubMed]
45. Paczesny, S.; Hanauer, D.; Sun, Y.; Reddy, P. New perspectives on the biology of acute GVHD. *Bone Marrow Transplant.* **2010**, *45*, 1–11. [CrossRef] [PubMed]
46. Zhao, F.; Zhang, Y.; Wang, H.; Jin, M.; He, S.; Shi, Y.; Guo, Y.; Zhang, Y. Blockade of osteopontin reduces alloreactive CD8$^+$ T cell-mediated graft-versus-host disease. *Blood* **2011**, *117*, 1723–1733. [CrossRef] [PubMed]
47. Weber, G.F.; Ashkar, S.; Glimcher, M.J.; Cantor, H. Receptor-ligand interaction between CD44 and osteopontin (Eta-1). *Science* **1996**, *271*, 509–512. [CrossRef] [PubMed]
48. Gagliani, N.; Magnani, C.F.; Huber, S.; Gianolini, M.E.; Pala, M.; Licona-Limon, P.; Guo, B.; Herbert, D.R.; Bulfone, A.; Trentini, F.; et al. Coexpression of CD49b and LAG-3 identifies human and mouse T regulatory type 1 cells. *Nat. Med.* **2013**, *19*, 739–746. [CrossRef] [PubMed]
49. Carsetti, R.; Rosado, M.M.; Wardmann, H. Peripheral development of B cells in mouse and man. *Immunol. Rev.* **2004**, *197*, 179–191. [CrossRef] [PubMed]
50. Agematsu, K.; Nagumo, H.; Yang, F.C.; Nakazawa, T.; Fukushima, K.; Ito, S.; Sugita, K.; Mori, T.; Kobata, T.; Morimoto, C.; et al. B cell subpopulations separated by CD27 and crucial collaboration of CD27$^+$ B cells and helper T cells in immunoglobulin production. *Eur. J. Immunol.* **1997**, *27*, 2073–2079. [CrossRef] [PubMed]
51. Sennels, H.P.; Jacobsen, S.; Jensen, T.; Hansen, M.S.; Ostergaard, M.; Nielsen, H.J.; Sorensen, S. Biological variation and reference intervals for circulating osteopontin, osteoprotegerin, total soluble receptor activator of nuclear factor κB ligand and high-sensitivity C-reactive protein. *Scand. J. Clin. Lab. Investig.* **2007**, *67*, 821–835. [CrossRef] [PubMed]

52. Ng, L.; Wan, T.M.; Lam, C.S.; Chow, A.K.; Wong, S.K.; Man, J.H.; Li, H.S.; Cheng, N.S.; Pak, R.C.; Cheung, A.H.; et al. Post-operative plasma osteopontin predicts distant metastasis in human colorectal cancer. *PLoS ONE* **2015**, *10*, e0126219. [CrossRef] [PubMed]
53. Kurata, M.; Okura, T.; Irita, J.; Enomoto, D.; Nagao, T.; Jotoku, M.; Miyoshi, K.; Desilva, V.R.; Higaki, J. Angiotensin II receptor blockade with valsartan decreases plasma osteopontin levels in patients with essential hypertension. *J. Hum. Hypertens.* **2011**, *25*, 334–339. [CrossRef] [PubMed]
54. Abdel-Azeez, H.A.; Al-Zaky, M. Plasma osteopontin as a predictor of coronary artery disease: Association with echocardiographic characteristics of atherosclerosis. *J. Clin. Lab. Anal.* **2010**, *24*, 201–206. [CrossRef] [PubMed]
55. Sfiridaki, A.; Miyakis, S.; Pappa, C.; Tsirakis, G.; Alegakis, A.; Kotsis, V.; Stathopoulos, E.; Alexandrakis, M. Circulating osteopontin: A dual marker of bone destruction and angiogenesis in patients with multiple myeloma. *J. Hematol. Oncol.* **2011**, *4*, 22. [CrossRef] [PubMed]
56. Patel, C.G.; Yee, A.J.; Scullen, T.A.; Nemani, N.; Santo, L.; Richardson, P.G.; Laubach, J.P.; Ghobrial, I.M.; Schlossman, R.L.; Munshi, N.C.; et al. Biomarkers of bone remodeling in multiple myeloma patients to tailor bisphosphonate therapy. *Clin. Cancer Res.* **2014**, *20*, 3955–3961. [CrossRef] [PubMed]
57. Szmigielska-Kaplon, A.; Krawczynska, A.; Czemerska, M.; Pluta, A.; Cebula-Obrzut, B.; Szmigielska, K.; Stepka, K.; Smolewski, P.; Robak, T.; Wierzbowska, A. Angiopoietins in haematopoietic stem cell mobilisation in patients with haematological malignancies. *Blood Transfus.* **2015**, *13*, 102–108. [PubMed]
58. Hagberg, I.A.; Akkok, C.A.; Lyberg, T.; Kjeldsen-Kragh, J. Apheresis-induced platelet activation: Comparison of three types of cell separators. *Transfusion* **2000**, *40*, 182–192. [CrossRef] [PubMed]
59. Macher, S.; Sipurzynski-Budrass, S.; Rosskopf, K.; Rohde, E.; Griesbacher, A.; Groselj-Strele, A.; Lanzer, G.; Schallmoser, K. Function and activation state of platelets in vitro depend on apheresis modality. *Vox Sang.* **2010**, *99*, 332–340. [CrossRef] [PubMed]
60. Koh, A.; da Silva, A.P.; Bansal, A.K.; Bansal, M.; Sun, C.; Lee, H.; Glogauer, M.; Sodek, J.; Zohar, R. Role of osteopontin in neutrophil function. *Immunology* **2007**, *122*, 466–475. [CrossRef] [PubMed]
61. Szmigielska-Kaplon, A.; Szemraj, J.; Hamara, K.; Robak, M.; Wolska, A.; Pluta, A.; Czemerska, M.; Krawczynska, A.; Jamroziak, K.; Szmigielska, K.; et al. Polymorphism of CD44 influences the efficacy of $CD34^{+}$ cells mobilization in patients with hematological malignancies. *Biol. Blood Marrow Transplantat. J. Am. Soc. Blood Marrow Transplant.* **2014**, *20*, 986–991. [CrossRef] [PubMed]
62. Melve, G.K.; Ersvssr, E.; Kittang, A.O.; Bruserud, O. The chemokine system in allogeneic stem-cell transplantation: A possible therapeutic target? *Expert Rev. Hematol.* **2011**, *4*, 563–576. [CrossRef] [PubMed]
63. Apelseth, T.O.; Hervig, T.A.; Wentzel-Larsen, T.; Bruserud, O. Cytokine accumulation in photochemically treated and γ-irradiated platelet concentrates during storage. *Transfusion* **2006**, *46*, 800–810. [CrossRef] [PubMed]
64. Apelseth, T.; Hervig, T. Comments on "release of immune modulation factors from platelet concentrates during storage after photochemical pathogen inactivation treatment". *Transfusion* **2009**, *49*, 603–604. [PubMed]

IV

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ORIGINAL ARTICLE

Granulocyte colony-stimulating factor alters the systemic metabolomic profile in healthy donors

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Abstract
Introduction Peripheral blood stem cells mobilized by granulocyte colony-stimulating factor (G-CSF) from healthy donors are commonly used for allogeneic stem cell transplantation. The effect of G-CSF administration on global serum metabolite profiles has not been investigated before.
Objectives This study aims to examine the systemic metabolomic profiles prior to and following administration of G-CSF in healthy adults.
Methods Blood samples were collected from 15 healthy stem cell donors prior to and after administration of G-CSF 10 μg/kg/day for 4 days. Using a non-targeted metabolomics approach, metabolite levels in serum were determined using ultrahigh performance liquid chromatography-tandem mass spectrometry and gas chromatography/mass spectrometry.
Results Comparison of the metabolite profiles of donors before and after G-CSF treatment revealed 239 metabolites that were significantly altered. The major changes of the metabolite profiles following G-CSF administration included alteration of several fatty acids, including increased levels of several medium and long-chain fatty acids, as well as polyunsaturated fatty acids; while there were lower levels of other lipid metabolites such as phospholipids, lysolipids, sphingolipids. Furthermore, there were significantly lower levels of several amino acids and/or their metabolites, including several amino acids with known immunoregulatory functions (methionine, tryptophan, valine). Lastly, the levels of several nucleotides and nucleotide metabolites (guanosine, adenosine, inosine) were also decreased after G-CSF administration, while methylated products were increased. Some of these altered products/metabolites may potentially have angioregulatory effects whereas others may suggest altered intracellular epigenetic regulation.
Conclusion Our results show that G-CSF treatment alters biochemical serum profiles, in particular amino acid, lipid and nucleotide metabolism. Additional studies are needed to further evaluate the relevance of these changes in healthy donors.

Keywords Allogeneic stem cell transplantation · Biochemical · Granulocyte colony-stimulating factor · Metabolomics · Stem cell donor

1 Introduction

Peripheral blood stem cell (PBSC) grafts are commonly used for allogeneic hematopoietic stem cell transplantation for a wide range of hematologic malignancies. These grafts are generally prepared by administration of granulocyte colony-stimulating factor (G-CSF) to healthy donors which mobilizes hematopoietic stem cells (HSCs) into the blood before cells are harvested by leukapheresis. The clinical advantage of using PBSCs compared to bone marrow grafts

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includes accelerated neutrophil engraftment, which increases the likelihood to engraft, and in addition PBSC allografts contain a much larger number of T-cells compared to bone marrow grafts, which has been correlated with better outcome (Malard et al. 2016; Pabst et al. 2007; Rezvani et al. 2006).

The HSC mobilizing agent G-CSF is a glycoprotein with multiple functions, including effects on the production, migration, differentiation and proliferation of neutrophils, as well as affecting adaptive immune responses (Bendall and Bradstock 2014; Panopoulos and Watowich 2008). G-CSF may have both direct and indirect effects on immune cells, including monocytes, granulocytes, T-cells and dendritic cells, and can also alter the expression of various soluble factors, including cytokines, metalloproteinases and adhesion molecules which may themselves contribute to effects induced by G-CSF (Rutella et al. 2005). Recently, various metabolites and metabolic pathways have been found to be involved in cell signaling, also among immune cells; e.g. both amino acids as well as their metabolites can bind to specific receptors on immunocompetent cells and thereby induce activation and/or differentiation of these cells (Buck et al. 2015). Furthermore, certain metabolites have a key role in fundamental metabolic pathways, such as glycolysis or lipid metabolism, and their availability may thus affect immune cell functions. As reviewed by Buck et al. (2015), cellular metabolism is important in the regulation of immunocompetent cell growth, e.g. differentiation and activation of T-cells. Thus, the availability and uptake of metabolites may potentially affect immune cell fate. In this aspect, metabolomics has emerged as a powerful tool to identify and characterize the low molecular mass composition of biological samples. In this exploratory study, we therefore used non-targeted metabolomics to investigate the early effects of in vivo G-CSF treatment on the global serum metabolite profile of healthy stem cell donors.

2 Materials and methods

2.1 Stem cell donors and mobilization

The study was approved by the local ethics committee (REK Vest, 2011/996 and 2011/1237) and all samples were collected after written informed consent. Blood samples were collected from 15 consecutive healthy HLA-matched related allogeneic stem cell donors (10 males and 5 females), with a mean age of 47 years (range 25–64 years) (Table 1). Donors received the human non-glycosylated G-CSF analog Filgrastim (r-metHuG-CSF, Neupogen, Amgen) or Tevagrastim (biosimilar Filgrastim) in a dose of 5.4 µg/kg body weight (range 4.1–6.7 µg/kg) twice daily subcutaneously for four days to induce stem cell mobilization. Our hospital is responsible for all allogeneic stem cell transplantations in a defined geographic area, and this study included a consecutive group of donors younger than 65 years of age and achieving pre-harvest $CD34^+$ cell counts above 15×10^3/L. Thus, our donors should be regarded as representative of healthy adult stem cell donors because they are unselected (i.e. consecutive), mobilize sufficient stem cells for preparation of allografts and their age is also representative for donors used in routine clinical practice.

2.2 Processing of blood samples

Venous blood samples were collected into Vacuette Z Serum Clot Activator tubes with Gel Separator (Greiner Bio-One GmbH, Kremsmünster, Austria) from donors at two time points, (i) prior to administration of G-CSF and (ii) following G-CSF administration just before apheresis on day 4. All samples were collected at 9 am and were allowed to coagulate for 30 min at room temperature in upright position before being centrifuged at $1310 \times g$ for 10 min at room temperature. The serum supernatants were immediately apportioned into 0.5 mL aliquots in plastic cryotubes (Nunc™, Roskilde, Denmark) and stored frozen at −80°C until analysed.

2.3 Analysis of G-CSF levels

Levels of human G-CSF were measured using a Luminex assay (R&D Systems, Bio-techne, Abingdon, UK), and the minimal detectable level was 20 pg/mL.

2.4 Analysis of human serum metabolites

All mass spectrometry data were collected at Metabolon Inc (Durham, NC). Each serum sample was accessioned into the Metabolon LIMS system and was assigned a unique identifier by this system which was used to track all sample handling and results. All samples were prepared using the automated MicroLab STAR® system (Hamilton Company, Bonaduz, Switzerland). Briefly, samples were extracted using Metabolon's standard solvent extraction method (Evans et al. 2014). A recovery standard was added prior to the first step in the extraction process for quality control purposes. To remove protein, dissociate small molecules bound to protein or trapped in the precipitated protein matrix, and to recover chemically diverse metabolites, proteins were precipitated with methanol under vigorous shaking for 2 min followed by centrifugation. The resulting extract was divided into five fractions: (i) one for analysis by ultrahigh performance liquid chromatography–tandem mass spectrometry (UPLC–MS/MS)

Table 1 Characteristics of allogeneic stem cell donors

ID	Age (years)	Gender	BMI (kg/m^2)	G-CSF dosage (μg/kg)	G-CSF (pg/mL) at clinical examination[a]	G-CSF (pg/mL) before apheresis[a]	$CD34^+$ cell count (10^3/L) pre-harvest[b]	$CD34^+$ stem cell yield ($\times 10^6$/kg)[b]
1	60	F	30	6.1	<20	>15,000	40.2	5.5
2	25	M	24	6.1	<20	13,514	44.4	8.8
3	45	M	25	5.8	242	>15,000	30.4	4.3
4	51	M	47	4.9	37	>15,000	58.8	3.9
5	39	M	30	5.3	72	13,404	108.8	22.4
6	64	M	36	4.1	<20	9495	26.7	3.9
7	54	F	23	5.7	41	>15,000	34.1	5.2
8	25	M	26	5.7	111	>15,000	147.8	15.1
9	46	F	25	4.6	49	3939	57.6	7.2
10	62	M	27	5.6	24	>15,000	97.0	7.2
11	51	M	26	5.1	53	>15,000	17.4	3.1
12	40	F	34	5.3	39	>15,000	66.7	6.8
13	39	M	25	5.5	22	>15,000	111.1	7.8
14	45	F	26	6.7	82	>15,000	55.1	5.6
15	58	M	29	5.1	80	6776	44.7	4.9
Mean	47		29	5.4	61	>13,000	62.7	8.7
Range	25–64	10M/ 5F	23–47	4.1–6.7	<20–242	3939– > 15,000	17.4–147.8	3.1–22.4

M male; *F* female; *BMI* body mass index

[a] G-CSF plasma levels were measured in donor samples collected at clinical examination and after four days of treatment with G-CSF before apheresis

[b] $CD34^+$ cell counts were done immediately before stem cell harvest and the $CD34^+$ stem cell yield estimated per kg donor/weight

with positive ion mode electrospray ionization, (ii) one for analysis by UPLC–MS/MS with negative ion mode electrospray ionization, (iii) one for LC polar platform, (iv) one for analysis by gas chromatography/mass spectrometry (GC–MS) and (v) one sample was reserved for backup. Samples were placed briefly on a Zymark TurboVap® (McKinley Scientific, Sparta, NJ, USA) to remove the organic solvent. Then samples were either stored overnight under nitrogen for LC or dried under vacuum overnight for GC, before preparation for analysis. Experimental samples were randomized across the platform and run with appropriate quality control samples spaced evenly among the injections. Compounds were identified by comparison to library entries based upon retention time/index, mass to charge ratio (m/z) and chromatographic data (also MS/MS spectral data), and peaks were quantified using area-under-the curve.

2.5 Statistical analyses

Two types of statistical analysis were performed: (1) significance tests (t-tests) and (2) classification analysis. Random Forest analysis is a supervised classification technique that provides an unbiased estimate of how well individuals can be classified into each group in a new data set. Statistical analyses were performed with the program R (http://cran.r-project.org/).

3 Results

3.1 G-CSF treatment alters the global metabolomic profile of healthy individuals

Metabolites were analysed in all serum samples collected from the healthy donors (i) prior to G-CSF administration and (ii) on day 4 after G-CSF administration. In total, 641 metabolites were identified (for a complete list see Supplementary Table 1), where levels of 239 metabolites were significantly changed ($p \leq 0.05$); 149 metabolites had increased levels (62%) and 90 metabolites had decreased levels (38%) after G-CSF administration (Table 2). These significantly altered metabolites belong mainly to amino acid and lipid classes, while metabolites associated with the categories nucleotides, carbohydrates, energy metabolism, cofactors/vitamins and xenobiotics are also present. Furthermore, 39 of these metabolites have a p value < 0.0001 and are involved in amino acid (19/39),

Table 2 Metabolite classes significantly altered after G-CSF treatment

Metabolite classes/pathways	Total number of metabolites identified	Number of significantly altered metabolites		
		$P < 0.05$	$P < 0.001$	$P < 0.0001$
Amino acids	161	75	30	19
Peptide	28	16	1	1
Carbohydrates	25	8	5	2
Energy metabolism	9	2	–	–
Lipids	250	78	13	5
Nucleotides	34	21	11	10
Cofactors-vitamins	22	10	4	–
Xenobiotics	112	29	4	2
Total number of metabolites	641	239	68	39

nucleotide (10/39) or lipid (5/39) metabolism (Table 2). Among the significantly altered levels of metabolites ($n = 239$, $p \leq 0.05$) we would expect to see approximately 12 metabolites that meet our level of significance criteria by random chance, however, our data have a low false discovery rate (FDR) of less than 5% for all metabolites except one metabolite with 5.1%, indicating a high level of confidence in the results (Supplementary Table 2). All significantly altered metabolites ($p \leq 0.05$) and their group mean ratios (before vs. after G-CSF treatment) are shown in Supplementary Table 2.

We performed a principal component analysis (PCA) which showed that the samples before and after G-CSF administration were generally distinguishable from each other (Fig. 1); one G-CSF treated sample seemed to deviate from the other samples but no outlier samples were identified. This exceptional stem cell donor had the highest total leukocyte and platelet counts in peripheral blood, the lowest Hb level after G-CSF administration and the lowest G-CSF plasma level before apheresis, but did not otherwise differ from the others, and samples from this donor were included in all our analyses. Taken together, these results demonstrate that four days of G-CSF administration alters the systemic metabolomic profile of healthy individuals; and despite some heterogeneity between donors, an altered amino acid, lipid and nucleotide metabolism seems to be a common characteristic.

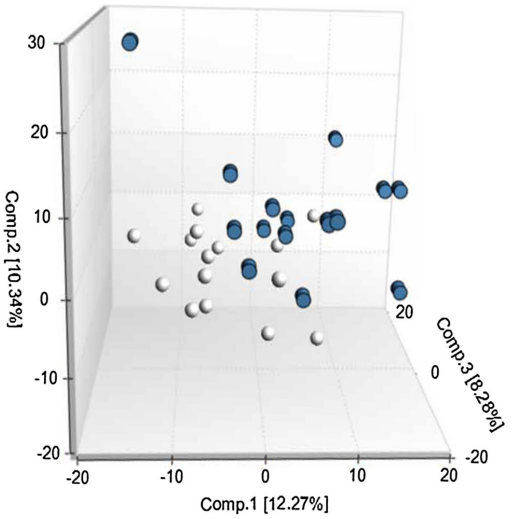

Fig. 1 Principal component analysis (PCA) scores plot based on the serum metabolome of healthy stem cell donors before and after G-CSF treatment. An overlap was seen between groups ($n = 15$ in each group, *open circles* before G-CSF treatment, *filled circles* after G-CSF treatment), but groups were generally distinguishable from each other

3.2 Alteration of single metabolites by G-CSF treatment

Random forest classification was used for further statistical analyses. Even though there was an overlap between samples collected before and during G-CSF therapy in the PCA plot (Fig. 1), the random forest classification demonstrated that G-CSF treated versus untreated samples could be distinguished with 97% predictive accuracy based on their overall metabolite profiles (Fig. 2). The 30 top-ranking metabolites that contributed most to separation of the samples are shown where the metabolites are ranked according to the mean decrease accuracy (%) (Fig. 2). These metabolites are involved in several pathways, with the majority of metabolites belonging to amino acid metabolism (9 metabolites) and nucleotide metabolism (9 metabolites), but lipid (4 metabolites), xenobiotics (3 metabolites), carbohydrate (2 metabolites), cofactors/

	Predictive		
Actual	Pre	Post	Class Error
Pre	14	1	0.067
Post	0	15	0.000
Predictive accuracy = 97%			

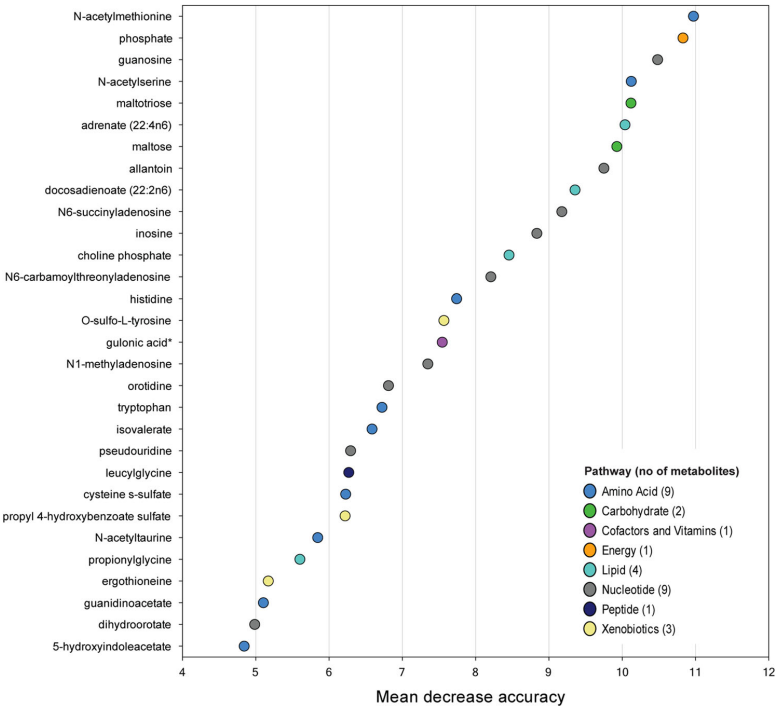

Fig. 2 Random forest analysis of the metabolic profiles in samples taken before and after administration of G-CSF in healthy stem cell donors. Random forest analysis could distinguish between the metabolic profiles of the two groups with a predictive accuracy of 97%. The variable importance plot shows the variable on the y-axis, and their importance for separation of the two groups on the x-axis. The top-ranked 30 metabolites are thus ordered top-to-bottom as most- to least-important based on their importance. The *inset table* shows the main signaling pathways where each metabolite belongs, reflected by the different colors in the plot. *Asterisk* indicates that the biochemical name is identified but has not been confirmed based on a standard

vitamin (1 metabolite) and peptides (1 metabolite) were also included among the 30 top-ranking metabolites in this analysis.

3.3 Alteration of lipid, amino acid and nucleotide metabolism by G-CSF treatment

Treatment with G-CSF resulted in altered systemic (i.e. serum) levels of a wide range of metabolites reflecting an alteration of different metabolic pathways during this treatment in healthy individuals:

- *Lipid metabolism* Among the main metabolites that distinguished between pre-and post-G-CSF administration groups, there was a consistent alteration of the amount of fatty acids indicating altered lipid metabolism (i.e. a change in fatty acid synthesis, lipid hydrolysis or mitochondrial β-oxidation) (Fig. 3a). The post-G-CSF group had significantly elevated levels of several long-chain fatty acids (such as myristate, palmitate, margarate, and stearate), as well as polyunsaturated fatty acids (adrenate, linoleate, linolenate, dihomolinoleate, docosadienoate, docosapentaenoate). Finally, levels of carnitine-conjugated lipids were increased.
- *Amino acid metabolism* was also altered; within the post-G-CSF samples there was a reduced level of dipeptides and amino acids including the aromatic amino acids tryptophan, phenylalanine and tyrosine and their metabolites, as well as branched-chain amino acids valine, isoleucine and leucine (Fig. 3b). The essential amino acid tryptophan can be metabolized by several pathways to give rise to serotonin or kynurenine, and levels of these degradation products as well as indoleacetate and 3-indoxyl sulfate were all significantly lower after G-CSF therapy.
- *Nucleic acid metabolism* Our results show a significantly lower level of purine nucleosides, including guanosine, adenosine and inosine in the post-G-CSF

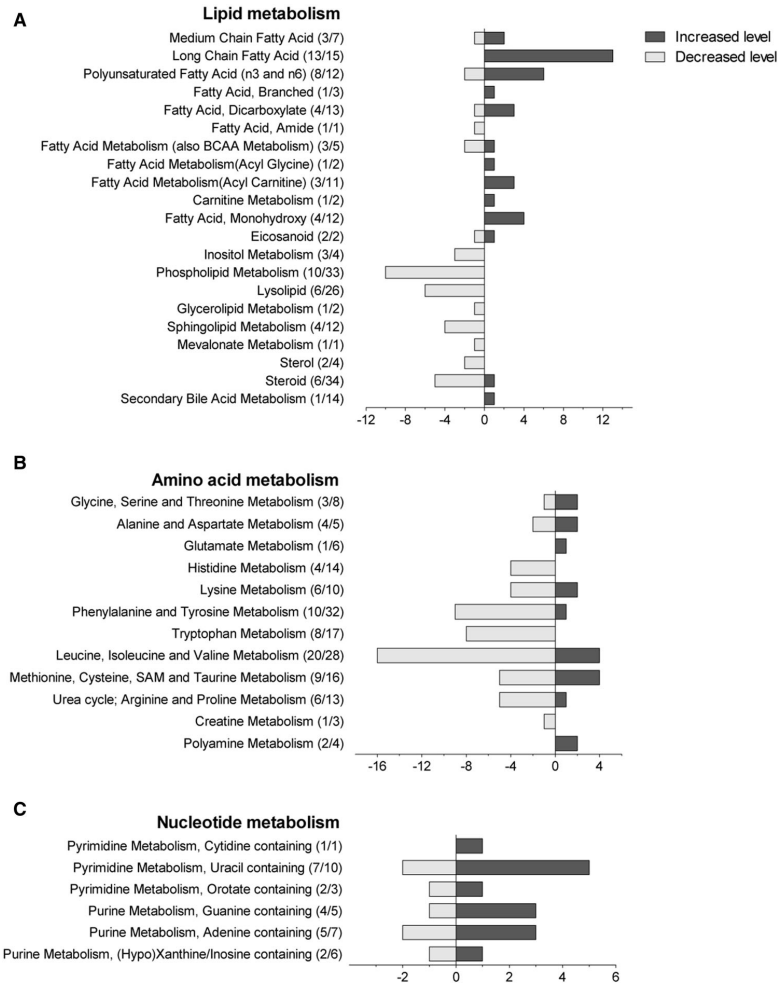

Fig. 3 Metabolite pathways altered after G-CSF administration among the classes lipids, amino acids and nucleotides. The *bar charts* show the different amounts of metabolites belonging to their different pathways within the three main classes: **a** lipid metabolism, **b** amino acid metabolism and **c** nucleotide metabolism, where metabolite levels were either increased (*dark grey* colored) or decreased (*light grey*)

samples, indicating altered nucleotide metabolism (Fig. 3c). Furthermore, several methylated products were increased.

3.4 G-CSF induced alteration of metabolic pathways

We performed a metabolomic pathway enrichment analysis based on significantly altered metabolites ($p < 0.001$), to identify pathways that contribute to the major differences when comparing samples taken before and after G-CSF administration (Fig. 4). This analysis identified altered glycogen metabolism as a major effect of G-CSF treatment, and metabolites belonging to pathways involved in nucleotide metabolism and amino sugar/acid metabolism were also over-represented in samples after G-CSF treatment.

3.5 The metabolic alterations induced during G-CSF therapy are not caused by acetaminophen

Musculoskeletal pain and flu-like symptoms are common during G-CSF therapy (Stroncek et al. 1996), and symptomatic relief can be achieved by acetaminophen (paracetamol). The detection of several metabolites of acetaminophen in the serum of stem cell donors indicates that paracetamol had been taken by some donors during G-CSF therapy. We therefore compared the metabolite

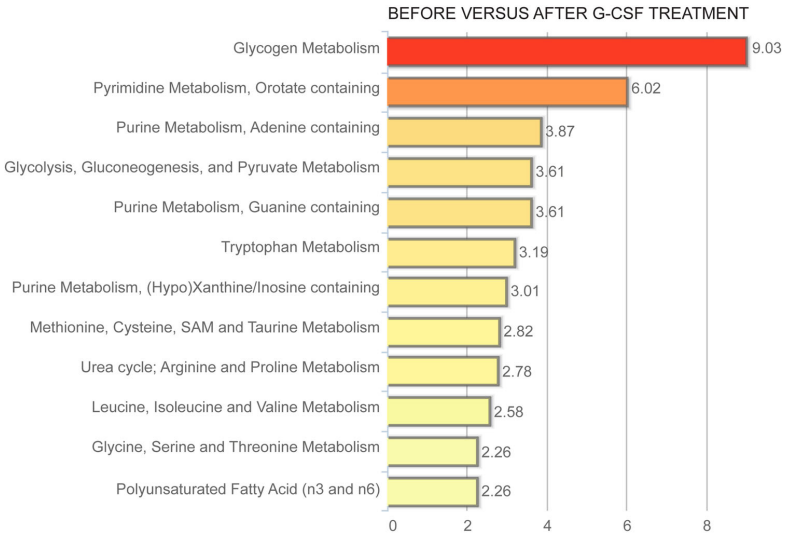

Fig. 4 Metabolite pathway enrichment analysis to identify pathways enriched after G-CSF administration in healthy donors. A pathway enrichment analysis was done based on significantly altered metabolites with $p < 0.001$. Only signaling pathways with an enrichment value greater than two and at least two metabolites within each pathway are shown in the figure. The most significant p-values are seen in *red*, while the least significant are in *yellow*

profile before and after G-CSF therapy for donors with high and low/undetectable levels of metabolites involved in acetaminophen metabolism. When comparing the levels of the 30 top-ranked metabolites (Fig. 2), we did not find any significant differences between the donor samples with high and low/undetectable paracetamol metabolite (data not shown). Thus, it seems unlikely that the paracetamol intake has a major impact on the metabolic modulation during G-CSF therapy.

4 Discussion

In this study, we investigated the early effects of G-CSF administration on the serum global metabolite profile of healthy stem cell donors that were younger than 65 years and had an adequate stem cell mobilization. G-CSF is mainly used as short-term therapy, generally requiring 4–6 days of treatment, for stem cell mobilization (Bendall and Bradstock 2014); however, it is also used as long-term treatment for patients with low-risk myelodysplastic syndrome (Jadersten et al. 2005) and especially for patients with chronic neutropenia (Dale 2016; Dale and Welte 2011; Donadieu et al. 2011; Zeidler et al. 2014). The suggested initial doses for congenital neutropenia are 3–5 μg/kg that are increased in steps of 5 μg/kg (Dale 2016; Donadieu et al. 2011), thus these G-CSF doses used in long-term therapy are also comparable to the doses used in our present study (5 μg/kg twice daily).

Our study included a relatively small number of samples, but our random forest classification analysis resulted in 97% predictive accuracy in differentiating the two groups, indicating that differences due to G-CSF administration were readily present. Several of the 30 top-ranking metabolites shown in Fig. 2 have been reported to be involved in biological processes such as regulation of immune responses, inflammation, vascular biology and epigenetic regulation (see Supplementary Table 3), though further studies will be needed to see if G-CSF has a long-term effect on these biological processes. In general, global metabolomics profiling revealed altered levels of lipids, amino acids, carbohydrates and nucleotides after administration with G-CSF.

One of the strongest changes in our dataset was the altered lipid metabolism, in particular the significantly higher levels of long-chain fatty acids as well as carnitine-conjugated lipids after G-CSF administration, indicating changes in fatty acid β-oxidation. Long-chain fatty acids are conjugated to carnitine to facilitate transport across the mitochondrial membrane, and the increased acyl carnitine levels may thus suggest increased β-oxidation. Moreover, glycerol, a marker of lipolysis, was significantly decreased in the G-CSF treated group. These alterations may be due to increased fatty acid β-oxidation or alternatively due to disturbance of fatty acid oxidation resulting in increased amounts of lipid precursors. In addition, we found a decline in the serum levels of sphingosine 1-phosphate (S1P) after G-CSF administration, which is in concordance with another study that measured S1P levels in donors undergoing G-CSF-induced mobilization (Juarez et al. 2012). Disruption of fatty acid signaling has been implicated in mobilization of stem cells, in particular S1P (Ratajczak

et al. 2010). To summarize, G-CSF treatment alters fatty acid metabolism and decreases the systemic levels of fatty acid metabolites involved in hydrolysis (phospholipid metabolism and lysolipids) whereas long/medium-chain fatty acids are generally increased.

In this study we found lower levels of branched chain amino acids and aromatic amino acids following G-CSF treatment. Among the aromatic amino acids we found altered levels of tryptophan and its degradation products which are shown to be associated with inflammation. Kynurenine plays a role in modulation of inflammation and the ratio of kynurenin/tryptophan has been suggested to be an indicator for the activity of indolamine-2,3-dioxygenase (IDO) (Widner et al. 1997), which can affect T cell functions. Branched chain amino acids can be substrates for both energy production and protein synthesis. They can be metabolized to give rise to intermediates for several metabolic pathways including the TCA cycle or fatty acid synthesis. Less amounts of the branched amino acids isoleucine, valine and leucine may thus potentially have an impact on energy metabolism by reduction of available metabolites. However, in addition to the lower levels of these amino acids, we also observed a general decrease in dipeptide levels after G-CSF administration which is supportive of reduced proteolysis.

Previous studies have examined effects of in vivo administration of G-CSF on normal peripheral blood mononuclear cells (PBMCs) using whole genome expression profiling. Changes in global gene expression profiles were then described both at early time points after G-CSF administration (up to 5 days) (Hernandez et al. 2005) and after 2–10 months (Amariglio et al. 2007), and even a year after G-CSF administration in $CD34^+$ progenitor cells (Baez et al. 2014). These studies have revealed that there seems to be both early responses to G-CSF, transient changes that are normalized over time and more long-lasting changes. In the recent study by Bàez et al., G-CSF-mobilized hematopoietic progenitors had a difference in the expression of six microRNAs and even one year after G-CSF administration over 2424 genes maintained their altered expression (Baez et al. 2014). Among the differentially expressed genes were genes involved in cellular growth, cell death and survival, protein synthesis, gene expression and nucleic acid metabolism. In another study of twenty stem cell donors, changes of DNA methyltransferase activity in peripheral blood cells were found after G-CSF administration, though these changes returned to baseline within a week after apheresis (Leitner et al. 2014). Thus, several studies of donor cells suggest epigenetic changes induced after G-CSF administration in healthy donors, though none of these studies have investigated the global metabolite profile of healthy donors after G-CSF treatment. Accordingly, in our study of the systemic metabolite profile, we observed altered nucleic acid metabolism by G-CSF therapy, including lower levels of the purine nucleosides. Furthermore, several methylated products were increased which could suggest a difference in methylation potential through treatment with G-CSF. These results should be interpreted with great care, but could suggest that G-CSF has the potential to influence epigenetics.

We have not performed any functional assays to evaluate the potential association between metabolites altered by G-CSF and immunomodulatory effects; however, G-CSF has been shown to induce several cellular and immunological changes in donor cells (Anderlini et al. 1996; Shaw et al. 2015), and several of the altered metabolites found in our study have known immunoregulatory and/or angioregulatory effects, or can be markers of altered regulation of epigenetic/gene expression that may contribute to the previously described long-lasting effects after G-CSF therapy (Baez et al. 2014). Altered metabolite levels may reflect the direct effects of G-CSF on different immune cell types, affecting cell proliferation, differentiation and function, but also indirect effects through e.g. upregulation of cytokine production may subsequently affect cells and lead to altered metabolite levels. G-CSF binds to the single high-affinity 140 kDa G-CSF receptor (G-CSFR), which is expressed on myeloid progenitor cells, mature granulocytes and monocytes, lymphocytes and endothelial cells (Demetri and Griffin 1991; Franzke et al. 2003) and can activate multiple signaling pathways including JAK-STAT and ERK/MAPK pathways. However, the expression of this receptor does not seem to be required for progenitor mobilization induced by G-CSF (Liu et al. 2000), and G-CSF-mediated effects may also occur independent of the G-CSF receptor. Further studies are needed to explore the mechanism inducing the metabolite changes after G-CSF treatment and their potential immunomodulatory effects and/or effects on other cell functions.

A possible explanation for our findings could be that they are secondary to an increased proliferation of immature hematopoietic cells to replace the cells that are lost from hematopoietic niches to the circulation. However, the $CD34^+$ cell number and the total peripheral blood leukocyte number is controlled daily during stem cell mobilization, and the duration of this altered compartmentalization of hematopoietic cells is therefore relatively short (<24 h). Furthermore, the increased levels of immature hematopoietic cells in the peripheral blood probably represent a minor part of the overall number of nucleated bone marrow cells, and one should also emphasize that the cells have not yet been harvested at the time of sampling for metabolite analysis. However, we cannot exclude the possibility that the metabolic changes

are secondary to an increased proliferation of bone marrow cells, but if so this is in our opinion most likely caused by direct G-CSF stimulated proliferation during the whole treatment period rather than being a compensatory mechanism to altered compartmentalization during the last hours before stem cell harvesting.

Several metabolites classified as xenobiotics were altered after G-CSF therapy. Many stem cell donors experience side effects during treatment (Stroncek et al. 1996), and acetaminophen is then recommended for pain relief. Some altered metabolites belonging to the xenobiotic class are a direct result of acetaminophen usage, and in our opinion some of the other metabolite changes may be associated with treatment toxicity and/or altered gastrointestinal function.

5 Concluding remarks

In totum, our results show that the level of several metabolites changed after G-CSF administration, primarily there were (i) increased levels of lipids indicating altered fatty acid metabolism, (ii) increased levels of methylated nucleosides, (iii) changes associated with energy metabolism, and (iv) altered levels of amino acids, including reduced peptide levels indicating decreased proteolysis and altered levels of acetylated peptides. Long-term follow up studies have concluded that the use of G-CSF to mobilize stem cells appears to be safe (Shaw et al. 2015). Our study shows distinct differences in the metabolite profiles between healthy donors before and after G-CSF administration; however this is only a snapshot of the metabolomic profile of donors at an early time point after G-CSF administration and further studies should examine the metabolite profiles over time, using a larger set of donors, to clarify whether G-CSF also has long-term effects on metabolite profiles and/or if patients on long-term G-CSF therapy should be monitored with regard to metabolic abnormalities.

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Compliance with ethical standards

Conflicts of interest All authors declare that they have no conflicts of interest related to this manuscript.

Ethical approval All the authors declare that the procedures in this study were in accordance with the ethical standards of the responsible committee, approved by the local ethics committee in Bergen, Norway (REK Vest, 2011/996 and 2011/1237).

Informed consent Informed written consent was obtained from all individuals for being included in this study.

References

Amariglio, N., et al. (2007). Changes in gene expression pattern following granulocyte colony-stimulating factor administration to normal stem cell sibling donors. *Acta Haematologica, 117*, 68–73.

Anderlini, P., Przepiorka, D., Champlin, R., & Korbling, M. (1996). Biologic and clinical effects of granulocyte colony-stimulating factor in normal individuals. *Blood, 88*, 2819–2825.

Baez, A., et al. (2014). Granulocyte colony-stimulating factor produces long-term changes in gene and microRNA expression profiles in $CD34^{+}$ cells from healthy donors. *Haematologica, 99*, 243–251.

Bendall, L. J., & Bradstock, K. F. (2014). G-CSF: From granulopoietic stimulant to bone marrow stem cell mobilizing agent. *Cytokine & Growth Factor Reviews, 25*, 355–367.

Buck, M. D., O'Sullivan, D., & Pearce, E. L. (2015). T cell metabolism drives immunity. *Journal of Experimental Medicine, 212*, 1345–1360.

Dale, D. C. (2016). How I diagnose and treat neutropenia. *Current Opinion in Hematology, 23*, 1–4.

Dale, D. C., & Welte, K. (2011). Cyclic and chronic neutropenia. *Cancer Treatment and Research, 157*, 97–108.

Demetri, G. D., & Griffin, J. D. (1991). Granulocyte colony-stimulating factor and its receptor. *Blood, 78*, 2791–2808.

Donadieu, J., Fenneteau, O., Beaupain, B., Mahlaoui, N., & Chantelot, C. B. (2011). Congenital neutropenia: Diagnosis, molecular bases and patient management. *Orphanet Journal of Rare Disease, 6*, 26.

Evans, A. M., et al. (2014). High resolution mass spectrometry improves data quantity and quality as compared to unit mass resolution mass spectrometry in high-throughput profiling metabolomics. *Metabolomics, 4*, 132.

Franzke, A., et al. (2003). G-CSF as immune regulator in T cells expressing the G-CSF receptor: Implications for transplantation and autoimmune diseases. *Blood, 102*, 734–739.

Hernandez, J. M., et al. (2005). Mobilisation with G-CSF in healthy donors promotes a high but temporal deregulation of genes. *Leukemia, 19*, 1088–1091.

Jadersten, M., Montgomery, S. M., Dybedal, I., Porwit-MacDonald, A., & Hellstrom-Lindberg, E. (2005). Long-term outcome of treatment of anemia in MDS with erythropoietin and G-CSF. *Blood, 106*, 803–811.

Juarez, J. G., et al. (2012). Sphingosine-1-phosphate facilitates trafficking of hematopoietic stem cells and their mobilization by CXCR4 antagonists in mice. *Blood, 119*, 707–716.

Leitner, G. C., Faschingbauer, M., Wenda, S., Weigel, G., & Fischer, G. (2014). Administration of recombinant human granulocyte-colony-stimulating factor does not induce long-lasting detectable epigenetic alterations in healthy donors. *Transfusion, 54*, 3121–3126.

Liu, F., Poursine-Laurent, J., & Link, D. C. (2000). Expression of the G-CSF receptor on hematopoietic progenitor cells is not required for their mobilization by G-CSF. *Blood, 95*, 3025–3031.

Malard, F., et al. (2016). Larger number of invariant natural killer T cells in PBSC allografts is associated with improved GVHD-free, progression-free survival. *Blood, 127*, 1828–1835.

Pabst, C., Schirutschke, H., Ehninger, G., Bornhauser, M., & Platzbecker, U. (2007). The graft content of donor T cells expressing gamma delta TCR+ and CD4+foxp3+ predicts the risk of acute graft versus host disease after transplantation of allogeneic peripheral blood stem cells from unrelated donors. *Clinical Cancer Research, 13*, 2916–2922.

Panopoulos, A. D., & Watowich, S. S. (2008). Granulocyte colony-stimulating factor: Molecular mechanisms of action during steady state and 'emergency' hematopoiesis. *Cytokine, 42*, 277–288.

Ratajczak, M. Z., et al. (2010). Novel insight into stem cell mobilization-plasma sphingosine-1-phosphate is a major chemoattractant that directs the egress of hematopoietic stem progenitor cells from the bone marrow and its level in peripheral blood increases during mobilization due to activation of complement cascade/membrane attack complex. *Leukemia, 24*, 976–985.

Rezvani, K., et al. (2006). High donor FOXP3-positive regulatory T-cell (Treg) content is associated with a low risk of GVHD following HLA-matched allogeneic SCT. *Blood, 108*, 1291–1297.

Rutella, S., Zavala, F., Danese, S., Kared, H., & Leone, G. (2005). Granulocyte colony-stimulating factor: A novel mediator of T cell tolerance. *Journal of Immunology, 175*, 7085–7091.

Shaw, B. E., Confer, D. L., Hwang, W., & Pulsipher, M. A. (2015). A review of the genetic and long-term effects of G-CSF injections in healthy donors: A reassuring lack of evidence for the development of haematological malignancies. *Bone Marrow Transplantation, 50*, 334–340.

Stroncek, D. F., et al. (1996). Treatment of normal individuals with granulocyte-colony-stimulating factor: Donor experiences and the effects on peripheral blood $CD34^{+}$ cell counts and on the collection of peripheral blood stem cells. *Transfusion, 36*, 601–610.

Widner, B., Werner, E. R., Schennach, H., Wachter, H., & Fuchs, D. (1997). Simultaneous measurement of serum tryptophan and kynurenine by HPLC. *Clinical Chemistry, 43*, 2424–2426.

Zeidler, C., et al. (2014). Outcome and management of pregnancies in severe chronic neutropenia patients by the European Branch of the Severe Chronic Neutropenia International Registry. *Haematologica, 99*, 1395–1402.

Supplementary Table 1. Complete list of all metabolites identified.

Comparison mean values significantly different:
- [red] $p \leq 0.05$, fold of change ≥ 1.00
- [dark green] $p \leq 0.05$, fold of change < 1.00

Comparison mean value difference approaching significance:
- [pink] $0.05 < p < 0.10$, fold of change ≥ 1.00
- [light green] $0.05 < p < 0.10$, fold of change < 1.00

Pathway Sort Order	Super Pathway	Sub Pathway	Biochemical Name	Platform	Mass	Comp ID	KEGG	HMDB	PubChem	Fold of Change, Paired t-Test: Post G-CSF / Pre G-CSF		Paired t-Test: Post G-CSF / Pre G-CSF		Mean Values	
										Mean Pairs Ratio	Group Mean Ratio	*p*-value	*q*-value	Pre G-CSF	Post G-CSF
1	Amino Acid	Glycine, Serine and Threonine Metabolism	glycine	LC/MS pos	263.14662	58	C00037	HMDB00123	750	1.03	1.03	0.5926	0.2889	0.9931	1.0186
2			N-acetylglycine	LC/MS neg	116.03531	27710		HMDB00532	10972	1.06	1.08	0.4135	0.2277	1.1664	1.2564
5			dimethylglycine	LC/MS pos	104.07061	5086	C01026	HMDB00092	673	1.05	1.05	0.2836	0.1777	1.0523	1.1063
6			betaine	LC/MS pos	118.08626	3141	C00719	HMDB00043	247	0.95	0.96	0.1464	0.1118	1.1138	1.0653
9			serine	LC/MS pos	106.04987	1648	C00065	HMDB00187	5951	0.79	0.79	0.0000	0.0003	1.1173	0.8779
10			N-acetylserine	LC/MS polar	146.04588	37076		HMDB02931	65249	1.63	1.61	0.0000	0.0000	0.8101	1.3054
14			threonine	LC/MS pos	120.06552	1284	C00188	HMDB00167	6288	0.92	0.90	0.0826	0.0743	1.0456	0.9420
15			N-acetylthreonine	LC/MS neg	160.06153	33939	C01118		152204	1.26	1.23	0.0184	0.0256	0.9305	1.1405
25		Alanine and Aspartate Metabolism	alanine	LC/MS pos	90.05496	1126	C00041	HMDB00161	5950	1.13	1.11	0.0097	0.0169	0.9654	1.0764
26			N-acetylalanine	LC/MS neg	130.05096	1585	C02847	HMDB00766	88064	1.29	1.28	0.0000	0.0001	0.8756	1.1198
28			aspartate	LC/MS polar	132.03023	443	C00049	HMDB00191	5960	0.64	0.60	0.0068	0.0132	1.3009	0.7755
30			asparagine	LC/MS pos	133.06077	512	C00152	HMDB00168	6267	0.94	0.93	0.0258	0.0310	1.0885	1.0128
32			N-acetylaspartate (NAA)	LC/MS polar	174.04079	22185	C01042	HMDB00812	65065	0.84	0.84	0.0667	0.0654	1.0369	0.8707
38		Glutamate Metabolism	glutamate	LC/MS pos	148.06044	57	C00025	HMDB00148	611	0.91	0.82	0.1030	0.0870	1.1249	0.9274
39			glutamine	LC/MS pos	147.07642	53	C00064	HMDB00641	5961	1.07	1.06	0.0387	0.0423	0.9626	1.0249
40			N-acetylglutamate	LC/MS polar	188.05644	15720	C00624	HMDB01138	70914	0.91	0.85	0.1624	0.1207	1.0550	0.8985
41			N-acetylglutamine	LC/MS pos	189.08699	33943	C02716	HMDB06029	182230	1.07	1.02	0.8441	0.3561	1.0729	1.0923
42			N-acetyl-aspartyl-glutamate (NAAG)	LC/MS pos	305.09795	35665	C12270	HMDB01067	5255	1.11	1.06	0.5458	0.2767	0.9585	1.0134
49			pyroglutamine*	LC/MS pos	129.06586	46225			134508	1.01	1.02	0.9421	0.3821	1.6535	1.6785
53		Histidine Metabolism	histidine	LC/MS neg	154.0622	59	C00135	HMDB00177	6274	0.84	0.82	0.0013	0.0042	1.0801	0.8873
54			N-acetylhistidine	LC/MS neg	196.07276	33946	C02997	HMDB32055	75619	0.78	0.81	0.0212	0.0283	1.0851	0.8799
55			1-methylhistidine	LC/MS pos	170.09241	30460	C01152	HMDB00001	92105	1.21	1.08	0.4340	0.2346	1.0495	1.1377
56			3-methylhistidine	LC/MS neg	168.07785	15677	C01152	HMDB00479	64969	1.58	0.82	0.8074	0.3470	1.2446	1.0239
57			N-acetyl-3-methylhistidine*	LC/MS neg	210.08841	43256			193270	2.45	1.49	0.6148	0.2952	0.8506	1.2711
58			N-acetyl-1-methylhistidine*	LC/MS pos	212.1028	43255				1.02	1.03	0.6978	0.3210	1.2117	1.2509
59			hydantoin-5-propionic acid	LC/MS polar	171.04113	40473	C05565	HMDB01212	782	1.16	1.10	0.6077	0.2924	1.2455	1.3746
60			trans-urocanate	LC/MS pos	139.05021	607	C00785	HMDB00301	736715	1.58	1.40	0.9219	0.3770	1.1521	1.6166
61			cis-urocanate	LC/MS pos	139.05021	40410			1549103	2.21	2.26	0.7392	0.3303	1.1927	2.6965
63			imidazole propionate	LC/MS pos	141.06586	40730		HMDB02271	70630	0.87	0.90	0.0344	0.0388	1.4112	1.2745
64			imidazole lactate	LC/MS pos	157.06077	15716	C05568	HMDB02320	440129	1.06	1.00	0.7503	0.3314	0.9544	0.9559
70			1-methylimidazoleacetate	LC/MS pos	141.06586	32350	C05828	HMDB02820	75810	1.95	1.06	0.2762	0.1754	0.9766	1.0325
71			4-imidazoleacetate	LC/MS polar	125.03565	32349	C02835	HMDB02024	96215	0.67	0.67	0.0022	0.0062	1.0848	0.7269
72			N-acetylhistamine	LC/MS pos	154.09749	48679	C05135	HMDB13253	69602	0.97	0.52	0.2202	0.1488	1.3101	0.6866
73		Lysine Metabolism	lysine	LC/MS pos	147.11281	1301	C00047	HMDB00182	5962	0.83	0.82	0.0002	0.0009	1.1419	0.9333
74			N2-acetyllysine	LC/MS pos	189.12337	36751	C12989	HMDB00446	92907	0.80	0.72	0.0133	0.0213	1.2041	0.8660
75			N6-acetyllysine	LC/MS pos	189.12337	36752	C02727	HMDB00206	92832	1.15	1.14	0.0030	0.0074	0.9215	1.0495
76			N-6-trimethyllysine	LC/MS pos	189.15976	1498	C03793	HMDB01325	440120	1.04	0.98	0.9011	0.3708	1.0179	0.9984
79			2-aminoadipate	LC/MS pos	162.07609	6146	C00956	HMDB00510	469	0.83	0.75	0.0157	0.0234	1.3280	0.9993
82			glutarate (pentanedioate)	LC/MS polar	131.03498	396	C00489	HMDB00661	743	0.66	0.62	0.0023	0.0064	1.7006	1.0553
83			glutarylcarnitine (C5)	LC/MS pos	276.14417	44664		HMDB13130	71464488	1.01	1.00	0.9426	0.3821	1.0277	1.0318
84			3-methylglutarylcarnitine (1)	LC/MS pos	290.15982	46547		HMDB00552	128145	2.23	2.19	0.0000	0.0000	0.9545	2.0915
85			3-methylglutarylcarnitine (2)	LC/MS pos	290.15982	46548		HMDB00552	128145	1.03	0.87	0.6702	0.3123	1.3498	1.1801
90			pipecolate	LC/MS pos	130.08626	1444	C00408	HMDB00070	849	0.98	0.97	0.3525	0.2017	1.1748	1.1358
98		Phenylalanine and Tyrosine Metabolism	phenylalanine	LC/MS pos	166.08626	64	C00079	HMDB00159	6140	0.88	0.87	0.0019	0.0057	1.0679	0.9320
99			N-acetylphenylalanine	LC/MS neg	206.08226	33950	C03519	HMDB00512	74839	0.97	0.96	0.3551	0.2028	1.1306	1.0799
102			phenylpyruvate	LC/MS neg	163.04007	566	C00166	HMDB00205	997	0.94	0.92	0.1332	0.1045	1.1377	1.0462
103			phenyllactate (PLA)	LC/MS neg	165.05571	22130	C05607	HMDB00779	3848	1.10	1.03	0.5923	0.2889	1.2251	1.2564
106			phenylacetate	LC/MS neg	135.04515	15958	C07086	HMDB00209	999	0.78	0.71	0.0179	0.0251	1.3119	0.9367
107			4-hydroxyphenylacetate	LC/MS neg	151.04007	541	C00642	HMDB00020	127	0.91	0.84	0.1092	0.0910	1.3172	1.1110
110			phenylacetylglutamine	LC/MS pos	265.11829	35126	C04148	HMDB06344	92258	1.02	0.81	0.3858	0.2164	1.3564	1.0965
111			tyrosine	LC/MS pos	182.08117	1299	C00082	HMDB00158	6057	0.78	0.77	0.0000	0.0001	1.1540	0.8850
112			N-acetyltyrosine	LC/MS neg	222.07718	32390		HMDB00866	68310	0.72	0.73	0.0002	0.0013	1.1960	0.8752
120			4-hydroxyphenylpyruvate	LC/MS neg	179.03498	1669	C01179	HMDB00707	979	0.99	0.93	0.4110	0.2270	0.9760	0.9054
121			3-(4-hydroxyphenyl)lactate	LC/MS neg	181.05063	32197	C03672	HMDB00755	9378	1.00	0.96	0.6507	0.3072	1.1211	1.0818
126			phenol sulfate	LC/MS neg	172.9914	32553	C02180	HMDB60015	74426	0.98	0.92	0.3907	0.2186	2.1406	1.9601
128			p-cresol sulfate	LC/MS neg	187.00705	36103	C01468	HMDB11635	4615423	1.84	0.88	0.4789	0.2524	1.2436	1.0977
134			o-cresol sulfate	LC/MS neg	187.00705	36845			11615528	1.53	1.42	0.0629	0.0628	0.9383	1.3313
139			vanillylmandelate (VMA)	LC/MS neg	197.04555	1567	C05584	HMDB00291	1245	0.93	0.94	0.1767	0.1280	1.0534	0.9886
141			3-methoxytyrosine	LC/MS pos	212.09174	12017		HMDB01434	1670	1.11	1.11	0.0027	0.0072	1.0033	1.1138
143			3-methoxytyramine sulfate	LC/MS neg	246.04416	44618				0.93	0.67	0.1658	0.1222	1.0819	0.7269
148			homovanillate (HVA)	LC/MS neg	181.05063	1101	C05582	HMDB00118	1738	0.76	0.77	0.0062	0.0125	1.1547	0.8859
163			gentisate	LC/MS polar	153.01933	18280	C00628	HMDB00152	3469	0.96	0.74	0.1138	0.0937	1.2615	0.9341
167			3-[3-(sulfooxy)phenyl]propanoic acid	LC/MS neg	245.01253	45415			187488	1.47	0.49	0.1331	0.1045	1.8998	0.9335
171			3-(3-hydroxyphenyl)propionate	LC/MS neg	165.05571	35635	C11457	HMDB00375	91	2.84	0.70	0.1546	0.1167	1.2430	0.8666
172			3-(4-hydroxyphenyl)propionate	LC/MS neg	165.05571	39587	C01744	HMDB02199	10394	0.94	0.78	0.1292	0.1024	0.8551	0.6685
174			3-phenylpropionate (hydrocinnamate)	LC/MS neg	149.0608	15749	C05629	HMDB00764	107	0.81	0.67	0.0326	0.0373	2.1967	1.4678
179			thyroxine	LC/MS neg	775.67944	2761	C01829	HMDB01918	5819	0.95	0.94	0.0296	0.0344	1.0667	1.0056
180			5-hydroxymethyl-2-furoic acid	LC/MS neg	141.01933	42040	C20448	HMDB02432	80642	0.82	0.54	0.0227	0.0289	1.6842	0.9148
181			2-hydroxyphenylacetate	LC/MS neg	151.04006	1432	C05852	HMDB00669	11970	0.94	0.86	0.2415	0.1590	1.2094	1.0369
183			phenylacetylcarnitine	LC/MS pos	280.15434	48425				1.01	0.85	0.1935	0.1370	1.1813	1.0027
184			dopamine sulfate (1)	LC/MS neg	232.02848	48406				1.06	0.75	0.5368	0.2733	1.6253	1.2190
185			dopamine sulfate (2)	LC/MS neg	232.02845	48407				1.02	0.75	0.4566	0.2432	1.4719	1.1076
186			p-cresol-glucuronide*	LC/MS neg	283.08232	48841		HMDB11686	154035	3.75	0.99	0.2788	0.1758	1.1125	1.0986
187			tyramine O-sulfate	LC/MS neg	216.0336	48408		HMDB06409	153005	3.42	0.86	0.5074	0.2627	2.1093	1.8182
188			N-formylphenylalanine	LC/MS neg	192.06661	48433			759256	0.78	0.61	0.0178	0.0251	1.2909	0.7914
193		Tryptophan Metabolism	tryptophan	LC/MS pos	205.09716	54	C00078	HMDB00929	6305	0.75	0.75	0.0000	0.0000	1.1807	0.8829
194			N-acetyltryptophan	LC/MS neg	245.09316	33959	C03137	HMDB13713	700653	0.75	0.73	0.0016	0.0050	1.2745	0.9310
197			indolelactate	LC/MS pos	206.08117	18349	C02043	HMDB00671	92904	0.94	0.92	0.1440	0.1107	1.2943	1.1847
198			indoleacetate	LC/MS pos	176.07061	27513	C00954	HMDB00197	802	0.86	0.82	0.0250	0.0301	1.4667	1.1978
200			indolepropionate	LC/MS pos	190.08626	32405		HMDB02302	3744	0.92	0.78	0.1525	0.1157	1.2646	0.9878
202			3-indoxyl sulfate	LC/MS neg	212.0023	27672		HMDB00682	10258	0.91	0.90	0.0814	0.0737	1.1600	1.0452
203			kynurenine	LC/MS pos	209.09207	15140	C00328	HMDB00684	161166	0.84	0.83	0.0004	0.0020	1.1147	0.9269
204			kynurenate	LC/MS neg	188.03531	1417	C01717	HMDB00715	3845	0.77	0.77	0.0007	0.0027	1.2853	0.9840
211			picolinate	LC/MS pos	124.03931	1512	C10164	HMDB02243	1018	0.62	0.60	0.0000	0.0001	1.3651	0.8190
218			5-hydroxyindoleacetate	LC/MS neg	190.05096	437	C05635	HMDB00763	1826	0.63	0.49	0.0033	0.0078	1.5830	0.7758
219			serotonin	LC/MS pos	177.10224	2342	C00780	HMDB00259	5202	0.76	0.69	0.0911	0.0796	1.3318	0.9224
224			indolebutyrate	LC/MS pos	204.10191	34402	C11284	HMDB02096	8617	1.07	1.08	0.5051	0.2627	0.7080	0.7641
226			indoleacetylglutamine	LC/MS neg	302.11463	42087		HMDB13240	25200879	1.00	0.92	0.4150	0.2277	1.3199	1.2157
227			tryptophan betaine	LC/MS pos	247.14411	37097	C09213	HMDB61115	442106	0.55	0.60	0.0000	0.0000	3.6167	2.1754
233			indole-3-carboxylic acid	LC/MS neg	160.0404	38116	C19837	HMDB03320	69867	1.15	1.03	0.6661	0.3114	1.0085	1.0392
234			C-glycosyltryptophan	LC/MS pos	367.14998	48782			10981970	1.10	1.09	0.1094	0.0910	0.9702	1.0534
235			N-acetylkynurenine (2)	LC/MS neg	249.08808	48757				0.68	0.60	0.0010	0.0035	1.1806	0.7137
237		Leucine, Isoleucine and Valine Metabolism	leucine	LC/MS pos	132.10191	60	C00123	HMDB00687	6106	0.81	0.80	0.0002	0.0012	1.1029	0.8828
238			N-acetylleucine	LC/MS neg	172.09791	1587	C02710	HMDB11756	70912	0.83	0.83	0.0153	0.0234	1.0607	0.8766
240			4-methyl-2-oxopentanoate	LC/MS neg	129.05571	22116	C00233	HMDB00695	70	0.86	0.86	0.0157	0.0234	1.1075	0.9495
243			isovalerate	LC/MS neg	101.0608	44656	C08262	HMDB00718	10430	0.60	0.58	0.0000	0.0001	1.2311	0.7200
244			isovalerylglycine	LC/MS neg	158.08226	35107		HMDB00678	546304	0.54	0.55	0.0008	0.0030	1.2632	0.6927
245			isovalerylcarnitine	LC/MS pos	246.16999	34407		HMDB00688	6426851	0.74	0.74	0.0005	0.0022	1.1596	0.8548
248			beta-hydroxyisovalerate	LC/MS neg	117.05572	12129		HMDB00754	69362	0.83	0.82	0.0012	0.0041	1.1749	0.9671
249			beta-hydroxyisovaleroylcarnitine	LC/MS pos	262.1649	35433				0.88	0.88	0.0444	0.0477	1.1032	0.9744
252			3-methylglutaconate	LC/MS polar	143.03498	38667		HMDB00522	1551553	0.94	0.96	0.2008	0.1398	1.1269	1.0768
256			alpha-hydroxyisovalerate	LC/MS neg	117.05571	33937		HMDB00407	99823	1.21	1.27	0.0071	0.0134	1.0033	1.2732
258			methylsuccinate	LC/MS polar	131.03498	15745		HMDB01844	10349	0.93	0.92	0.1095	0.0910	1.0555	0.9691
266			isoleucine	LC/MS pos	132.10191	1125	C00407	HMDB00172	6306	0.80	0.78	0.0005	0.0024	1.1221	0.8719
267			allo-isoleucine	GC/MS	274.22357	47034			6950182;99288	1.31	1.25	0.0085	0.0155	0.9935	1.2390
268			N-acetylisoleucine	LC/MS neg	172.09791	33967			2802421	0.95	0.93	0.2237	0.1504	0.9721	0.9087
269			3-methyl-2-oxovalerate	LC/MS neg	129.05572	15676	C00671	HMDB03736	47	0.86	0.86	0.0105	0.0176	1.1373	0.9769
270			2-methylbutyrylcarnitine (C5)	LC/MS pos	246.16999	45095		HMDB00378	6426901	0.89	0.86	0.0219	0.0285	1.0657	0.9210
272			tiglyl carnitine	LC/MS pos	244.15434	35428		HMDB02366	22833596	1.03	0.99	0.9726	0.3895	1.0775	1.0638
273			tigloylglycine	LC/MS pos	158.08117	1598		HMDB00959	6441567	0.61	0.57	0.0001	0.0006	1.2313	0.6963
275			2-hydroxy-3-methylvalerate	LC/MS neg	131.07136	36746		HMDB00317	164623	1.20	1.07	0.4459	0.2385	0.9196	0.9816
276			3-hydroxy-2-ethylpropionate	LC/MS neg	117.05571	32397		HMDB00396	188979	1.02	1.01	0.6731	0.3127	1.0507	1.0572
277			ethylmalonate	LC/MS polar	131.03498	15765		HMDB00622	11756	1.26	1.24	0.0047	0.0099	1.1462	1.4161
278			valine	LC/MS pos	118.08626	1649	C00183	HMDB00883	6287	0.81	0.80	0.0000	0.0001	1.0966	0.8766
279			N-acetylvaline	LC/MS neg	158.08226	1591		HMDB11757	66789	1.09	1.08	0.0175	0.0248	0.9730	1.0539
281			3-methyl-2-oxobutyrate	LC/MS neg	115.04006	44526	C00141	HMDB00019	49	1.07	1.06	0.5078	0.2627	1.0511	1.1160
282			isobutyrylcarnitine	LC/MS pos	232.15434	33441		HMDB00736	168379	0.82	0.78	0.0056	0.0116	1.2666	0.9868
283			isobutyrylglycine	LC/MS neg	144.06661	35437		HMDB00730	10855600	0.57	0.55	0.0001	0.0004	1.4584	0.8001
284			3-hydroxyisobutyrate	LC/MS polar	103.04007	1549	C06001	HMDB00336	87	0.86	0.81	0.0243	0.0299	1.1686	0.9447
285			alpha-hydroxyisocaproate	LC/MS neg	131.07136	22132	C03264	HMDB00746	83697	0.95	0.93	0.2323	0.1552	0.9515	0.8831
290		Methionine, Cysteine, SAM and Taurine Metabolism	methionine	LC/MS pos	150.05833	1302	C00073	HMDB00696	6137	0.77	0.75	0.0001	0.0005	1.1628	0.8752
291			N-acetylmethionine	LC/MS neg	190.05434	1589	C02712	HMDB11745	448580	1.89	1.88	0.0000	0.0000	0.7976	1.4971
292			N-formylmethionine	LC/MS neg	176.03869	2829	C03145	HMDB01015	439750	1.05	1.06	0.5808	0.2862	1.0324	1.0930
294			methionine sulfone	LC/MS polar	180.0336	44878			69961	1.04	1.04	0.6819	0.3158	1.0787	1.1183
295			methionine sulfoxide	LC/MS polar	164.03869	18374	C02989	HMDB02005	158980	0.94	0.90	0.2070	0.1418	1.0534	0.9517
298			S-adenosylhomocysteine (SAH)	LC/MS neg	383.11431	42382	C00021	HMDB00939	439155	1.33	1.18	0.4639	0.2465	0.7005	0.8258
303			alpha-ketobutyrate	LC/MS polar	101.02442	4968	C00109	HMDB00005	58	1.75	1.48	0.0300	0.0347	0.9656	1.4328
305			2-aminobutyrate	LC/MS pos	104.07061	42374	C02261	HMDB00650	439691	1.01	0.99	0.8186	0.3494	1.0219	1.0119
306			2-hydroxybutyrate (AHB)	LC/MS polar	103.04006	21044	C05984	HMDB00008	440864	1.41	1.30	0.0170	0.0245	0.9659	1.2542
307			cysteine	GC/MS	218	31453	C00097	HMDB00574	5862	0.69	0.66	0.0005	0.0022	1.2676	0.8375
309			cystine	LC/MS pos	241.03113	56	C00491	HMDB00192	67678	0.67	0.65	0.0000	0.0000	1.2565	0.8189
310			S-methylcysteine	LC/MS neg	134.02812	39592		HMDB02108	24417	1.03	0.93	0.5986	0.2896	1.1396	1.0628
312			cysteine s-sulfate	LC/MS neg	199.96928	22176	C05824	HMDB00731	115015	0.69	0.54	0.0030	0.0074	1.2694	0.6871
316			hypotaurine	LC/MS polar	108.01247	590	C00519	HMDB00965	107812	1.04	0.91	0.6069	0.2924	1.2855	1.1651
317			taurine	LC/MS neg	124.00739	2125	C00245	HMDB00251	1123	0.78	0.73	0.0077	0.0144	1.2349	0.9064
318			N-acetyltaurine	LC/MS neg	166.01795	48187			159864	1.62	1.60	0.0000	0.0001	0.9214	1.4727
325		Urea cycle; Arginine and Proline Metabolism	arginine	LC/MS pos	175.11896	1638	C00062	HMDB00517	232	0.85	0.84	0.0031	0.0075	1.1366	0.9536
326			urea	LC/MS pos	121.07201	1670	C00086	HMDB00294	1176	0.84	0.82	0.0040	0.0087	1.1318	0.9297
327			ornithine	LC/MS polar	131.0826	1493	C00077	HMDB03374	6262	0.68	0.67	0.0001	0.0007	1.2163	0.8201
328			proline	LC/MS pos	116.07061	1898	C00148	HMDB00162	145742	1.01	1.00	0.9388	0.3821	1.0226	1.0219
329			citrulline	LC/MS pos	176.10297	2132	C00327	HMDB00904	9750	0.94	0.92	0.1759	0.1280	1.0654	0.9769
332			homocitrulline	LC/MS pos	190.11862	22138	C02427	HMDB00679	65072	1.31	1.26	0.0570	0.0585	1.0883	1.3693
336			dimethylarginine (SDMA + ADMA)	LC/MS pos	203.15026	36808	C03626	HMDB01539	123831	1.26	1.25	0.0001	0.0004	0.8930	1.1130
337			N-acetylarginine	LC/MS pos	217.12952	33953	C02562	HMDB04620	67427	0.65	0.66	0.0000	0.0000	1.4152	0.9292
340			N-delta-acetylornithine	LC/MS pos	175.10772	43249			9920500	0.91	0.91	0.0953	0.0824	1.0595	0.9639
344			N-methylproline	LC/MS pos	130.08626	37431			557	1.36	0.83	0.5715	0.2847	2.3253	1.9318
346			trans-4-hydroxyproline	LC/MS pos	132.06552	32306	C01157	HMDB00725	5810	1.04	0.94	0.7143	0.3239	1.1325	1.0696
348			pro-hydroxy-pro	LC/MS pos	229.11829	35127		HMDB06695	11673055	1.07	1.06	0.5858	0.2872	1.0023	1.0618
352			N-acetylcitrulline	LC/MS neg	216.09898	48434	C15532	HMDB00856	656979	0.59	0.60	0.0005	0.0022	1.9575	1.1697
353		Creatine Metabolism	creatine	LC/MS pos	132.07676	27718	C00300	HMDB00064	586	0.98	0.97	0.4960	0.2587	1.0013	0.9676
354			creatinine	LC/MS pos	114.06619	513	C00791	HMDB00562	588	1.08	1.07	0.0697	0.0667	0.9715	1.0420
359			guanidinoacetate	LC/MS polar	116.04655	43802	C00581	HMDB00128	763	0.72	0.71	0.0000	0.0002	1.1968	0.8516
362		Polyamine Metabolism	acisoga	LC/MS pos	185.12846	43258			129397	1.33	1.25	0.0060	0.0121	0.8772	1.1006
366			5-methylthioadenosine (MTA)	LC/MS neg	296.0823	1419	C00170	HMDB01173	439176	1.19	1.18	0.0012	0.0041	0.9092	1.0733
369			N-acetylputrescine	LC/MS pos	131.11789	37496	C02714	HMDB02064	122356	1.62	1.05	0.5801	0.2862	0.9360	0.9811
370			4-acetamidobutanoate	LC/MS pos	146.08117	1558	C02946	HMDB03681	18189	0.95	0.95	0.2156	0.1462	1.0295	0.9786
375		Guanidino and Acetamido Metabolism	4-guanidinobutanoate	LC/MS pos	146.09241	15681	C01035	HMDB03464	500	0.93	0.92	0.1895	0.1346	1.1762	1.0778
376			guanidinosuccinate	LC/MS polar	174.05203	32446	C03139	HMDB03157	97856	1.06	1.09	0.2840	0.1777	0.6731	0.7356
380		Glutathione Metabolism	cysteine-glutathione disulfide	LC/MS pos	214.05123	35159		HMDB00656	4247235	0.95	0.89	0.2246	0.1506	0.9960	0.8907
384			cys-gly, oxidized	LC/MS neg	353.0595	18368			333293	1.16	1.15	0.1324	0.1045	1.0214	1.1729
385			5-oxoproline	LC/MS neg	128.03531	1494	C01879	HMDB00267	7405	0.94	0.93	0.0930	0.0808	1.0355	0.9641
393		Gamma-glutamyl Amino Acid	gamma-glutamylalanine	LC/MS pos	219.09755	37063		HMDB29142	440103	1.01	1.00	0.8125	0.3473	1.0106	1.0076
395			gamma-glutamylglutamate	LC/MS pos	277.10303	36738	C05282	HMDB11737	92865	0.80	0.77	0.0367	0.0406	1.2617	0.9684
396			gamma-glutamylglutamine	LC/MS pos	276.11902	2730	C05283	HMDB11738	150914	0.92	0.91	0.0717	0.0680	1.0731	0.9790
397			gamma-glutamylglycine	LC/MS neg	203.06734	33949		HMDB11667	165527	0.67	0.50	0.0068	0.0132	1.7954	0.9043
398			gamma-glutamylhistidine	LC/MS neg	283.10479	18245			7017195	0.90	0.87	0.0921	0.0802	1.3621	1.1883
399			gamma-glutamylisoleucine*	LC/MS pos	261.1445	34456		HMDB11170	14253342	0.87	0.81	0.0342	0.0387	1.1889	0.9599
400			gamma-glutamylleucine	LC/MS pos	261.1445	18369		HMDB11171	151023	0.86	0.83	0.0132	0.0213	1.1059	0.9147
401			gamma-glutamyl-epsilon-lysine	LC/MS pos	276.1554	33934		HMDB03869	65254;14284565	0.98	0.93	0.4439	0.2384	1.0674	0.9955
402			gamma-glutamylmethionine	LC/MS neg	277.08636	44872		HMDB29155	7009567	0.78	0.74	0.0029	0.0073	1.2240	0.9080

Comparison mean values significantly different:

$p \le 0.05$, fold of change ≥ 1.00

$p \le 0.05$, fold of change < 1.00

Comparison mean value difference approaching significance:

$0.05 < p < 0.10$, fold of change ≥ 1.00

$0.05 < p < 0.10$, fold of change < 1.00

Pathway Sort Order	Super Pathway	Sub Pathway	Biochemical Name	Platform	Mass	Comp ID	KEGG	HMDB	PubChem	Fold of Change, Paired t-Test: Post G-CSF / Pre G-CSF		Paired t-Test: Post G-CSF / Pre G-CSF		Mean Values	
										Mean Pairs Ratio	Group Mean Ratio	*p*-value	*q*-value	Pre G-CSF	Post G-CSF
403	Peptide		gamma-glutamylphenylalanine	LC/MS pos	295.12885	33422		HMDB00594	111299	0.98	0.96	0.4448	0.2384	1.0615	1.0153
404			gamma-glutamylthreonine*	LC/MS pos	249.10812	33364		HMDB29159		0.90	0.90	0.0482	0.0511	1.0908	0.9776
405			gamma-glutamyltryptophan	LC/MS pos	334.13975	33947		HMDB29160	3989307	0.80	0.78	0.0017	0.0053	1.1941	0.9356
406			gamma-glutamyltyrosine	LC/MS pos	311.12377	2734		HMDB11741	94340	0.85	0.82	0.0166	0.0244	1.0817	0.8885
407			gamma-glutamylvaline	LC/MS pos	247.12885	43829		HMDB11172	7015683	0.88	0.86	0.0147	0.0228	1.0957	0.9398
408			gamma-glutamyl-2-aminobutyrate	LC/MS pos	233.1132	37092				1.18	1.10	0.7803	0.3405	0.8844	0.9689
410		Dipeptide Derivative	N-acetylcarnosine	LC/MS pos	269.12444	43488		HMDB12881	9903482	1.00	0.98	0.6322	0.3013	1.0450	1.0260
517		Dipeptide	cyclo(val-val)	LC/MS pos	199.14411	49796			519728	1.00	0.86	0.5622	0.2822	1.1562	0.9924
545			glycylvaline	LC/MS pos	175.10772	18357		HMDB28854	97417	0.56	0.15	0.0034	0.0079	6.9270	1.0565
569			isoleucylglycine	LC/MS pos	189.12337	40008			342532	0.82	0.75	0.0157	0.0234	1.1828	0.8889
587			leucylglycine	LC/MS pos	189.12337	40045			79070	0.51	0.51	0.0001	0.0004	1.1721	0.5961
656			prolylglycine	LC/MS pos	173.09207	40703			7408076;6426709	0.89	0.80	0.0837	0.0744	1.0835	0.8663
696			threonylphenylalanine	LC/MS pos	267.13394	31530			4099799;4099798	0.81	0.73	0.0449	0.0480	1.1720	0.8526
735			valylglycine	LC/MS pos	175.10772	40475			136487	0.71	0.57	0.0054	0.0112	1.2603	0.7244
738			valylleucine	LC/MS pos	231.17032	39994			352039	0.80	0.78	0.0039	0.0087	1.1095	0.8613
751		Polypeptide	bradykinin, des-arg(9)	LC/MS pos	452.73742	34420	C00306	HMDB04246	105044	0.84	0.68	0.0329	0.0374	1.5525	1.0516
755			HWESASXX*	LC/MS pos	471.73762	32836				0.83	0.80	0.0598	0.0599	1.3486	1.0734
781		Fibrinogen Cleavage Peptide	ADSGEGDFXAEGGGVR*	LC/MS pos	768.84989	33084			16133137	2.00	1.18	0.3276	0.1945	0.9326	1.1044
782			DSGEGDFXAEGGGVR*	LC/MS pos	733.33133	31548				2.08	1.20	0.7362	0.3303	0.9152	1.1005
793	Carbohydrate	Glycolysis, Gluconeogenesis, and Pyruvate Metabolism	1,5-anhydroglucitol (1,5-AG)	LC/MS neg	163.06119	20675	C07326	HMDB02712	64960	0.91	0.92	0.0028	0.0073	0.9963	0.9126
796			glucose	LC/MS polar	179.05611	20488	C00031	HMDB00122	79025	1.00	0.99	0.7329	0.3294	1.0467	1.0349
812			pyruvate	LC/MS polar	87.00877	48990	C00022	HMDB00243	1060	1.60	1.42	0.0019	0.0057	0.9167	1.3015
813			lactate	LC/MS polar	89.02442	527	C00186	HMDB00190	612	1.46	1.41	0.0002	0.0009	0.8890	1.2513
816			glycerate	LC/MS polar	105.01933	1572	C00258	HMDB00139	752	0.83	0.83	0.0008	0.0032	1.0458	0.8642
831		Pentose Metabolism	ribose	GC/MS	204	12083	C00121	HMDB00283	5779	1.14	1.06	0.5913	0.2889	0.9277	0.9788
832			ribitol	LC/MS polar	151.06119	15772	C00474	HMDB00508	6912	1.03	1.02	0.9083	0.3726	1.0396	1.0571
833			ribonate	LC/MS polar	165.04046	27731	C01685	HMDB00867	5460677	0.95	0.94	0.2785	0.1758	1.0076	0.9487
836			xylonate	GC/MS	292	35638	C05411	HMDB60256	6602431	1.08	1.00	0.9999	0.3961	1.0565	1.0544
837			xylose	GC/MS	204	15835	C00181	HMDB00098	135191	1.31	1.19	0.5196	0.2672	1.0809	1.2824
838			xylitol	GC/MS	307.2	4966	C00379	HMDB02917	6912	1.27	1.20	0.1646	0.1217	0.9197	1.1026
839			arabinose	GC/MS	217	575	C00216	HMDB00646	66308	1.25	1.17	0.3259	0.1944	1.0208	1.1953
842			threitol	GC/MS	217.1	35854	C16884	HMDB04136	169019	1.16	1.14	0.2020	0.1398	1.0166	1.1617
844			arabitol	GC/MS	307.1	38075	C01904	HMDB00568	94154	1.00	0.94	0.5603	0.2821	1.0551	0.9881
850			fucose	GC/MS	204	15821	C01018	HMDB00174	19466	1.13	1.10	0.2856	0.1779	1.0383	1.1442
861		Glycogen Metabolism	maltotriose	LC/MS neg	503.16175	44688	C01835	HMDB01262	439586	39.18	16.27	0.0000	0.0000	0.0782	1.2721
864			maltose	LC/MS polar	387.11442	15586	C00208	HMDB00163	10991489	8.72	6.67	0.0000	0.0000	0.4175	2.7867
886		Disaccharides and Oligosaccharides	sucrose	LC/MS neg	341.10894	1519	C00089	HMDB00258	5988	1.60	0.82	0.6396	0.3040	1.9134	1.5684
900		Fructose, Mannose and Galactose Metabolism	fructose	LC/MS polar	179.05611	577	C00095	HMDB00660	5984	0.92	0.67	0.0818	0.0738	1.4563	0.9765
906			mannose	LC/MS polar	179.05611	584	C00159	HMDB00169	18950	1.40	1.23	0.0197	0.0270	0.9723	1.1979
910			mannitol	GC/MS	319.1	15335	C00392	HMDB00765	6251	1.34	1.03	0.8009	0.3453	1.1207	1.1490
924			galactonate	LC/MS polar	195.05102	27719	C00880	HMDB00565	128869	1.19	0.77	0.4280	0.2323	1.6234	1.2481
950		Aminosugar Metabolism	glucuronate	LC/MS polar	193.03537	15443	C00191	HMDB00127	444791	1.02	1.00	0.5349	0.2733	1.0362	1.0334
961			N-acetylneuraminate	LC/MS polar	308.0987	32377	C00270	HMDB00230	439197	1.02	1.00	0.7713	0.3371	1.0008	0.9985
973			erythronate*	LC/MS polar	135.02989	42420		HMDB00613	2781043	1.26	1.26	0.0003	0.0016	0.9399	1.1804
980	Energy	TCA Cycle	citrate	LC/MS neg	191.01973	1564	C00158	HMDB00094	311	6.51	5.51	0.1201	0.0970	0.9461	5.2152
982			aconitate [cis or trans]	LC/MS neg	173.00916	46173	C00417	HMDB00072		1.29	1.22	0.0264	0.0315	0.9360	1.1391
985			alpha-ketoglutarate	LC/MS polar	145.01425	528	C00026	HMDB00208	51	0.98	0.96	0.4248	0.2316	1.0262	0.9807
987			succinylcarnitine	LC/MS pos	262.12852	37058				1.10	1.10	0.0557	0.0577	1.0311	1.1354
988			succinate	LC/MS polar	117.01933	1437	C00042	HMDB00254	1110	0.88	0.87	0.0704	0.0671	1.0640	0.9279
989			fumarate	LC/MS polar	115.00368	1643	C00122	HMDB00134	444972	1.10	1.04	0.5064	0.2627	1.0186	1.0570
990			malate	LC/MS polar	133.01425	1303	C00149	HMDB00156	525	1.02	0.97	0.6297	0.3006	1.1418	1.1068
1000			2-methylcitrate/homocitrate	LC/MS neg	205.03538	52282				0.94	0.91	0.1793	0.1291	1.0576	0.9598
1003		Oxidative Phosphorylation	phosphate	LC/MS neg	96.96962	42109	C00009	HMDB01429	1061	0.81	0.81	0.0175	0.0248	1.0886	0.8828
1004		Short Chain Fatty Acid	valerate	LC/MS neg	101.0608	33443	C00803	HMDB00892	7991	0.95	0.84	0.2023	0.1398	1.0622	0.8885
1005		Medium Chain Fatty Acid	caproate (6:0)	LC/MS neg	115.07645	32489	C01585	HMDB00535	8892	1.08	1.05	0.7504	0.3314	1.0679	1.1209
1006			heptanoate (7:0)	LC/MS neg	129.0921	1644	C17714	HMDB00666	8094	0.86	0.85	0.0157	0.0234	1.1133	0.9497
1007			caprylate (8:0)	LC/MS neg	143.10775	32492	C06423	HMDB00482	379	1.22	1.21	0.2506	0.1637	1.0863	1.3186
1009			caprate (10:0)	LC/MS neg	171.13905	1642	C01571	HMDB00511	2969	1.65	1.48	0.0274	0.0325	1.0080	1.4871
1011			10-undecenoate (11:1n1)	LC/MS neg	183.13905	32497				1.15	1.04	0.4756	0.2512	1.1526	1.1938
1012			laurate (12:0)	LC/MS neg	199.17035	1645	C02679	HMDB00638	3893	1.49	1.39	0.0146	0.0228	0.9576	1.3346
1013			5-dodecenoate (12:1n7)	LC/MS neg	197.1547	33968		HMDB00529	5312378	1.32	1.34	0.1766	0.1280	1.1826	1.5857
1015		Long Chain Fatty Acid	myristate (14:0)	LC/MS neg	227.20165	1365	C06424	HMDB00806	11005	1.64	1.51	0.0247	0.0300	1.0213	1.5461
1016			myristoleate (14:1n5)	LC/MS neg	225.186	32418	C08322	HMDB02000	5281119	1.80	1.64	0.0233	0.0293	1.3317	2.1785
1017			pentadecanoate (15:0)	LC/MS neg	241.2173	1361	C16537	HMDB00826	13849	1.40	1.36	0.0022	0.0062	1.0312	1.3977
1018			palmitate (16:0)	LC/MS neg	255.23295	1336	C00249	HMDB00220	985	1.24	1.24	0.0193	0.0267	1.0105	1.2489
1019			palmitoleate (16:1n7)	LC/MS neg	253.2173	33447	C08362	HMDB03229	445638	1.85	1.73	0.0754	0.0696	1.3848	2.3988
1021			margarate (17:0)	LC/MS neg	269.2486	1121		HMDB02259	10465	1.55	1.50	0.0064	0.0127	1.0301	1.5499
1022			10-heptadecenoate (17:1n7)	LC/MS neg	267.23295	33971		HMDB60038	5312435	1.77	1.65	0.0198	0.0270	1.0032	1.6548
1023			stearate (18:0)	LC/MS neg	283.26425	1358	C01530	HMDB00827	5281	1.23	1.23	0.0202	0.0273	0.9912	1.2197
1025			oleate (18:1n9)	GC/MS	339.2	1359	C00712	HMDB00207	445639	1.70	1.61	0.0086	0.0156	1.0068	1.6250
1027			cis-vaccenate (18:1n7)	GC/MS	339.3	33970	C08367	HMDB03231	5282761	1.58	1.38	0.0110	0.0182	0.9850	1.3596
1031			nonadecanoate (19:0)	LC/MS neg	297.2799	1356	C16535	HMDB00772	12591	1.34	1.35	0.0008	0.0032	0.9524	1.2840
1032			10-nonadecenoate (19:1n9)	LC/MS neg	295.26425	33972		HMDB13622	5312513	1.88	1.74	0.0044	0.0094	0.9312	1.6195
1034			arachidate (20:0)	LC/MS neg	311.29555	1118	C06425	HMDB02212	10467	1.31	1.18	0.1587	0.1189	1.0427	1.2306
1037			eicosenoate (20:1)	LC/MS neg	309.2799	33587		HMDB02231	5282768	2.04	1.97	0.0001	0.0005	0.8493	1.6754
1041			erucate (22:1n9)	LC/MS neg	337.3112	1552	C08316	HMDB02068	5281116	2.39	1.38	0.0463	0.0493	1.0137	1.4032
1050		Polyunsaturated Fatty Acid (n3 and n6)	stearidonate (18:4n3)	LC/MS neg	275.20165	33969	C16300	HMDB06547	5312508	1.33	1.23	0.1590	0.1189	1.2865	1.5873
1051			eicosapentaenoate (EPA; 20:5n3)	LC/MS neg	301.2173	18467	C06428	HMDB01999	446284	0.83	0.79	0.0596	0.0599	1.6164	1.2783
1052			docosapentaenoate (n3 DPA; 22:5n3)	LC/MS neg	329.2486	32504	C16513	HMDB01976	6441454	1.45	1.37	0.0277	0.0326	0.9218	1.2634
1053			docosahexaenoate (DHA; 22:6n3)	LC/MS neg	327.23295	44675	C06429	HMDB02183	445580	0.96	0.90	0.3363	0.1955	1.3251	1.1889
1057			linoleate (18:2n6)	LC/MS neg	279.23295	1105	C01595	HMDB00673	5280450	1.42	1.38	0.0222	0.0288	1.0030	1.3855
1059			linolenate [alpha or gamma; (18:3n3 or 6)]	LC/MS neg	277.2173	34035	C06426	HMDB03073	5280934	1.80	1.70	0.0101	0.0173	0.9509	1.6206
1061			dihomo-linolenate (20:3n3 or n6)	LC/MS neg	305.2486	35718	C03242	HMDB02925	5280581	0.90	0.86	0.1201	0.0970	1.1769	1.0136
1062			arachidonate (20:4n6)	LC/MS neg	303.23295	1110	C00219	HMDB01043	444899	0.76	0.73	0.0093	0.0168	1.1828	0.8666
1063			adrenate (22:4n6)	LC/MS neg	331.26425	32980	C16527	HMDB02226	5497181	2.36	2.25	0.0000	0.0001	0.7346	1.6524
1064			docosapentaenoate (n6 DPA; 22:5n6)	LC/MS neg	329.2486	37478	C16513	HMDB01976	6441454	0.86	0.81	0.0397	0.0432	1.2608	1.0182
1065			docosadienoate (22:2n6)	LC/MS neg	335.29555	32415	C16533	HMDB61714	5282807	2.96	2.79	0.0000	0.0000	0.6261	1.7463
1066			dihomo-linoleate (20:2n6)	LC/MS neg	307.26425	17805	C16525	HMDB05060	6439848	1.90	1.77	0.0002	0.0010	0.8468	1.4958
1116		Fatty Acid, Branched	13-methylmyristic acid	LC/MS neg	241.2173	38293			151014	1.40	1.28	0.0813	0.0737	0.9630	1.2317
1119			15-methylpalmitate (isobar with 2-methylpalmitate)	LC/MS neg	269.24868	38768			17903417	1.34	1.32	0.3212	0.1930	1.0557	1.3975
1122			17-methylstearate	LC/MS neg	297.2799	38296			3083779	1.71	1.63	0.0019	0.0057	0.7957	1.2957
1133		Fatty Acid, Dicarboxylate	2-hydroxyglutarate	LC/MS polar	147.02989	37253	C02630	HMDB00606	43	1.71	1.61	0.0214	0.0284	0.9920	1.5942
1136			2-hydroxyadipate	LC/MS polar	161.04554	31934	C02360	HMDB00321	193530	0.88	0.68	0.0558	0.0577	1.1531	0.7814
1137			3-methyladipate	LC/MS polar	159.06628	36749		HMDB00555	12292	1.45	1.35	0.1395	0.1088	0.9878	1.3318
1139			maleate	LC/MS polar	115.00368	20676	C01384	HMDB00176	444266	0.77	0.73	0.0070	0.0134	1.3816	1.0110
1142			suberate (octanedioate)	LC/MS polar	173.08193	15730	C08278	HMDB00893	10457	1.23	0.79	0.3338	0.1953	2.0104	1.5913
1145			azelate (nonanedioate)	LC/MS neg	187.09758	18362	C08261	HMDB00784	2266	1.00	0.75	0.1780	0.1286	1.8739	1.4075
1146			sebacate (decanedioate)	LC/MS neg	201.11323	32398	C08277	HMDB00792	5192	1.37	1.31	0.0662	0.0654	0.9876	1.2948
1149			dodecanedioate	LC/MS neg	229.14453	32388	C02678	HMDB00623	12736	1.28	1.23	0.0541	0.0564	1.0110	1.2428
1150			tetradecanedioate	LC/MS neg	257.17583	35669		HMDB00872	13185	1.12	1.08	0.3204	0.1930	1.2258	1.3216
1151			hexadecanedioate	LC/MS neg	285.20713	35678	C19615	HMDB00672	10459	1.71	1.68	0.0009	0.0034	0.9444	1.5827
1152			octadecanedioate	LC/MS neg	313.23843	36754		HMDB00782	70095	2.08	1.94	0.0003	0.0013	0.9011	1.7485
1153			eicosanodioate	LC/MS neg	341.26973	39831			75502	0.95	0.78	0.2812	0.1769	1.3290	1.0409
1155			3-carboxy-4-methyl-5-propyl-2-furanpropanoate (CMPF)	LC/MS neg	239.09249	31787		HMDB61112	123979	0.89	0.96	0.1013	0.0862	2.0180	1.9357
1180		Fatty Acid, Amide	oleamide	LC/MS neg	280.26458	32458	C19670	HMDB02117	5283387	0.69	0.37	0.0105	0.0176	2.6343	0.9708
1186		Fatty Acid, Amino	2-aminoheptanoate	LC/MS pos	146.11756	43761			227939	1.00	0.94	0.4756	0.2512	1.0619	0.9952
1191			2-aminooctanoate	LC/MS pos	160.13321	43343		HMDB00991	69522	1.16	0.94	0.9291	0.3793	1.1555	1.0818
1199		Fatty Acid Synthesis	malonylcarnitine	LC/MS pos	248.11287	37059		HMDB02095	22833583	1.05	0.99	0.8270	0.3524	0.9712	0.9581
1200			malonate	LC/MS polar	103.00368	15872	C00383	HMDB00691	867	2.34	2.12	0.1487	0.1132	1.0365	2.1925
1201			2-methylmalonyl carnitine	LC/MS pos	262.12852	35482		HMDB13133	53481628	1.05	1.02	0.8324	0.3532	0.9749	0.9922
1213		Fatty Acid Metabolism (also BCAA Metabolism)	butyrylcarnitine	LC/MS pos	232.15434	32412	C02862	HMDB02013	439829	1.29	1.25	0.0010	0.0035	1.0910	1.3637
1214			butyrylglycine	LC/MS neg	144.06661	31850		HMDB00808	88412	0.96	0.90	0.3037	0.1860	1.5664	1.4076
1216			propionylcarnitine	LC/MS pos	218.13869	32452	C03017	HMDB00824	107738	0.84	0.84	0.0021	0.0061	1.0682	0.8925
1217			propionylglycine	LC/MS pos	132.06552	31932		HMDB00783	98681	0.78	0.77	0.0349	0.0391	1.0967	0.8400
1219			methylmalonate (MMA)	LC/MS polar	117.01933	1496	C02170	HMDB00202	487	0.91	0.92	0.1045	0.0880	1.0177	0.9355
1222		Fatty Acid Metabolism(Acyl Glycine)	hexanoylglycine	LC/MS neg	172.09791	35436		HMDB00701	99463	1.38	1.45	0.0237	0.0294	0.9499	1.3790
1225			N-palmitoyl glycine	LC/MS neg	312.25441	42092			151008	1.23	1.18	0.1112	0.0921	0.9318	1.1001
1230		Fatty Acid Metabolism(Acyl Carnitine)	acetylcarnitine	LC/MS pos	204.12304	32198	C02571	HMDB00201	1	1.09	1.08	0.2524	0.1645	0.9316	1.0068
1231			hydroxybutyrylcarnitine*	LC/MS pos	248.14925	43264		HMDB13127	53481617	1.49	1.44	0.0006	0.0025	0.8635	1.2458
1233			hexanoylcarnitine	LC/MS pos	260.18564	32328		HMDB00705	6426853	1.39	1.37	0.0010	0.0035	1.0121	1.3885
1234			octanoylcarnitine	LC/MS pos	288.21694	33936	C02838	HMDB00791	123701	1.32	1.12	0.0998	0.0852	1.0252	1.1431
1236			decanoylcarnitine	LC/MS pos	316.24824	33941		HMDB00651	10245190	1.42	1.13	0.0880	0.0777	0.9643	1.0899
1238			cis-4-decenoyl carnitine	LC/MS pos	314.23259	38178				1.05	1.05	0.9411	0.3821	0.9371	0.9856
1239			laurylcarnitine	LC/MS pos	344.27954	34534		HMDB02250	10427569	2.86	1.82	0.0696	0.0667	0.7475	1.3612
1242			palmitoylcarnitine	LC/MS pos	400.34214	44681	C02990	HMDB00222	461	2.77	2.31	0.2703	0.1735	0.9507	2.1947
1244			linoleoylcarnitine*	LC/MS pos	424.34214	46223		HMDB06469	6450015	4.73	3.60	0.2368	0.1575	0.3817	1.3723
1245			oleoylcarnitine	LC/MS pos	426.35779	35160		HMDB05065	6441392;53477789	3.77	2.98	0.0586	0.0596	0.7766	2.3145
1247			myristoleoylcarnitine*	LC/MS pos	370.29471	48182				3.62	2.54	0.0247	0.0300	0.6640	1.6882
1248		Carnitine Metabolism	deoxycarnitine	LC/MS pos	146.11756	36747	C01181	HMDB01161	134	1.02	1.02	0.8811	0.3674	1.0017	1.0174
1249			carnitine	LC/MS pos	162.11247	15500	C00318	HMDB00062	10917	1.07	1.07	0.0034	0.0079	0.9669	1.0327
1252		Ketone Bodies	acetoacetate	LC/MS pos	103.03898	33963	C00164	HMDB00060	96	1.20	0.97	0.7890	0.3418	1.1123	1.0767
1254			3-hydroxybutyrate (BHBA)	LC/MS polar	103.04007	542	C01089	HMDB00357	441	1.03	1.00	0.7415	0.3304	1.1796	1.1834
1257		Fatty Acid, Monohydroxy	alpha-hydroxycaproate	LC/MS neg	131.07136	37073		HMDB01624	99824	1.19	1.23	0.5691	0.2840	1.0678	1.3089
1258			2-hydroxyoctanoate	LC/MS neg	159.10266	22036		HMDB02264	94180	0.94	0.85	0.2385	0.1579	1.1505	0.9768
1259			2-hydroxydecanoate	LC/MS neg	187.13396	42489			21488	1.18	0.89	0.5437	0.2762	1.2932	1.1506
1261			2-hydroxypalmitate	LC/MS neg	271.22786	35675		HMDB31057	92836	0.90	0.85	0.0878	0.0777	1.0830	0.9220
1262			2-hydroxystearate	LC/MS neg	299.25917	17945	C03045		69417	0.88	0.83	0.0525	0.0554	1.1743	0.9778
1265			3-hydroxyoctanoate	LC/MS neg	159.10266	22001		HMDB01954	26613	1.31	1.19	0.1614	0.1203	1.0634	1.2665
1266			3-hydroxydecanoate	LC/MS neg	187.13396	22053		HMDB02203	26612	1.37	1.25	0.0175	0.0248	1.0661	1.3361
1267			3-hydroxysebacate	LC/MS neg	217.10814	31943		HMDB00350	3017884	2.15	1.72	0.0041	0.0089	0.9934	1.7070
1268			3-hydroxylaurate	LC/MS neg	215.16526	32457		HMDB00387	94216	1.57	1.36	0.0234	0.0293	0.9687	1.3179
1273			5-hydroxyhexanoate	LC/MS neg	131.07136	31938		HMDB00525	170748	1.16	1.07	0.4059	0.2255	1.0430	1.1150
1280			16-hydroxypalmitate	LC/MS neg	271.22786	39609	C18218	HMDB06294	10466	1.44	1.39	0.0246	0.0300	0.9760	1.3539
1285			13-HODE + 9-HODE	LC/MS neg	295.22825	37752			43013	1.12	0.96	0.9724	0.3895	1.3972	1.3377
1295		Fatty Acid, Dihydroxy	12,13-DiHOME	LC/MS neg	313.23843	38395	C14829	HMDB04705	10236635	1.60	1.22	0.3483	0.2002	0.9774	1.1947
1296			9,10-DiHOME	LC/MS neg	313.23843	38399	C14828	HMDB04704	9966640	1.12	1.10	0.2207	0.1488	1.1846	1.3070
1361		Eicosanoid	5-HETE	LC/MS neg	319.22786	37372	C04805	HMDB11134	5280733	1.99	1.77	0.0187	0.0259	0.6777	1.1989
1366			12-HETE	LC/MS neg	319.22786	37536		HMDB06111	5312983	0.60	0.31	0.0011	0.0036	2.9009	0.9121
1376		Endocannabinoid	oleic ethanolamide	LC/MS neg	324.2908	38102		HMDB02088	5283454	1.14	1.08	0.7185	0.3253	1.0167	1.0956
1387			N-stearoyltaurine	LC/MS neg	390.26835	39730			168274	1.26	1.01	0.9724	0.3895	0.9774	0.9827
1391		Inositol Metabolism	myo-inositol	LC/MS polar	225.06159	1124	C00137	HMDB00211	892	0.87	0.88	0.0293	0.0342	1.0789	0.9455
1392			chiro-inositol	GC/MS	318.1	37112	C19891	HMDB34220		3.00	1.12	0.8301	0.3531	1.5734	1.7646
1394			scyllo-inositol	GC/MS	318.2	32379	C06153	HMDB06088	892	0.79	0.71	0.0096	0.0169	1.5570	1.0997
1399			inositol 1-phosphate (I1P)	GC/MS	318.1	1481	C04006	HMDB00213	440194	0.78	0.75	0.0029	0.0073	1.1882	0.8856
1418		Phospholipid Metabolism	choline	LC/MS pos	104.10699	15506	C00114	HMDB00097	305	0.89	0.89	0.0065	0.0127	1.0740	0.9552
1419			choline phosphate	LC/MS pos	184.07332	34396	C00588	HMDB01565	1014	0.38	0.24	0.0000	0.0002	1.5486	0.3782
1421			glycerophosphorylcholine (GPC)	LC/MS pos	258.1101	15990	C00670	HMDB00086	71920	0.77	0.76	0.0017	0.0051	1.1454	0.8651
1424			ethanolamine	GC/MS	174.1	1497	C00189	HMDB00149	700	1.02	1.00	0.8639	0.3620	1.0190	1.0169
1427			glycerophosphoethanolamine	LC/MS polar	214.04859	37455	C01233	HMDB00114	123874	0.98	0.97	0.4561	0.2432	0.9595	0.9343
1428			trimethylamine N-oxide	LC/MS pos	151.14411	40406	C01104	HMDB00925	1145	2.34	2.51	0.7706	0.3371	1.7278	4.3427
1429			glycerophosphoinositol*	LC/MS polar	333.05922	47155				1.71	1.29	0.6174	0.2959	0.9448	1.2208
1430			1-palmitoyl-2-oleoyl-GPC (16:0/18:1)*	LC/MS polar	804.57604	52461			6436017	0.97	0.96	0.3560	0.2028	1.0222	0.9765
1431			1-stearoyl-2-arachidonoyl-GPC (18:0/20:4)	LC/MS polar	854.59091	42450			16219824	0.90	0.86	0.0830	0.0743	1.2047	1.0307
1432			1-palmitoyl-2-linoleoyl-GPC (16:0/18:2)*	LC/MS polar	802.56018	52460			5287971	0.93	0.93	0.0722	0.0680	1.0673	0.9914
1433			1,2-dipalmitoyl-GPC (16:0/16:0)	LC/MS polar	778.5604	19130		HMDB00564	452110	1.10	1.09	0.3692	0.2090	0.9167	0.9994
1434			1-stearoyl-2-oleoyl-GPC (18:0/18:1)	LC/MS polar	832.6073	52438				0.91	0.88	0.1237	0.0992	1.0690	0.9435
1436			1-palmitoyl-2-arachidonoyl-GPC (16:0/20:4)*	LC/MS polar	826.56034	52459			10747814	0.88	0.87	0.0036	0.0081	1.0829	0.9405
1437			1-stearoyl-2-linoleoyl-GPC (18:0/18:2)*	LC/MS polar	830.59165	52452				0.89	0.87	0.0594	0.0599	1.0246	0.8948
1440			1-palmitoyl-2-palmitoleoyl-GPC (16:0/16:1)*	LC/MS polar	776.5447	52470				0.96	0.85	0.3125	0.1900	1.2584	1.0713
1443			1-(1-enyl-palmitoyl)-2-oleoyl-GPC (P-16:0/18:1)*	LC/MS polar	788.58109	52478				1.26	0.93	0.7901	0.3418	0.8943	0.8284
1444			1-stearoyl-2-arachidonoyl-GPI (18:0/20:4)*	LC/MS polar	885.54948	52449				0.86	0.82	0.0051	0.0108	1.2290	1.0081
1445			1-palmitoyl-2-linoleoyl-GPI (16:0/18:2)*	LC/MS polar	833.51845	52450				0.78	0.76	0.0029	0.0073	1.1804	0.8935
1446			1-oleoyl-2-linoleoyl-GPI (18:1/18:2)*	LC/MS polar	859.53393	52451				0.74	0.70	0.0006	0.0025	1.2817	0.8955
1447			1-palmitoyl-2-arachidonoyl-GPI (16:0/20:4)*	LC/MS polar	857.51855	52467				0.70	0.66	0.0010	0.0035	1.2151	0.7965
1448			1-stearoyl-2-linoleoyl-GPI (18:0/18:2)*	LC/MS polar	861.54985	52468				0.79	0.78	0.0031	0.0075	1.1257	0.8800
1451			1-palmitoyl-2-oleoyl-GPG (16:0/18:1)*	LC/MS polar	747.51853	52448			5283509	1.10	1.04	0.7099	0.3231	1.1149	1.1559

Comparison mean values significantly different:
- (red) $p \le 0.05$, fold of change ≥ 1.00
- (dark green) $p \le 0.05$, fold of change < 1.00

Comparison mean value difference approaching significance:
- (pink) $0.05 < p < 0.10$, fold of change ≥ 1.00
- (light green) $0.05 < p < 0.10$, fold of change < 1.00

Pathway Sort Order	Super Pathway	Sub Pathway	Biochemical Name	Platform	Mass	Comp ID	KEGG	HMDB	PubChem	Fold of Change, Paired t-Test: Post G-CSF / Pre G-CSF — Mean Pairs Ratio	Group Mean Ratio	Paired t-Test: Post G-CSF / Pre G-CSF — *p*-value	*q*-value	Mean Values: Pre G-CSF	Post G-CSF
1453	Lipid		1-(1-enyl-stearoyl)-2-arachidonoyl-GPE (P-18:0/20:4)*	LC/MS polar	750.54431	52475				0.96	0.93	0.3066	0.1873	1.1298	1.0458
1454			1-(1-enyl-stearoyl)-2-docosahexaenoyl-GPE (P-18:0/22:6)*	LC/MS polar	774.54431	52476				1.08	1.00	0.9702	0.3895	1.1649	1.1669
1456			1-palmitoyl-2-oleoyl-GPE (16:0/18:1)	LC/MS polar	716.52357	19263		HMDB05320	5283496	0.99	0.88	0.3681	0.2090	1.0473	0.9226
1458			1-stearoyl-2-arachidonoyl-GPE (18:0/20:4)*	LC/MS polar	766.53922	52447				1.00	0.96	0.6628	0.3112	1.0365	0.9950
1459			1-stearoyl-2-docosahexaenoyl-GPE (18:0/22:6)*	LC/MS polar	790.53922	52466				1.11	0.95	0.6260	0.2994	1.2806	1.2189
1460			1-stearoyl-2-oleoyl-GPE (18:0/18:1)	LC/MS polar	744.55488	42448				1.83	1.23	0.1075	0.0902	0.7743	0.9548
1461			1-(1-enyl-palmitoyl)-GPC (P-16:0)*	LC/MS polar	524.33577	52474			10917802	0.79	0.77	0.0003	0.0016	1.1293	0.8739
1462			1-palmitoyl-2-arachidonoyl-GPE (16:0/20:4)*	LC/MS polar	738.50792	52464				1.03	0.95	0.7670	0.3364	1.0369	0.9799
1463			1-palmitoyl-2-docosahexaenoyl-GPE (16:0/22:6)*	LC/MS polar	762.50792	52465				1.12	0.96	0.7135	0.3239	1.4741	1.4083
1464			1-palmitoyl-2-linoleoyl-GPE (16:0/18:2)	LC/MS polar	714.50792	42449		HMDB05322	9546747	1.12	0.99	0.7200	0.3253	1.0925	1.0797
1465			1-stearoyl-2-linoleoyl-GPE (18:0/18:2)*	LC/MS polar	742.5392	52446				0.93	0.92	0.1404	0.1089	1.1628	1.0644
1471		Lysolipid	1-palmitoyl-GPC (16:0)	LC/MS pos	496.33977	33955		HMDB10382	86554	1.06	0.87	0.1800	0.1292	1.3091	1.1433
1472			2-palmitoyl-GPC (16:0)*	LC/MS pos	496.33977	35253		HMDB61702	15061532	1.08	0.87	0.1967	0.1389	1.3901	1.2148
1473			1-palmitoleoyl-GPC (16:1)*	LC/MS pos	494.32412	33230		HMDB10383	24779461	0.90	0.79	0.0667	0.0654	1.4601	1.1500
1474			2-palmitoleoyl-GPC (16:1)*	LC/MS pos	494.32412	35819				1.12	0.91	0.4853	0.2547	1.0618	0.9612
1477			1-stearoyl-GPC (18:0)	LC/MS pos	524.37107	33961		HMDB10384	497299	1.15	0.85	0.2012	0.1398	1.6766	1.4276
1478			2-stearoyl-GPC (18:0)*	LC/MS pos	524.37107	35255			10208382	1.26	0.85	0.1133	0.0936	1.6241	1.3864
1479			1-oleoyl-GPC (18:1)	LC/MS pos	522.35542	48258		HMDB02815	16081932	1.13	1.01	0.3288	0.1948	1.3328	1.3464
1482			1-linoleoyl-GPC (18:2)	LC/MS pos	520.33977	34419	C04100	HMDB10386	11988421	1.17	0.92	0.4091	0.2265	1.4350	1.3218
1484			1-linolenoyl-GPC (18:3)*	LC/MS pos	518.32412	45951				1.08	0.85	0.2002	0.1398	1.4876	1.2706
1494			1-arachidonoyl-GPC (20:4)*	LC/MS pos	544.33977	33228	C05208	HMDB10395		1.23	1.17	0.8088	0.3470	1.1068	1.3001
1505			1-(1-enyl-palmitoyl)-GPE (P-16:0)*	LC/MS neg	436.28334	39270				1.54	1.06	0.7613	0.3345	1.3034	1.3826
1506			1-(1-enyl-stearoyl)-GPE (P-18:0)*	LC/MS neg	464.31464	39271				0.90	0.84	0.0884	0.0778	1.1594	0.9687
1507			1-(1-enyl-oleoyl)-GPE (P-18:1)*	LC/MS neg	462.29899	44621				2.23	1.27	0.3766	0.2122	1.3984	1.7765
1510			1-palmitoyl-GPE (16:0)	LC/MS neg	452.27826	35631		HMDB11503	9547069	0.77	0.73	0.0030	0.0073	1.2350	0.9027
1513			1-stearoyl-GPE (18:0)	LC/MS neg	480.30956	42398		HMDB11130	9547068	2.73	0.89	0.7224	0.3259	2.2349	1.9810
1514			2-stearoyl-GPE (18:0)*	LC/MS neg	480.30956	41220				1.20	1.01	0.6858	0.3167	1.2215	1.2307
1515			1-oleoyl-GPE (18:1)	LC/MS neg	478.29391	35628		HMDB11506	9547071	1.12	0.86	0.5551	0.2809	1.1738	1.0091
1519			1-linoleoyl-GPE (18:2)*	LC/MS neg	476.27826	32635		HMDB11507	52925130	0.84	0.76	0.0228	0.0289	1.1630	0.8872
1523			1-arachidonoyl-GPE (20:4)*	LC/MS neg	500.27826	35186		HMDB11517	42607465	0.96	0.90	0.2710	0.1735	1.1057	0.9908
1533			1-palmitoyl-GPI (16:0)*	LC/MS neg	571.28888	35305		HMDB61695		0.68	0.61	0.0058	0.0119	1.3543	0.8292
1536			1-stearoyl-GPI (18:0)	LC/MS neg	599.32018	19324		HMDB61696		0.85	0.73	0.0535	0.0560	1.3270	0.9626
1538			1-oleoyl-GPI (18:1)*	LC/MS neg	597.30453	36602				0.59	0.55	0.0005	0.0022	1.3719	0.7493
1540			1-linoleoyl-GPI (18:2)*	LC/MS neg	595.28888	36594				0.82	0.78	0.0326	0.0373	1.1479	0.8917
1544			1-arachidonoyl-GPI (20:4)*	LC/MS neg	619.28888	34214		HMDB61690		0.91	0.84	0.1368	0.1070	1.0692	0.8930
1555			1-arachidonoyl-GPA (20:4)	LC/MS neg	457.23606	46325				0.68	0.59	0.0070	0.0134	1.3556	0.7951
1560			1-stearoyl-GPG (18:0)	LC/MS neg	511.30414	34437				1.02	0.79	0.2733	0.1746	0.8553	0.6729
1595		Glycerolipid Metabolism	glycerol	LC/MS neg	91.04007	15122	C00116	HMDB00131	753	0.67	0.71	0.0024	0.0067	1.3120	0.9366
1596			glycerol 3-phosphate	GC/MS	357.1	15365	C00093	HMDB00126	754	1.22	0.59	0.0745	0.0694	3.5547	2.1042
1602		Monoacylglycerol	1-myristoylglycerol (14:0)	LC/MS neg	227.20163	35625	C01885	HMDB11561	79050	1.18	1.02	0.9995	0.3961	1.1593	1.1828
1606			1-palmitoylglycerol (16:0)	LC/MS neg	255.23293	21127		HMDB31074	14900	1.50	1.11	0.7831	0.3409	1.1446	1.2650
1607			2-palmitoylglycerol (16:0)	LC/MS neg	255.23293	33419		HMDB11533	123409	1.13	0.55	0.1293	0.1024	1.0680	0.5908
1611			1-oleoylglycerol (18:1)	LC/MS pos	374.32649	21184		HMDB11567	5283468	1.58	1.57	0.1875	0.1335	1.0427	1.6322
1612			2-oleoylglycerol (18:1)	LC/MS neg	281.24858	21232			5319879	2.48	1.09	0.9940	0.3961	0.9554	1.0369
1613			1-linoleoylglycerol (18:2)	LC/MS neg	279.23293	27447			5283469	1.36	1.16	0.2659	0.1715	0.8844	1.0280
1614			2-linoleoylglycerol (18:2)	LC/MS neg	279.23293	32506		HMDB11538	5365676	1.41	1.07	0.7523	0.3317	0.9901	1.0612
1615			1-linolenoylglycerol (18:3)	LC/MS neg	277.21728	34393		HMDB11569	53480978	1.57	1.12	0.7006	0.3212	1.0108	1.1313
1616			1-arachidonylglycerol (20:4)	LC/MS neg	303.23293	34397	C13857	HMDB11572	5282281	1.48	1.08	0.7480	0.3314	1.2854	1.3867
1617			2-arachidonoylglycerol (20:4)	LC/MS neg	303.23293	19266	C13856	HMDB04666	5282280	2.65	1.50	0.1823	0.1302	0.7387	1.1068
1621			1-docosahexaenoylglycerol (22:6)	LC/MS neg	309.22236	35153		HMDB11587		1.45	1.07	0.6735	0.3127	1.4517	1.5595
1623			1-dihomo-linolenylglycerol (alpha, gamma)	LC/MS neg	305.24858	48341				1.58	0.98	0.7840	0.3409	1.2670	1.2413
1624			oleoyl-linoleoyl-glycerol	LC/MS polar	663.52055	48424				1.86	0.63	0.3136	0.1901	0.9718	0.6148
1625			2-docosahexaenoylglcyerol*	LC/MS neg	309.22236	48675		HMDB11557		1.13	0.85	0.4065	0.2255	1.4629	1.2405
1626			1-palmitoleoylglycerol (16:1)*	LC/MS neg	253.21727	52431				1.72	1.01	0.9970	0.3961	1.1795	1.1964
1627			2-palmitoleoylglycerol (16:1)*	LC/MS neg	253.21727	52432				1.33	1.03	0.8113	0.3473	0.8085	0.8366
1638		Sphingolipid Metabolism	palmitoyl sphingomyelin (d18:1/16:0)	LC/MS polar	747.56577	37506			9939941	0.94	0.94	0.1546	0.1167	1.0367	0.9742
1639			stearoyl sphingomyelin (d18:1/18:0)	LC/MS polar	775.59707	19503	C00550	HMDB01348	6453725	0.99	0.97	0.6438	0.3051	0.9985	0.9697
1640			sphingomyelin (d18:1/18:1, d18:2/18:0)	LC/MS polar	773.58142	37529			6443882	0.95	0.92	0.2148	0.1462	1.1196	1.0306
1643			sphingosine 1-phosphate	LC/MS polar	378.24148	34445	C06124	HMDB00277	5283560	0.84	0.82	0.0078	0.0144	1.0986	0.9009
1654			sphingomyelin (d18:1/14:0, d16:1/16:0)*	LC/MS polar	719.53447	42463			11433862	0.87	0.88	0.0155	0.0234	1.0413	0.9144
1655			sphingomyelin (d18:2/14:0, d18:1/14:1)*	LC/MS polar	717.51882	47154				0.78	0.77	0.0027	0.0072	1.1159	0.8560
1656			sphingomyelin (d18:1/24:1, d18:2/24:0)*	LC/MS polar	857.67533	47153				1.05	1.04	0.5966	0.2892	0.9530	0.9938
1657			sphingomyelin (d18:2/16:0, d18:1/16:1)*	LC/MS polar	745.55012	42459				0.86	0.86	0.0025	0.0068	1.0752	0.9201
1658			sphingomyelin (d18:1/20:1, d18:2/20:0)*	LC/MS polar	801.61272	48491				0.95	0.93	0.2481	0.1625	1.0335	0.9604
1659			behenoyl sphingomyelin (d18:1/22:0)*	LC/MS polar	831.65967	48492				1.07	1.00	0.9536	0.3856	0.9538	0.9550
1660			sphingomyelin (d18:1/22:1, d18:2/22:0, d16:1/24:1)*	LC/MS polar	829.64402	48493				0.96	0.95	0.2966	0.1838	1.0023	0.9560
1661			sphingomyelin (d18:1/20:0, d16:1/22:0)*	LC/MS polar	803.62837	48490				1.04	1.00	0.9156	0.3750	1.0199	1.0157
1664		Mevalonate Metabolism	3-hydroxy-3-methylglutarate	LC/MS polar	161.04555	531	C03761	HMDB00355	1662	0.85	0.80	0.0205	0.0275	1.1788	0.9375
1674		Sterol	lathosterol	GC/MS	255.2	39864	C01189	HMDB01170	65728	0.99	0.91	0.5099	0.2633	1.0795	0.9857
1676			cholesterol	GC/MS	329.3	63	C00187	HMDB00067	11025495	0.82	0.82	0.0000	0.0001	1.1012	0.9015
1686			7-alpha-hydroxy-3-oxo-4-cholestenoate (7-Hoca)	LC/MS neg	429.30103	36776	C17337	HMDB12458	3081085	1.11	1.10	0.0770	0.0707	0.9698	1.0627
1699			campesterol	GC/MS	343.4	39511	C01789	HMDB02869	173183	0.87	0.81	0.0241	0.0298	1.1340	0.9240
1720		Steroid	pregnenolone sulfate	LC/MS neg	395.18976	38170		HMDB00774	105074	0.90	0.92	0.1245	0.0995	1.4959	1.3824
1728			21-hydroxypregnenolone disulfate	LC/MS neg	245.06711	46115	C05485	HMDB04026	134595	1.02	1.01	0.8085	0.3470	1.1006	1.1098
1736			5-pregnen-3b, 17-diol-20-one 3-sulfate	LC/MS neg	411.18468	37482		HMDB00416	152971	1.17	1.23	0.1142	0.0937	0.6391	0.7846
1738			5alpha-pregnan-3beta,20beta-diol monosulfate (1)	LC/MS neg	399.22106	37196				1.11	1.03	0.3774	0.2122	1.0087	1.0414
1741			5alpha-pregnan-3beta,20alpha-diol monosulfate (2)	LC/MS neg	399.22106	37200				1.16	1.11	0.3471	0.2000	1.0967	1.2166
1744			5alpha-pregnan-3beta,20alpha-diol disulfate	LC/MS neg	239.0853	37198				1.25	1.13	0.9809	0.3922	0.9300	1.0531
1745			5alpha-pregnan-3(alpha or beta),20beta-diol disulfate	LC/MS neg	239.0853	46172			5127902	1.04	0.85	0.4829	0.2540	1.0012	0.8517
1747			pregnen-diol disulfate*	LC/MS neg	238.07747	32562				1.04	1.02	0.8627	0.3620	1.0242	1.0481
1748			pregn steroid monosulfate*	LC/MS neg	397.20541	32619				1.09	1.05	0.1978	0.1393	1.2464	1.3148
1749			pregnanediol-3-glucuronide	LC/MS neg	495.29634	40708			123796	1.14	1.12	0.6403	0.3040	0.9874	1.1021
1757			cortisol	LC/MS neg	361.20204	1712	C00735	HMDB00063	5754	0.99	0.96	0.4397	0.2372	0.9891	0.9510
1759			corticosterone	LC/MS neg	345.20713	5983	C02140	HMDB01547	5753	1.48	1.10	0.9035	0.3712	0.8957	0.9842
1762			cortisone	LC/MS neg	359.18639	1769	C00762	HMDB02802	222786	0.85	0.82	0.0138	0.0220	1.1471	0.9461
1767			dehydroisoandrosterone sulfate (DHEA-S)	LC/MS neg	367.15846	32425	C04555	HMDB01032	12594	0.97	0.93	0.3141	0.1901	1.1466	1.0715
1768			16a-hydroxy DHEA 3-sulfate	LC/MS neg	383.15338	38168				0.97	0.94	0.3431	0.1981	1.0935	1.0309
1770			epiandrosterone sulfate	LC/MS neg	369.17411	33973	C07635	HMDB00365		1.01	1.00	0.7441	0.3309	1.0730	1.0727
1773			androsterone sulfate	LC/MS neg	369.17411	31591		HMDB02759	159663	1.08	1.02	0.2394	0.1580	1.2290	1.2593
1774			4-androsten-3beta,17beta-diol monosulfate (1)	LC/MS neg	369.17411	37211		HMDB03818		1.22	1.18	0.0755	0.0696	1.0317	1.2170
1775			4-androsten-3beta,17beta-diol monosulfate (2)	LC/MS neg	369.17411	37210				0.84	0.75	0.0668	0.0654	1.2791	0.9582
1778			4-androsten-3alpha,17alpha-diol monosulfate (2)	LC/MS neg	369.17411	37207				1.10	1.10	0.9967	0.3961	1.3812	1.5208
1779			4-androsten-3alpha,17alpha-diol monosulfate (3)	LC/MS neg	369.17411	37209				1.09	1.08	0.2557	0.1658	1.1219	1.2077
1783			4-androsten-3beta,17beta-diol disulfate (1)	LC/MS neg	224.06182	37202	C04295	HMDB03818	10634	0.94	0.94	0.0997	0.0852	1.0630	0.9950
1784			4-androsten-3beta,17beta-diol disulfate (2)	LC/MS neg	224.0624	37203	C04295	HMDB03818	10634	0.79	0.82	0.0203	0.0274	1.2465	1.0254
1788			5alpha-androstan-3alpha,17beta-diol monosulfate (1)	LC/MS neg	371.18976	37186				1.32	1.26	0.0026	0.0069	1.1409	1.4394
1789			5alpha-androstan-3alpha,17alpha-diol monosulfate	LC/MS neg	371.18976	37183				1.34	1.06	0.3260	0.1944	0.7442	0.7917
1791			5alpha-androstan-3beta,17beta-diol monosulfate (2)	LC/MS neg	371.18976	37192				1.25	1.12	0.0569	0.0585	1.0768	1.2012
1796			5alpha-androstan-3beta,17alpha-diol disulfate	LC/MS neg	225.06965	37187				1.33	0.93	0.5113	0.2635	1.0675	0.9882
1797			5alpha-androstan-3alpha,17beta-diol disulfate	LC/MS neg	225.06965	37184				1.07	1.03	0.6671	0.3114	0.6755	0.6929
1798			5alpha-androstan-3beta,17beta-diol disulfate	LC/MS neg	225.06965	37190	C12525	HMDB00493	242332	0.86	0.87	0.0375	0.0413	1.4057	1.2287
1803			andro steroid monosulfate (1)*	LC/MS neg	383.1534	32827	C04555	HMDB02759		0.93	0.90	0.1590	0.1189	1.0725	0.9699
1826			etiocholanolone glucuronide	LC/MS neg	465.24939	47112				0.88	0.93	0.0401	0.0434	1.4135	1.3203
1828			5alpha-androstan-3alpha,17beta-diol monosulfate (2)	LC/MS neg	371.18976	37185				0.98	0.93	0.4230	0.2311	1.3318	1.2430
1829			17alpha-hydroxypregnanolone glucuronide	LC/MS neg	509.2756	47403				1.19	1.12	0.2941	0.1827	0.9477	1.0567
1834			pregnanolone/allopregnanolone sulfate	LC/MS neg	397.20541	48394				0.79	0.56	0.0216	0.0284	1.0092	0.5669
1841		Primary Bile Acid Metabolism	cholate	LC/MS neg	407.28029	22842	C00695	HMDB00619	221493	1.20	1.12	0.2545	0.1655	2.6735	3.0064
1842			glycocholate	LC/MS neg	464.30176	18476	C01921	HMDB00138	10140	1.73	1.07	0.2695	0.1734	1.3238	1.4101
1843			taurocholate	LC/MS neg	514.28439	18497	C05122	HMDB00036	6675	2.02	0.81	0.3405	0.1971	1.7445	1.4159
1844			chenodeoxycholate	LC/MS neg	391.28538	1563	C02528	HMDB00518	10133	1.51	0.86	0.5609	0.2821	1.2556	1.0758
1845			glycochenodeoxycholate	LC/MS neg	448.30684	32346	C05466	HMDB00637	12544	1.20	0.81	0.7292	0.3283	1.5177	1.2302
1846			taurochenodeoxycholate	LC/MS neg	498.28948	18494	C05465	HMDB00951	387316	1.33	0.71	0.7397	0.3303	2.1697	1.5304
1850			tauro-beta-muricholate	LC/MS neg	514.28439	33983		HMDB00932	168408	1.49	0.84	0.7556	0.3326	1.4203	1.1961
1852		Secondary Bile Acid Metabolism	deoxycholate	LC/MS neg	391.28538	1114	C04483	HMDB00626	222528	1.19	0.95	0.7400	0.3303	1.2145	1.1520
1853			glycodeoxycholate	LC/MS neg	448.30684	18477	C05464	HMDB00631	3035026	1.79	1.44	0.0386	0.0423	0.9031	1.3019
1854			taurodeoxycholate	LC/MS neg	498.28948	12261	C05463	HMDB00896	2733768	1.48	0.97	0.2592	0.1676	1.2745	1.2357
1858			glycolithocholate	LC/MS neg	432.31193	31912	C15557	HMDB00698	115245	1.48	1.16	0.0727	0.0682	0.8704	1.0108
1859			glycolithocholate sulfate*	LC/MS neg	255.63073	32620	C11301	HMDB02639	72222	1.84	1.35	0.1176	0.0959	0.8721	1.1776
1860			taurolithocholate	LC/MS neg	482.29456	31889	C02592	HMDB00722	10595	1.08	1.06	0.3524	0.2017	0.8723	0.9241
1861			taurolithocholate 3-sulfate	LC/MS neg	280.62205	36850	C03642	HMDB02580	440071	1.78	0.95	0.8332	0.3532	1.3687	1.3063
1863			ursodeoxycholate	LC/MS neg	391.28538	1605	C07880	HMDB00946	31401	0.92	0.68	0.0694	0.0667	1.3801	0.9317
1864			glycoursodeoxycholate	LC/MS neg	448.30684	39379		HMDB00708	12310288	1.22	0.97	0.9978	0.3961	1.4790	1.4325
1865			tauroursodeoxycholate	LC/MS neg	498.28948	39378		HMDB00874	9848818	1.92	0.70	0.8530	0.3592	1.8828	1.3244
1872			glycohyocholate	LC/MS neg	464.30176	42574				1.51	1.22	0.1674	0.1230	1.0591	1.2917
1881			glycocholenate sulfate*	LC/MS neg	254.62291	32599				1.04	0.94	0.2846	0.1777	1.1210	1.0563
1882			taurocholenate sulfate	LC/MS neg	279.61422	32807				0.91	0.76	0.0982	0.0844	1.6493	1.2503
1886			3b-hydroxy-5-cholenoic acid	LC/MS neg	373.27482	43507		HMDB00308	92997	1.08	0.85	0.4214	0.2307	1.1839	1.0086
2430	Nucleotide	Purine Metabolism, (Hypo)Xanthine/Inosine containing	inosine	LC/MS neg	267.07349	1123	C00294	HMDB00195	6021	0.34	0.25	0.0000	0.0002	2.2074	0.5410
2431			hypoxanthine	LC/MS neg	135.03123	3127	C00262	HMDB00157	790	1.22	1.16	0.4043	0.2253	0.9047	1.0515
2432			xanthine	LC/MS pos	153.04071	3147	C00385	HMDB00292	1188	1.18	1.06	0.5955	0.2892	0.9284	0.9849
2434			xanthosine	LC/MS pos	285.08296	15136	C01762	HMDB00299	64959	1.36	1.24	0.6936	0.3197	0.8634	1.0725
2439			urate	LC/MS neg	167.02106	1604	C00366	HMDB00289	1175	1.05	1.04	0.1231	0.0990	1.0240	1.0686
2440			allantoin	LC/MS polar	157.03671	1107	C02350	HMDB00462	204	2.22	2.13	0.0000	0.0001	0.7023	1.4963
2448		Purine Metabolism, Adenine containing	adenosine 3',5'-cyclic monophosphate (cAMP)	LC/MS neg	328.04524	2831	C00575	HMDB00058	6076	0.81	0.77	0.0217	0.0284	1.1050	0.8499
2452			adenosine	LC/MS neg	134.0472	555	C00212	HMDB00050	60961	0.78	0.29	0.0215	0.0284	3.4216	0.9964
2453			adenine	LC/MS pos	136.06178	554	C00147	HMDB00034	190	0.89	0.83	0.0811	0.0737	1.1237	0.9381
2458			N1-methyladenosine	LC/MS pos	282.11969	15650	C02494	HMDB03331	27476	1.14	1.15	0.8697	0.3632	0.9297	1.0679
2459			N6-methyladenosine	LC/MS neg	148.06285	37114		HMDB04044	102175	1.45	1.40	0.0000	0.0003	0.8416	1.1763
2468			N6-carbamoylthreonyladenosine	LC/MS neg	411.12698	35157		HMDB41623	161466	1.51	1.47	0.0000	0.0001	0.8161	1.2014
2477			N6-succinyladenosine	LC/MS neg	382.10043	48130		HMDB00912		2.10	1.99	0.0000	0.0002	0.7039	1.4013
2485		Purine Metabolism, Guanine containing	guanosine	LC/MS pos	284.09895	1573	C00387	HMDB00133	6802	0.31	0.22	0.0000	0.0002	2.2781	0.5097
2488			7-methylguanine	LC/MS pos	166.07234	35114	C02242	HMDB00897	11361	1.09	1.09	0.5806	0.2862	0.9617	1.0524
2492			N1-methylguanosine	LC/MS pos	298.1146	31609		HMDB01563	96373	1.16	1.15	0.0122	0.0200	0.9447	1.0890
2493			N2-methylguanosine	LC/MS neg	296.10004	35133		HMDB05862	3035422	1.35	1.32	0.0016	0.0051	0.8647	1.1409
2494			N2,N2-dimethylguanosine	LC/MS pos	312.13025	35137		HMDB04824	92919	1.28	1.25	0.0001	0.0006	0.9081	1.1346
2505		Pyrimidine Metabolism, Orotate containing	dihydroorotate	LC/MS polar	157.02548	601	C00337	HMDB03349	648	0.73	0.72	0.0000	0.0000	1.2239	0.8836
2506			orotate	LC/MS polar	155.00983	1505	C00295	HMDB00226	967	1.27	1.08	0.2068	0.1418	1.0800	1.1654
2508			orotidine	LC/MS polar	287.05209	35172	C01103	HMDB00788	92751	1.89	1.90	0.0000	0.0000	0.8196	1.5540
2518		Pyrimidine Metabolism, Uracil containing	uridine	LC/MS neg	243.06226	606	C00299	HMDB00296	6029	1.03	1.02	0.6472	0.3061	1.0040	1.0221
2519			uracil	LC/MS neg	111.02	605	C00106	HMDB00300	1174	1.34	1.30	0.0139	0.0220	0.9029	1.1707
2520			pseudouridine	LC/MS pos	245.07682	33442	C02067	HMDB00767	15047	1.26	1.26	0.0000	0.0000	0.9344	1.1812
2521			5-methyluridine (ribothymidine)	LC/MS neg	257.07791	35136		HMDB00884	445408	0.84	0.83	0.0228	0.0289	1.0719	0.8916
2528			5,6-dihydrouracil	LC/MS pos	115.05021	1559	C00429	HMDB00076	649	1.18	1.19	0.0071	0.0134	0.9634	1.1445
2532			2'-deoxyuridine	LC/MS neg	111.01997	1412	C00526	HMDB00012	13712	1.54	1.44	0.0105	0.0176	0.8843	1.2728
2533			4-ureidobutyrate	LC/MS polar	145.06186	22118			1571307	0.72	0.71	0.0025	0.0068	1.2137	0.8620
2534			3-ureidopropionate	LC/MS pos	133.06077	3155	C02642	HMDB00026	111	1.75	1.64	0.0033	0.0077	0.8271	1.3524
2535			beta-alanine	GC/MS	334.1	35838	C00099	HMDB00056	239	1.27	1.04	0.6052	0.2922	1.0703	1.1088
2536			N-acetyl-beta-alanine	LC/MS pos	132.06552	37432	C01073		76406	1.02	0.98	0.8902	0.3677	1.1099	1.0896
2544		Pyrimidine Metabolism, Cytidine containing	cytidine	LC/MS neg	242.07824	514	C00475	HMDB00089	6175	1.03	0.73	0.3225	0.1933	1.4520	1.0532
2548			N4-acetylcytidine	LC/MS pos	286.10337	35130		HMDB05923	107461	1.46	1.45	0.0002	0.0013	0.9307	1.3468
2562		Pyrimidine Metabolism, Thymine containing	5,6-dihydrothymine	LC/MS pos	129.06586	1418	C00906	HMDB00079	93556	0.90	0.88	0.0532	0.0560	1.1117	0.9749
2566	Cofactors and Vitamins	Nicotinate and Nicotinamide Metabolism	quinolinate	LC/MS polar	166.01458	1899	C03722	HMDB00232	1066	0.98	0.94	0.4144	0.2277	0.9752	0.9195
2570			nicotinamide	LC/MS pos	123.05529	594	C00153	HMDB01406	936	1.40	1.17	0.8360	0.3538	0.9529	1.1141
2582			1-methylnicotinamide	LC/MS pos	137.07094	27665	C02918	HMDB00699	10129985	0.66	0.56	0.0006	0.0025	1.2096	0.6797
2587			trigonelline (N'-methylnicotinate)	LC/MS pos	138.05496	32401	C01004	HMDB00875	5570	1.00	0.70	0.2016	0.1398	1.8939	1.3334
2589			N1-Methyl-2-pyridone-5-carboxamide	LC/MS pos	153.06586	40469	C05842	HMDB04193	69698	0.67	0.62	0.0010	0.0035	1.2544	0.7773
2595		Pantothenate and CoA Metabolism	pantothenate	LC/MS neg	218.10339	1508	C00864	HMDB00210	6613	1.10	1.02	0.4289	0.2323	1.0451	1.0701
2603		Ascorbate and Aldarate Metabolism	ascorbate (Vitamin C)	GC/MS	332.1	1640	C00072	HMDB00044		2.05	1.59	0.8849	0.3677	1.1762	1.8725
2605			threonate	LC/MS polar	135.02989	27738	C01620	HMDB00943	151152	0.78	0.76	0.0012	0.0041	1.0236	0.7782
2606			arabonate	GC/MS	292.1	37516	C00878	HMDB00539	122045	1.03	0.96	0.8671	0.3627	1.0812	1.0392
2607			oxalate (ethanedioate)	LC/MS neg	88.98803	20694	C00209	HMDB02329	971	0.65	0.64	0.0166	0.0244	1.1510	0.7406
2608			gulonic acid*	LC/MS polar	195.05102	46957			9794176	2.06	1.75	0.0001	0.0008	0.6970	1.2204
2609		Tocopherol Metabolism	alpha-tocopherol	GC/MS	502.5	1561	C02477	HMDB01893	14985	0.82	0.81	0.0005	0.0023	1.0892	0.8851
2611			beta-tocopherol	GC/MS	488.3	35702	C14152	HMDB06335	6857447	1.15	0.82	0.5367	0.2733	1.0853	0.8921
2614			gamma-tocopherol	GC/MS	488.4	33420	C02483	HMDB01492	14986	0.82	0.66	0.0359	0.0401	1.4611	0.9659
2617			gamma-CEHC	LC/MS neg	263.12888	44876		HMDB01931	133098	0.89	0.76	0.0785	0.0718	1.3059	0.9880
2618			gamma-CEHC glucuronide*	LC/MS neg	439.16097	42381				1.24	0.93	0.4958	0.2587	0.9912	0.9227
2620			alpha-CEHC sulfate	LC/MS neg	357.10134	47666				1.46	1.25	0.4440	0.2384	0.9307	1.1635
2644			bilirubin (Z,Z)	LC/MS neg	583.2562	43807	C00486	HMDB00054	5280352	0.56	0.51	0.0001	0.0008	1.7259	0.8726

Comparison mean values significantly different:

$p \leq 0.05$, fold of change ≥ 1.00

$p \leq 0.05$, fold of change < 1.00

Comparison mean value difference approaching significance:

$0.05 < p < 0.10$, fold of change ≥ 1.00

$0.05 < p < 0.10$, fold of change < 1.00

Pathway Sort Order	Super Pathway	Sub Pathway	Biochemical Name	Platform	Mass	Comp ID	KEGG	HMDB	PubChem	Fold of Change, Paired t-Test: Post G-CSF / Pre G-CSF Mean Values Paired Ratio	Fold of Change, Paired t-Test: Post G-CSF / Pre G-CSF Mean Values Group Mean Ratio	Paired t-Test: Post G-CSF / Pre G-CSF p-value	Paired t-Test: Post G-CSF / Pre G-CSF q-value	Mean Values: Pre G-CSF	Mean Values: Post G-CSF
2645		Hemoglobin and Porphyrin Metabolism	bilirubin (E,E)*	LC/MS neg	583.2562	32586			5315464	0.74	0.75	0.0042	0.0091	1.3280	0.9198
2647			biliverdin	LC/MS neg	581.24090	2137	C00500	HMDB03006	5353439	0.88	0.75	0.0695	0.0667	1.4135	1.0622
2648			I-urobilinogen	LC/MS neg	591.31881	32426	C05785	HMDB04157	26818	1.38	0.63	0.5673	0.2837	1.3546	0.8574
2670		Vitamin B6 Metabolism	pyridoxate	LC/MS neg	182.04588	31555	C00847	HMDB00017	6723	0.81	0.80	0.0021	0.0061	1.4754	1.1353
2672			4-pyridoxate	LC/MS neg	178.05096	15753	C03186	HMDB00714	464	1.23	0.67	0.2450	0.1609	1.6526	1.2397
2676			2-hydroxyhippurate (salicylurate)	LC/MS neg	194.04588	18261	C07588	HMDB00840	10253	1.45	0.72	0.7075	0.3226	1.4091	1.0133
2677			3-hydroxyhippurate	LC/MS neg	194.04588	39930		HMDB06116	450288	0.96	0.67	0.0596	0.0569	1.2752	0.8462
2678			4-hydroxyhippurate	LC/MS neg	194.04588	35527		HMDB13678	151012	1.56	1.22	0.8892	0.3677	1.4274	1.7445
2684			benzoate	LC/MS neg	121.0295	15778	C00180	HMDB01870	243	1.01	0.99	0.8878	0.3677	0.9957	0.9902
2693			4-hydroxybenzoate	LC/MS neg	137.02441	21133	C00156	HMDB00500	135	1.93	1.72	0.1819	0.1302	0.9442	1.6219
2701			catechol sulfate	LC/MS neg	188.99313	35320	C00090	HMDB59724	3083679	0.85	0.71	0.0743	0.0604	1.2579	0.8891
2703			O-methylcatechol sulfate	LC/MS neg	203.00198	46111			22473	0.94	0.72	0.1158	0.0947	1.6539	1.1962
2704			3-methyl catechol sulfate (1)	LC/MS neg	203.00198	46165				0.72	0.71	0.0229	0.0289	1.4830	0.7879
2705			3-methyl catechol sulfate (2)	LC/MS neg	203.00198	46164				0.72	0.72	0.0158	0.0224	1.3245	0.6851
2706			4-methylcatechol sulfate	LC/MS neg	203.00198	46146				1.40	1.00	0.3081	0.1878	1.4099	1.4078
2710		Benzoate Metabolism	methyl-4-hydroxybenzoate	LC/MS polar	151.04509	34386		HMDB12572	7456	40.24	39.61	0.0363	0.0404	0.9339	28.3049
2714			2-methylphenylsulfate	LC/MS neg	201.0227	36847				1.67	0.93	0.9712	0.3895	1.9349	1.8018
2715			3-methylphenylsulfate	LC/MS neg	201.0227	36846	C14386			3.11	0.80	0.3012	0.1860	1.8728	1.4896
2716			4-methylphenylsulfate	LC/MS neg	201.0227	36099	C13937			1.43	0.78	0.9712	0.3895	1.6244	1.2733
2717			4-ethylphenol sulfate	LC/MS neg	199.00705	36098	C05427	HMDB04072	6426786	6.05	0.93	0.0116	0.0191	3.2546	2.3007
2729			3-methoxycatechol sulfate (1)	LC/MS neg	218.99585	48763				1.06	0.93	0.3731	0.2849	0.8722	0.8089
2730			3-methoxycatechol sulfate (2)	LC/MS neg	218.99585	48752				1.03	0.78	0.1419	0.1094	1.7490	1.3576
2731			methyl-4-hydroxybenzoate sulfate	LC/MS neg	230.99585	48429				45.71	2.93	0.3213	0.1930	2.5108	1.3622
2732			propyl 4-hydroxybenzoate sulfate	LC/MS neg	259.02818	48460				3.85	3.63	0.0009	0.0034	0.6908	1.2675
2735			caffeine	LC/MS pos	195.08765	569	C07481	HMDB01847	2519	1.11	0.80	0.7071	0.3228	1.2373	0.9896
2736			paraxanthine	LC/MS pos	181.07201	18254	C13167	HMDB01860	4687	1.09	0.81	0.5850	0.2872	1.2918	1.0507
2737			theobromine	LC/MS pos	181.07201	18392	C07480	HMDB02825	5429	1.12	0.88	0.3978	0.2221	1.2205	1.0723
2738			theophylline	LC/MS pos	181.07201	18394	C07130	HMDB01889	2153	1.08	0.87	0.5508	0.2521	1.2503	1.1239
2739			1-methyluric acid	LC/MS pos	183.05127	34395	C16359	HMDB03099	69726	0.87	0.63	0.0575	0.0588	1.5026	0.9466
2741			1,3-dimethyluric acid	LC/MS pos	195.05236	32391		HMDB02817	70346	0.90	0.78	0.1190	0.0870	1.3652	1.0687
2742			1,7-dimethyluric acid	LC/MS pos	195.05236	34400	C16356	HMDB11103	91611	1.08	0.82	0.3272	0.1945	1.1517	0.9452
2743			3,7-dimethyluric acid	LC/MS pos	195.05236	34399	C16360	HMDB01982	83128	0.89	0.77	0.0085	0.0155	1.7071	1.3823
2744		Xanthine Metabolism	1,3,7-trimethyluric acid	LC/MS pos	209.06801	34404	C16361	HMDB02123	79437	0.98	0.83	0.3356	0.1955	1.3519	1.1184
2745			1-methylxanthine	LC/MS pos	167.05636	34389	C16358	HMDB03738	80220	0.89	0.89	0.0041	0.0089	1.7840	0.9756
2746			3-methylxanthine	LC/MS pos	167.05636	32445	C16357	HMDB03886	70639	0.97	0.80	0.1759	0.1280	1.2181	0.9847
2747			7-methylxanthine	LC/MS pos	167.05636	34390	C16353	HMDB03991	68374	0.92	0.87	0.0290	0.0340	1.4468	0.8764
2748			5-acetylamino-6-amino-3-methyluracil	LC/MS pos	169.08257	34424	C16366	HMDB04400	88299	1.48	0.52	0.2326	0.1552	1.7199	0.8979
2749			5-acetylamino-6-formylamino-3-methyluracil	LC/MS pos	227.07748	34401	C16365	HMDB13105	105214	0.72	0.61	0.0304	0.0350	1.2637	0.6425
2751			cotinine	LC/MS pos	177.10224	553		HMDB00846	854019	0.91	0.80	0.2006	0.1398	4.9467	3.9566
2752		Tobacco Metabolite	hydroxycotinine	LC/MS pos	193.09716	38661		HMDB13190	10219774	0.93	0.82	0.3037	0.1860	0.1734	0.1420
2753			cotinine N-oxide	LC/MS pos	193.09716	38662		HMDB54411	9815514	1.00	1.00	0.3343	0.1953	0.9699	0.9663
2781			2-piperidinone	LC/MS pos	100.07593	43400			12868	1.00	1.00	0.3012	0.1953	1.4596	1.3942
2833			levulinate (4-oxovalerate)	LC/MS pos	99.04406	22177		HMDB00720	11579	0.94	0.89	0.2054	0.1415	1.1401	1.0113
2844			1,6-anhydroglucose	GC/MS	204.1	21040		HMDB00640	2724705	1.77	1.45	0.3694	0.2090	1.1835	1.7715
2847			2,3-dihydroxyisovalerate	LC/MS polar	133.05063	38276	C04039	HMDB12141	677	1.17	0.92	0.7490	0.3314	1.3236	1.2239
2853			2-isopropylmalate	LC/MS polar	175.08119	15987	C02504	HMDB00402	77	1.33	0.91	0.8870	0.3677	1.1364	1.0373
2864			3-hydroxyindolin-2-one	LC/MS pos	132.0444	42561	C11130		6097	1.32	1.17	0.2768	0.1754	1.0098	1.1811
2871			betonicine	LC/MS pos	160.09682	38100	C08269	HMDB29412	164642	2.13	0.71	0.5291	0.2715	4.4510	3.1706
2875			glucuronate	LC/MS polar	195.05102	587	C00257	HMDB00625	10690	1.09	1.04	0.7001	0.3212	1.0182	1.1084
2885			N-acetylalitilin	LC/MS pos	218.04925	45404			1.56	0.87	0.6694	0.3114	0.7451	0.6463
2915			cinnamoylglycine	LC/MS pos	204.06581	38637		HMDB11621	709625	0.86	0.67	0.0692	0.0667	1.8104	1.2175
2932			dihydroferulic acid	LC/MS neg	195.06828	40481			14340	0.97	0.78	0.0909	0.0795	1.6383	1.2442
2941			ergothioneine	LC/MS neg	228.08122	37459	C05570	HMDB03045	3032311			0.0000	0.0000	1.5383	0.9496
2943			erythritol	GC/MS	217	20699	C00503	HMDB02994	222285	1.13	1.11	0.0833	0.0743	0.9812	1.0929
2946			ferulic acid 4-sulfate	LC/MS neg	273.00744	47114		HMDB29200	6305574	0.87	0.86	0.0747	0.0694	1.8641	1.0487
2965			homoisoleucine*	LC/MS pos	158.11755	33009	C08263	HMDB13633	441447	0.97	0.82	0.3027	0.1860	1.1571	1.1204
2969			indolin-2-one	LC/MS pos	134.06004	43374	C12332		321710	1.34	0.94	0.8925	0.3679	1.1583	1.0841
2991		Food Component/Plant	methyl indole-3-acetate	LC/MS pos	190.08626	1584		HMDB29738	74706	1.11	1.01	0.8926	0.3677	1.4215	1.4244
2994			N-(2-furoyl)glycine	LC/MS neg	168.02925	31536		HMDB06839	21863	0.73	0.67	0.0411	0.0443	2.3108	0.9588
3017			piperine	LC/MS pos	286.14377	33935	C09882	HMDB29377	638024	1.06	0.87	0.2148	0.1462	1.5315	1.3035
3027			quinate	LC/MS polar	191.05611	18335	C00296	HMDB00772	6508	1.11	0.68	0.5045	0.2892	1.3111	1.8037
3031			saccharin	LC/MS neg	181.99173	21151	D01085	HMDB29723	5143	1.34	0.79	0.2376	0.1578	0.7700	0.6058
3034			S-allylcysteine	LC/MS pos	162.05833	43239			96080	1.11	0.93	0.0225	0.0265	1.3827	1.5351
3049			stachydrine	LC/MS pos	144.10191	34384	C10172	HMDB04827	115244	1.75	0.75	0.9436	0.3821	2.1440	1.6021
3054			tartaric acid	LC/MS neg	149.00916	15338	C00898	HMDB00956	444305	1.72	1.66	0.1455	0.1114	1.7360	2.8763
3055			theanine	LC/MS pos	175.10772	22206	C03497	HMDB34005	439378	0.99	1.07	0.6583	0.3102	0.2744	0.2928
3057			thymol sulfate	LC/MS neg	229.054	36085	C09508	HMDB01878		1.29	0.48	0.1021	0.0865	3.8456	1.8532
3070			4-allylphenol sulfate	LC/MS neg	213.0227	37181				1.67	0.93	0.9712	0.3895	2.5218	2.3454
3071			methyl glucopyranoxide (alpha + beta)	LC/MS pos	212.11268	46144				1.31	0.80	0.4872	0.2552	1.9362	1.5398
3088			4-vinylguaiacol sulfate	LC/MS neg	229.01761	48442				1.11	0.82	0.0155	0.0224	1.4245	1.5795
3099			pyraline	LC/MS pos	255.13394	48428				1.28	1.14	0.4879	0.2482	0.9757	1.1162
3100			umbelliferone sulfate	LC/MS neg	240.99123	48674			129859	1.66	0.64	0.3018	0.1860	3.2189	1.5458
3102			eugenol sulfate	LC/MS neg	243.03326	48715			180632	1.19	0.15	0.1420	0.1094	12.2355	1.8394
3108			isoeugenol sulfate	LC/MS neg	243.03326	48697				3.78	0.87	0.4262	0.2323	1.9474	1.6002
3126		Bacterial/Fungal	tartronate (hydroxymalonate)	LC/MS neg	118.99559	20950	C02287	HMDB35227	45	1.11	0.68	0.0035	0.0079	1.4044	1.8873
3196			2-hydroxyacetaminophen sulfate*	LC/MS neg	246.00778	33173				787.95	13.37	0.0007	0.0169	4.4804	59.9252
3157			2-methoxyacetaminophen sulfate*	LC/MS neg	260.02343	47031				42.16	19.75	0.0038	0.0088	0.0561	1.1518
3158			3-cysteinl-S-yl)acetaminophen*	LC/MS pos	271.07471	34365			5233914	25.59	9.80	0.0146	0.0228	0.6845	0.8285
3159			3-(N-acetyl-L-cysteinl-S-yl) acetaminophen	LC/MS neg	311.07072	47221			83967	15.96	5.63	0.0170	0.0245	0.0803	0.5241
3160			4-acetaminophen sulfate	LC/MS neg	230.01287	37475	C05824	HMDB59911	83939	1304.51	11.32	0.0005	0.0169	0.7187	8.1123
3161			4-acetamidophenol	LC/MS neg	150.05605	12032	C05824	HMDB01859	1983	297.79	14.07	0.0102	0.0175	0.0985	1.3577
3162			4-acetamidophenylglucuronide	LC/MS neg	326.08514	15736		HMDB35316	83944	561.77	11.34	0.0098	0.0175	0.0098	0.2280
3163			2-methoxyacetaminophen glucuronide*	LC/MS neg	356.09857	33161				80.04	23.62	0.0128	0.0208	0.0618	1.2742
3166			ibuprofen	LC/MS pos	224.16451	17798	D00126	HMDB03925	3672	6.10	4.11	0.6827	0.3212	0.1768	0.7295
3167			2-hydroxyibuprofen	LC/MS pos	205.12231	43339			1.73	1.60	0.5743	0.2849	0.4312	0.6901
3169			carboxyibuprofen	LC/MS pos	235.09756	43333		10444113	6.34	2.93	0.7914	0.3418	0.1183	0.3468
3181		Drug	4-acetylphenol sulfate	LC/MS neg	215.00196	44620			4684006	0.94	0.78	0.0719	0.0680	1.1673	0.9059
3188			6-oxopiperidine-2-carboxylic acid	LC/MS neg	142.05586	43231			3014237	0.92	0.86	0.1628	0.1207	1.1532	0.9940
3226			4-hydroxycoumarin	LC/MS neg	161.02447	37445	C20454		54682000	1.42	0.67	0.7080	0.3228	1.1857	0.7970
3258			hydrochlorothiazide	LC/MS neg	295.95719	39625	C07081	HMDB00928	3639	0.99	0.99	0.3343	0.1953	0.9481	0.9401
3260			hydroquinone sulfate	LC/MS neg	188.98631	35322	C00530	HMDB03414	161209	1.48	1.01	0.8564	0.3801	1.2080	1.2189
3278			lidocaine	LC/MS pos	235.18049	35661	D00358	HMDB14626	3676	1.42	1.42	0.0970	0.0836	0.3582	0.5084
3284			metformin	LC/MS pos	130.10873	38326	C07351	HMDB01921	4091	0.93	0.14	0.3343	0.1953	0.1525	0.0221
3288			metoprolol	LC/MS pos	268.19072	18037	D02358	HMDB00932	4171	0.99	0.98	0.3343	0.1953	0.8511	0.8743
3289			metoprolol acid metabolite*	LC/MS pos	268.15434	34109			92936	0.98	0.98	0.3343	0.1953	0.8924	0.8736
3290			N-ethylbiclavamidide	LC/MS pos	207.14916	33586	C16581	HMDB06656	24415	1.17	1.17	0.1284	0.1094	0.8140	0.9540
3307			omeprazole	LC/MS neg	344.10743	38600	C07326	HMDB02613	4594	1.00	1.00			1.0000	1.0000
3375			salicylate	LC/MS neg	137.02442	1515	C00805	HMDB01895	338	0.88	0.57	0.0671	0.0654	1.6004	0.9720
3376			2-acetamidophenol sulfate	LC/MS neg	230.01288	48580			181671	1.02	0.51	0.2743	0.1747	1.7075	0.8723
3421			diglycerol	LC/MS neg	167.08414	40702			42653	1.53	1.08	0.2157	0.1462	1.3040	1.4121
3422			1,2-propanediol	GC/MS	117	38002	C00583	HMDB01881	1030	1.43	1.04	0.8440	0.3581	1.3576	1.4136
3429			2-pyrrolidinone	LC/MS pos	86.06004	31675		HMDB02039	12025	1.01	0.90	0.3403	0.1971	1.0221	0.9225
3430			sulfate*	LC/MS neg	96.9601	46560	C00059	HMDB03488	1118	1.11	0.67	0.0168	0.0224	1.4245	1.5347
3433			O-sulfo-L-tyrosine	LC/MS neg	260.02343	45413			514186	1.42	1.42	0.0000	0.0000	0.6221	1.1480
3434			2-aminophenol sulfate	LC/MS neg	188.0023	43286		HMDB04116	181670	0.85	0.67	0.0668	0.0667	2.2961	1.5958
3492			dimethyl sulfone	LC/MS pos	95.01613	43424	C11142	HMDB04883	6213	1.20	1.15	0.1404	0.1089	1.0538	1.2138
3494			ectoine	LC/MS pos	143.08151	33651	C06231		129041	1.58	1.26	0.5850	0.2851	0.9692	2.2851
3520			phenylacetamilne*	LC/MS pos	266.13899	43268				1.08	0.86	0.5815	0.2892	1.3880	1.1878
3536			N-methylpipecolate	LC/MS pos	144.10191	47101			11862129;11288529	1.01	1.07	0.6826	0.3158	0.9976	1.0682
3538			4-methylbenzenesulfonate	LC/MS neg	171.01215	43502	C06077		8101	0.76	0.76	0.0058	0.0118	1.3533	1.0330
3541			4-hydroxychlorothalonil	LC/MS neg	244.90817	48441			34217	0.67	0.67	0.0006	0.0025	1.5026	1.2861
3545			1,2,3-benzenetriol sulfate (2)	LC/MS neg	204.98132	48762				1.64	1.44	0.7892	0.3418	1.3003	2.7573
3546			2-methoxyestrone/catechol sulfate	LC/MS neg	218.99588	48445				1.39	1.08	0.3717	0.2099	1.4372	1.5579
3547			3-hydroxypyridine sulfate	LC/MS neg	173.98585	46648						0.0110	0.0182	1.2264	0.7809
3550			cyclohexanebutanoic acid	LC/MS neg	169.1234	48776			75200	1.35	1.09	0.5350	0.2733	0.5974	1.0850
3553			ethyl paraben sulfate	LC/MS neg	245.01253	48463				1.11	0.69	0.1767	0.1280	9.3006	6.3786

Biochemical Name(s) Indicates compounds that have not been officially confirmed based on a standard, but are are confident in its identity.

Supplementary Table 2. All significantly altered metabolites (ps0.05), sorted according to their p-value.

Comparison mean values significantly different:

$p \leq 0.05$, fold of change ≥ 1.00

$p \leq 0.05$, fold of change < 1.00

Pathway Sort Order	Super Pathway	Sub Pathway	Biochemical Name	Fold of Change, Paired t-Test: Post G-CSF Pre G-CSF — Mean Pairs Ratio	Fold of Change, Paired t-Test: Post G-CSF Pre G-CSF — Group Mean Ratio	Paired t-Test: Post G-CSF / Pre G-CSF — p -value	Paired t-Test: Post G-CSF / Pre G-CSF — q -value	Mean Values: Pre G-CSF	Mean Values: Post G-CSF
10	Amino Acid	Glycine, Serine and Threonine Metabolism	N-acetylserine	1.83	1.81	0.0000	0.0000	0.8101	1.3054
1065	Lipid	Polyunsaturated Fatty Acid (n3 and n6)	docosadienoate (22:2n6)	2.96	2.79	0.0000	0.0000	0.6261	1.7463
2941	Xenobiotics	Food Component/Plant	ergothioneine	0.46	0.51	0.0000	0.0000	1.8383	0.9406
291	Amino Acid	Methionine, Cysteine, SAM and Taurine Metabolism	N-acetylmethionine	1.89	1.88	0.0000	0.0000	0.7976	1.4971
2508	Nucleotide	Pyrimidine Metabolism, Orotate containing	orotidine	1.69	1.90	0.0000	0.0000	0.8196	1.5540
227	Amino Acid	Tryptophan Metabolism	tryptophan betaine	1.65	1.60	0.0000	0.0000	3.6167	2.1754
309	Amino Acid	Methionine, Cysteine, SAM and Taurine Metabolism	cystine	0.67	0.65	0.0000	0.0000	1.2565	0.8189
2505	Nucleotide	Pyrimidine Metabolism, Orotate containing	dihydroorotate	0.73	0.72	0.0000	0.0000	1.2239	0.8836
84	Amino Acid	Lysine Metabolism	3-methylglutarylcarnitine (1)	2.23	2.19	0.0000	0.0000	0.9545	2.0915
861	Carbohydrate	Glycogen Metabolism	maltotriose	39.18	16.27	0.0000	0.0000	0.0782	1.2721
864	Carbohydrate	Glycogen Metabolism	maltose	8.72	6.67	0.0000	0.0000	0.4175	2.7867
337	Amino Acid	Urea cycle; Arginine and Proline Metabolism	N-acetylarginine	0.65	0.66	0.0000	0.0000	1.4152	0.9292
2520	Nucleotide	Pyrimidine Metabolism, Uracil containing	pseudouridine	1.26	1.26	0.0000	0.0000	0.9344	1.1812
193	Amino Acid	Tryptophan Metabolism	tryptophan	0.35	0.75	0.0000	0.0000	1.1807	0.8829
3433	Xenobiotics	Chemical	O-sulfo-L-tyrosine	1.40	1.40	0.0000	0.0000	0.8221	1.1480
1676	Lipid	Sterol	cholesterol	0.82	0.82	0.0000	0.0001	1.1012	0.9015
318	Amino Acid	Methionine, Cysteine, SAM and Taurine Metabolism	N-acetyltaurine	1.62	1.60	0.0000	0.0001	0.9214	1.4727
1063	Lipid	Polyunsaturated Fatty Acid (n3 and n6)	adrenate (22:4n6)	2.36	2.25	0.0000	0.0001	0.7346	1.6524
278	Amino Acid	Leucine, Isoleucine and Valine Metabolism	valine	0.61	0.60	0.0000	0.0001	1.0966	0.6766
243	Amino Acid	Leucine, Isoleucine and Valine Metabolism	isovalerate	0.60	0.58	0.0000	0.0001	1.2311	0.7200
111	Amino Acid	Phenylalanine and Tyrosine Metabolism	tyrosine	0.78	0.77	0.0000	0.0001	1.1540	0.8850
211	Amino Acid	Tryptophan Metabolism	picolinate	0.62	0.60	0.0000	0.0001	1.3651	0.8190
2468	Nucleotide	Purine Metabolism, Adenine containing	N6-carbamoylthreonyladenosine	1.51	1.47	0.0000	0.0001	0.8161	1.2014
2440	Nucleotide	Purine Metabolism, (Hypo)Xanthine/Inosine containing	allantoin	2.22	2.13	0.0000	0.0001	0.7023	1.4963
26	Amino Acid	Alanine and Aspartate Metabolism	N-acetylalanine	1.29	1.28	0.0000	0.0001	0.8756	1.1198
2477	Nucleotide	Purine Metabolism, Adenine containing	N6-succinyladenosine	2.10	1.99	0.0000	0.0002	0.7039	1.4013
2430	Nucleotide	Purine Metabolism, (Hypo)Xanthine/Inosine containing	inosine	0.34	0.25	0.0000	0.0002	2.2074	0.5410
359	Amino Acid	Creatine Metabolism	guanidinoacetate	0.72	0.71	0.0000	0.0002	1.1968	0.8516
2485	Nucleotide	Purine Metabolism, Guanine containing	guanosine	0.31	0.22	0.0000	0.0002	2.2781	0.5097
1419	Lipid	Phospholipid Metabolism	choline phosphate	0.38	0.24	0.0000	0.0002	1.5486	0.3782
2459	Nucleotide	Purine Metabolism, Adenine containing	N6-methyladenosine	1.45	1.40	0.0000	0.0003	0.8416	1.1763
9	Amino Acid	Glycine, Serine and Threonine Metabolism	serine	0.79	0.79	0.0000	0.0003	1.1173	0.8779
336	Amino Acid	Urea cycle; Arginine and Proline Metabolism	dimethylarginine (SDMA + ADMA)	1.26	1.25	0.0001	0.0004	0.8938	1.1130
283	Amino Acid	Leucine, Isoleucine and Valine Metabolism	isobutyrylglycine	0.57	0.55	0.0001	0.0004	1.4584	0.8001
587	Peptide	Dipeptide	leucylglycine	0.51	0.51	0.0001	0.0004	1.1721	0.5961
1037	Lipid	Long Chain Fatty Acid	eicosenoate (20:1)	2.04	1.97	0.0001	0.0005	0.8493	1.6754
290	Amino Acid	Methionine, Cysteine, SAM and Taurine Metabolism	methionine	0.77	0.76	0.0001	0.0005	1.1628	0.8752
2494	Nucleotide	Purine Metabolism, Guanine containing	N2,N2-dimethylguanosine	1.28	1.25	0.0001	0.0006	0.9081	1.1346
273	Amino Acid	Leucine, Isoleucine and Valine Metabolism	tigloylglycine	0.61	0.57	0.0001	0.0006	1.2313	0.6963
327	Amino Acid	Urea cycle; Arginine and Proline Metabolism	ornithine	0.68	0.62	0.0001	0.0007	1.2163	0.8201
2608	ofactors and Vitamin	Ascorbate and Aldarate Metabolism	quilonic acid*	2.06	1.75	0.0001	0.0008	0.6970	1.2204
2644	ofactors and Vitamin	Hemoglobin and Porphyrin Metabolism	bilirubin (Z,Z)	0.56	0.51	0.0001	0.0008	1.7259	0.8726
73	Amino Acid	Lysine Metabolism	lysine	0.83	0.82	0.0002	0.0009	1.1419	0.9333
112	Amino Acid	Phenylalanine and Tyrosine Metabolism	N-acetyltyrosine	0.72	0.73	0.0002	0.0013	1.1960	0.8752
237	Amino Acid	Leucine, Isoleucine and Valine Metabolism	leucine	0.61	0.59	0.0002	0.0012	1.1029	0.8828
813	Carbohydrate	Glycolysis, Gluconeogenesis, and Pyruvate Metabolism	lactate	1.46	1.41	0.0002	0.0009	0.8890	1.2513
1066	Lipid	Polyunsaturated Fatty Acid (n3 and n6)	dihomo-linoleate (20:2n6)	1.90	1.77	0.0002	0.0010	0.8468	1.4958
2548	Nucleotide	Pyrimidine Metabolism, Cytidine containing	N4-acetylcytidine	1.46	1.45	0.0002	0.0013	0.9307	1.3468
973	Carbohydrate	Aminosugar Metabolism	erythronate*	1.26	1.26	0.0003	0.0016	0.9399	1.1804
1152	Lipid	Fatty Acid, Dicarboxylate	octadecanedioate	2.08	1.94	0.0003	0.0013	0.9011	1.7485
1461	Lipid	Phospholipid Metabolism	1-(1-enyl-palmitoyl)-GPC (P-16:0)*	0.79	0.77	0.0003	0.0016	1.1293	0.8739
203	Amino Acid	Tryptophan Metabolism	kynurenine	0.84	0.83	0.0004	0.0020	1.1147	0.9269
245	Amino Acid	Leucine, Isoleucine and Valine Metabolism	isovalerylcarnitine	0.74	0.74	0.0005	0.0022	1.1596	0.8548
266	Amino Acid	Leucine, Isoleucine and Valine Metabolism	isoleucine	0.80	0.78	0.0005	0.0024	1.1221	0.8719
307	Amino Acid	Methionine, Cysteine, SAM and Taurine Metabolism	cysteine	0.69	0.66	0.0005	0.0022	1.2678	0.8375
352	Amino Acid	Urea cycle; Arginine and Proline Metabolism	N-acetylcitrulline	0.59	0.60	0.0005	0.0022	1.9575	1.1697
1538	Lipid	Lysolipid	1-oleoyl-GPI (18:1)*	0.59	0.55	0.0005	0.0022	1.3719	0.7493
2609	ofactors and Vitamin	Tocopherol Metabolism	alpha-tocopherol	0.82	0.81	0.0005	0.0023	1.0892	0.8851
1231	Lipid	Fatty Acid Metabolism(Acyl Carnitine)	hydroxybutyrylcarnitine*	1.49	1.44	0.0006	0.0025	0.8635	1.2458
1446	Lipid	Phospholipid Metabolism	1-oleoyl-2-linoleoyl-GPI (18:1/18:2)*	0.74	0.70	0.0006	0.0025	1.2817	0.8955
2582	ofactors and Vitamin	Nicotinate and Nicotinamide Metabolism	1-methylnicotinamide	0.66	0.56	0.0006	0.0025	1.2096	0.6797
3541	Xenobiotics	Chemical	4-hydroxychlorothalonil	0.87	0.86	0.0006	0.0025	1.5026	1.2861
204	Amino Acid	Tryptophan Metabolism	kynurenate	0.77	0.77	0.0007	0.0027	1.2853	0.9840
244	Amino Acid	Leucine, Isoleucine and Valine Metabolism	isovalerylglycine	0.54	0.55	0.0008	0.0030	1.2632	0.6927
816	Carbohydrate	Glycolysis, Gluconeogenesis, and Pyruvate Metabolism	glycerate	0.83	0.83	0.0008	0.0032	1.0458	0.8642
1031	Lipid	Long Chain Fatty Acid	nonadecanoate (19:0)	1.34	1.35	0.0008	0.0032	0.9524	1.2840
1151	Lipid	Fatty Acid, Dicarboxylate	hexadecanedioate	1.71	1.68	0.0009	0.0034	0.9444	1.5827
2732	Xenobiotics	Benzoate Metabolism	propyl 4-hydroxybenzoate sulfate	2.04	1.89	0.0009	0.0034	0.6908	1.2675
235	Amino Acid	Tryptophan Metabolism	N-acetylkynurenine (2)	0.88	0.86	0.0010	0.0035	1.1806	0.7137
1213	Lipid	Fatty Acid Metabolism (also BCAA Metabolism)	butyrylcarnitine	1.29	1.25	0.0010	0.0035	1.0910	1.3637
1233	Lipid	Fatty Acid Metabolism(Acyl Carnitine)	hexanoylcarnitine	1.39	1.37	0.0010	0.0035	1.0121	1.3885
1447	Lipid	Phospholipid Metabolism	1-palmitoyl-2-arachidonoyl-GPI (16:0/20:4)*	0.70	0.68	0.0010	0.0035	1.2151	0.7965
2589	ofactors and Vitamin	Nicotinate and Nicotinamide Metabolism	N1-Methyl-2-pyridone-5-carboxamide	0.65	0.62	0.0010	0.0035	1.2544	0.7773
1366	Lipid	Eicosanoid	12-HETE	0.60	0.31	0.0011	0.0036	2.9009	0.9121
248	Amino Acid	Leucine, Isoleucine and Valine Metabolism	beta-hydroxyisovalerate	0.83	0.82	0.0012	0.0041	1.1749	0.9671
366	Amino Acid	Polyamine Metabolism	5-methylthioadenosine (MTA)	1.19	1.18	0.0012	0.0041	0.9092	1.0733
2605	ofactors and Vitamin	Ascorbate and Aldarate Metabolism	threonate	0.71	0.67	0.0012	0.0041	1.0236	0.7782
2761	Xenobiotics	Food Component/Plant	2-piperidinone	0.73	0.67	0.0012	0.0041	1.4596	0.9842
53	Amino Acid	Histidine Metabolism	histidine	0.84	0.82	0.0013	0.0042	1.0801	0.8873
194	Amino Acid	Tryptophan Metabolism	N-acetyltryptophan	0.75	0.73	0.0016	0.0050	1.2745	0.9310
2493	Nucleotide	Purine Metabolism, Guanine containing	N2-methylguanosine	1.35	1.32	0.0016	0.0051	0.8647	1.1409
405	Peptide	Gamma-glutamyl Amino Acid	gamma-glutamyltryptophan	0.80	0.76	0.0017	0.0053	1.1941	0.9356
1421	Lipid	Phospholipid Metabolism	glycerophosphorylcholine (GPC)	0.77	0.76	0.0017	0.0051	1.1454	0.8651
98	Amino Acid	Phenylalanine and Tyrosine Metabolism	phenylalanine	0.88	0.87	0.0019	0.0057	1.0679	0.9320
812	Carbohydrate	Glycolysis, Gluconeogenesis, and Pyruvate Metabolism	pyruvate	1.60	1.42	0.0019	0.0057	0.9167	1.3015
1122	Lipid	Fatty Acid, Branched	17-methylstearate	1.71	1.63	0.0019	0.0057	0.7957	1.2957
1216	Lipid	Fatty Acid Metabolism (also BCAA Metabolism)	propionylcarnitine	0.84	0.84	0.0021	0.0061	1.0682	0.8925
2670	ofactors and Vitamin	Vitamin B6 Metabolism	pyridoxate	0.77	0.70	0.0021	0.0061	1.4754	1.0353
71	Amino Acid	Histidine Metabolism	4-imidazoleacetate	0.67	0.67	0.0022	0.0062	1.0848	0.7269
1017	Lipid	Long Chain Fatty Acid	pentadecanoate (15:0)	1.40	1.36	0.0022	0.0062	1.0312	1.3977
82	Amino Acid	Lysine Metabolism	glutarate (pentanedioate)	0.66	0.62	0.0023	0.0064	1.7006	1.0553
1595	Lipid	Glycerolipid Metabolism	glycerol	0.67	0.71	0.0024	0.0067	1.3120	0.9366
1657	Lipid	Sphingolipid Metabolism	sphingomyelin (d18:2/16:0, d18:1/16:1)*	0.86	0.86	0.0025	0.0068	1.0752	0.9201
2533	Nucleotide	Pyrimidine Metabolism, Uracil containing	4-ureidobutyrate	0.72	0.76	0.0025	0.0068	1.2137	0.8620
1788	Lipid	Steroid	5alpha-androstan-3alpha,17beta-diol monosulfate (1)	1.32	1.26	0.0026	0.0069	1.1409	1.4394
141	Amino Acid	Phenylalanine and Tyrosine Metabolism	3-methoxytyrosine	1.11	1.11	0.0027	0.0072	1.0033	1.1138
1655	Lipid	Sphingolipid Metabolism	sphingomyelin (d18:2/14:0, d18:1/14:1)*	0.78	0.77	0.0027	0.0072	1.1159	0.8560
793	Carbohydrate	Glycolysis, Gluconeogenesis, and Pyruvate Metabolism	1,5-anhydroglucitol (1,5-AG)	0.91	0.92	0.0028	0.0073	0.9963	0.9126
402	Peptide	Gamma-glutamyl Amino Acid	gamma-glutamylmethionine	0.78	0.74	0.0029	0.0073	1.2240	0.9080
1399	Lipid	Inositol Metabolism	inositol 1-phosphate (I1P)	0.78	0.75	0.0029	0.0073	1.1882	0.8856
1445	Lipid	Phospholipid Metabolism	1-palmitoyl-2-linoleoyl-GPI (16:0/18:2)*	0.78	0.76	0.0029	0.0073	1.1804	0.8935
75	Amino Acid	Lysine Metabolism	N6-acetyllysine	1.15	1.14	0.0030	0.0074	0.9215	1.0495
312	Amino Acid	Methionine, Cysteine, SAM and Taurine Metabolism	cysteine s-sulfate	0.69	0.54	0.0030	0.0074	1.2694	0.6871
1510	Lipid	Lysolipid	1-palmitoyl-GPE (16:0)	0.77	0.73	0.0030	0.0073	1.2350	0.9027
325	Amino Acid	Urea cycle; Arginine and Proline Metabolism	arginine	0.85	0.84	0.0031	0.0075	1.1366	0.9536
1448	Lipid	Phospholipid Metabolism	1-stearoyl-2-linoleoyl-GPI (18:0/18:2)*	0.79	0.78	0.0031	0.0075	1.1257	0.8800
218	Amino Acid	Tryptophan Metabolism	5-hydroxyindoleacetate	0.63	0.49	0.0033	0.0078	1.5830	0.7758
2534	Nucleotide	Pyrimidine Metabolism, Uracil containing	3-ureidopropionate	1.75	1.64	0.0033	0.0077	0.8271	1.3524
545	Peptide	Dipeptide	glycylvaline	0.66	0.15	0.0034	0.0079	6.9270	1.0565
1249	Lipid	Carnitine Metabolism	carnitine	1.07	1.07	0.0034	0.0079	0.9669	1.0327
3126	Xenobiotics	Bacterial/Fungal	tartronate (hydroxymalonate)	0.50	0.48	0.0035	0.0079	1.4044	0.6673
1436	Lipid	Phospholipid Metabolism	1-palmitoyl-2-arachidonoyl-GPC (16:0/20:4)*	0.84	0.87	0.0036	0.0081	1.0829	0.9405
3157	Xenobiotics	Drug	2-methoxyacetaminophen sulfate*	432.16	18.75	0.0036	0.0080	0.0561	1.0516
738	Peptide	Dipeptide	valylleucine	0.80	0.78	0.0039	0.0087	1.1095	0.8613
326	Amino Acid	Urea cycle; Arginine and Proline Metabolism	urea	0.84	0.82	0.0040	0.0087	1.1318	0.9297
1267	Lipid	Fatty Acid, Monohydroxy	3-hydroxysebacate	2.15	1.72	0.0041	0.0089	0.9934	1.7070
2745	Xenobiotics	Xanthine Metabolism	1-methylxanthine	0.46	0.45	0.0041	0.0089	1.7840	0.8758
2645	ofactors and Vitamin	Hemoglobin and Porphyrin Metabolism	bilirubin (E,E)*	0.74	0.69	0.0042	0.0091	1.3280	0.9198
1032	Lipid	Long Chain Fatty Acid	10-nonadecenoate (19:1n9)	1.88	1.74	0.0044	0.0094	0.9312	1.6195
277	Amino Acid	Leucine, Isoleucine and Valine Metabolism	ethylmalonate	1.26	1.24	0.0047	0.0099	1.1462	1.4161
1444	Lipid	Phospholipid Metabolism	1-stearoyl-2-arachidonoyl-GPI (18:0/20:4)*	0.86	0.82	0.0051	0.0108	1.2290	1.0081
735	Peptide	Dipeptide	valylglycine	0.71	0.57	0.0054	0.0112	1.2603	0.7244
282	Amino Acid	Leucine, Isoleucine and Valine Metabolism	isobutyrylcarnitine	0.82	0.78	0.0056	0.0116	1.2666	0.9868
1533	Lipid	Lysolipid	1-palmitoyl-GPI (16:0)*	0.68	0.61	0.0058	0.0119	1.3543	0.8292
3538	Xenobiotics	Chemical	4-methylbenzenesulfonate	0.62	0.76	0.0058	0.0118	1.3533	1.0330
362	Amino Acid	Polyamine Metabolism	acisoga	1.33	1.25	0.0060	0.0121	0.8772	1.1006
148	Amino Acid	Phenylalanine and Tyrosine Metabolism	homovanillate (HVA)	0.76	0.72	0.0062	0.0125	1.1547	0.8859

$p \leq 0.05$, fold of change ≥ 1.00

$p \leq 0.05$, fold of change < 1.00

Pathway Sort Order	Super Pathway	Sub Pathway	Biochemical Name	Fold of Change, Paired t-Test: Post G-CSF Pre G-CSF Mean Pairs Ratio	Fold of Change, Paired t-Test: Post G-CSF Pre G-CSF Group Mean Ratio	Paired t-Test: Post G-CSF / Pre G-CSF p-value	Paired t-Test: Post G-CSF / Pre G-CSF q-value	Mean Values: Pre G-CSF	Mean Values: Post G-CSF
1021	Lipid	Long Chain Fatty Acid	margarate (17:0)	1.55	1.50	0.0064	0.0127	1.0301	1.5499
1418	Lipid	Phospholipid Metabolism	choline	0.69	0.69	0.0065	0.0127	1.0740	0.9552
28	Amino Acid	Alanine and Aspartate Metabolism	aspartate	0.64	0.60	0.0068	0.0132	1.3009	0.7755
397	Peptide	Gamma-glutamyl Amino Acid	gamma-glutamylglycine	0.67	0.50	0.0068	0.0132	1.7954	0.9043
1139	Lipid	Fatty Acid, Dicarboxylate	maleate	0.77	0.73	0.0070	0.0134	1.3816	1.0110
1555	Lipid	Lysolipid	1-arachidonoyl-GPA (20:4)	0.68	0.59	0.0070	0.0134	1.3556	0.7951
256	Amino Acid	Leucine, Isoleucine and Valine Metabolism	alpha-hydroxyisovalerate	1.21	1.27	0.0071	0.0134	1.0033	1.2732
2528	Nucleotide	Pyrimidine Metabolism, Uracil containing	5,6-dihydrouracil	1.18	1.19	0.0071	0.0134	0.9634	1.1445
317	Amino Acid	Methionine, Cysteine, SAM and Taurine Metabolism	taurine	0.78	0.73	0.0077	0.0144	1.2349	0.9064
1643	Lipid	Sphingolipid Metabolism	sphingosine 1-phosphate	0.84	0.82	0.0078	0.0144	1.0986	0.9009
267	Amino Acid	Leucine, Isoleucine and Valine Metabolism	allo-isoleucine	1.31	1.25	0.0085	0.0155	0.9935	1.2390
2743	Xenobiotics	Xanthine Metabolism	3,7-dimethylurate	0.73	0.63	0.0085	0.0155	1.7071	1.0823
1025	Lipid	Long Chain Fatty Acid	oleate (18:1n9)	1.70	1.61	0.0086	0.0156	1.0068	1.6250
1062	Lipid	Polyunsaturated Fatty Acid (n3 and n6)	arachidonate (20:4n6)	0.76	0.73	0.0093	0.0168	1.1828	0.8666
3160	Xenobiotics	Drug	4-acetaminophen sulfate	1306.61	61.32	0.0095	0.0169	0.7167	8.1123
1394	Lipid	Inositol Metabolism	scyllo-inositol	0.79	0.71	0.0096	0.0169	1.5570	1.0997
25	Amino Acid	Alanine and Aspartate Metabolism	alanine	1.13	1.11	0.0097	0.0169	0.9654	1.0764
3156	Xenobiotics	Drug	2-hydroxyacetaminophen sulfate*	787.95	13.37	0.0097	0.0169	4.4804	59.9252
3162	Xenobiotics	Drug	4-acetamidophenylglucuronide	381.77	12.34	0.0098	0.0170	0.0708	1.2280
1059	Lipid	Polyunsaturated Fatty Acid (n3 and n6)	linolenate [alpha or gamma; (18:3n3 or 6)]	1.80	1.70	0.0101	0.0173	0.9509	1.6206
3161	Xenobiotics	Drug	4-acetamidophenol	197.35	14.07	0.0102	0.0175	0.0965	1.3577
269	Amino Acid	Leucine, Isoleucine and Valine Metabolism	3-methyl-2-oxovalerate	0.86	0.86	0.0105	0.0176	1.1373	0.9769
1180	Lipid	Fatty Acid, Amide	oleamide	0.69	0.37	0.0105	0.0176	2.6343	0.9708
2532	Nucleotide	Pyrimidine Metabolism, Uracil containing	2'-deoxyuridine	1.54	1.44	0.0105	0.0176	0.8843	1.2728
1027	Lipid	Long Chain Fatty Acid	cis-vaccenate (18:1n7)	1.58	1.39	0.0110	0.0182	0.9850	1.3596
3547	Xenobiotics	Chemical	3-hydroxypyridine sulfate	0.72	0.64	0.0110	0.0182	1.2264	0.7808
2717	Xenobiotics	Benzoate Metabolism	4-vinylphenol sulfate	0.73	0.71	0.0116	0.0191	3.2346	2.3007
2492	Nucleotide	Purine Metabolism, Guanine containing	N1-methylguanosine	1.18	1.15	0.0122	0.0200	0.9447	1.0890
3163	Xenobiotics	Drug	2-methoxyacetaminophen glucuronide*	50.09	20.62	0.0128	0.0208	0.0618	1.2742
400	Peptide	Gamma-glutamyl Amino Acid	gamma-glutamylleucine	0.86	0.83	0.0132	0.0213	1.1059	0.9147
74	Amino Acid	Lysine Metabolism	N2-acetyllysine	0.80	0.72	0.0133	0.0213	1.2041	0.8660
1762	Lipid	Steroid	cortisone	0.85	0.82	0.0138	0.0220	1.1471	0.9461
2519	Nucleotide	Pyrimidine Metabolism, Uracil containing	uracil	1.34	1.30	0.0139	0.0220	0.9029	1.1707
1012	Lipid	Medium Chain Fatty Acid	laurate (12:0)	1.49	1.39	0.0146	0.0228	0.9576	1.3346
3158	Xenobiotics	Drug	3-(cystein-S-yl)acetaminophen*	25.58	9.80	0.0146	0.0228	0.0845	0.8285
407	Peptide	Gamma-glutamyl Amino Acid	gamma-glutamylvaline	0.88	0.86	0.0147	0.0228	1.0957	0.9398
238	Amino Acid	Leucine, Isoleucine and Valine Metabolism	N-acetylleucine	0.83	0.83	0.0153	0.0234	1.0607	0.8766
1654	Lipid	Sphingolipid Metabolism	sphingomyelin (d18:1/14:0, d16:1/16:0)*	0.87	0.88	0.0155	0.0234	1.0413	0.9144
3098	Xenobiotics	Food Component/Plant	4-vinylguaiacol sulfate	0.61	0.57	0.0155	0.0234	1.4245	0.8159
79	Amino Acid	Lysine Metabolism	2-aminoadipate	0.83	0.75	0.0157	0.0234	1.3280	0.9993
240	Amino Acid	Leucine, Isoleucine and Valine Metabolism	4-methyl-2-oxopentanoate	0.86	0.86	0.0157	0.0234	1.1075	0.9495
569	Peptide	Dipeptide	isoleucylglycine	0.82	0.75	0.0157	0.0234	1.1828	0.9889
1006	Lipid	Medium Chain Fatty Acid	heptanoate (7:0)	0.86	0.85	0.0157	0.0234	1.1133	0.9497
2705	Xenobiotics	Benzoate Metabolism	3-methyl catechol sulfate (2)	0.74	0.62	0.0158	0.0234	1.3245	0.6851
406	Peptide	Gamma-glutamyl Amino Acid	gamma-glutamyltyrosine	0.85	0.82	0.0166	0.0244	1.0817	0.8885
2607	dfactors and Vitamir	Ascorbate and Aldarate Metabolism	oxalate (ethanedioate)	0.65	0.64	0.0166	0.0244	1.1510	0.7406
3430	Xenobiotics	Chemical	sulfate*	0.76	0.76	0.0168	0.0245	1.0917	0.8347
306	Amino Acid	Methionine, Cysteine, SAM and Taurine Metabolism	2-hydroxybutyrate (AHB)	1.41	1.30	0.0170	0.0245	0.9659	1.2542
3159	Xenobiotics	Drug	3-(N-acetyl-L-cystein-S-yl) acetaminophen	15.96	5.53	0.0170	0.0245	0.0965	0.5341
279	Amino Acid	Leucine, Isoleucine and Valine Metabolism	N-acetylvaline	1.09	1.08	0.0175	0.0248	0.9730	1.0539
1003	Energy	Oxidative Phosphorylation	phosphate	0.91	0.91	0.0175	0.0248	1.0886	0.9828
1266	Lipid	Fatty Acid, Monohydroxy	3-hydroxydecanoate	1.37	1.25	0.0175	0.0248	1.0661	1.3361
188	Amino Acid	Phenylalanine and Tyrosine Metabolism	N-formylphenylalanine	0.78	0.61	0.0178	0.0251	1.2909	0.7914
106	Amino Acid	Phenylalanine and Tyrosine Metabolism	phenylacetate	0.78	0.71	0.0179	0.0251	1.3119	0.9367
15	Amino Acid	Glycine, Serine and Threonine Metabolism	N-acetylthreonine	1.26	1.23	0.0184	0.0256	0.9305	1.1405
1361	Lipid	Eicosanoid	5-HETE	1.59	1.77	0.0187	0.0259	0.6777	1.1989
1018	Lipid	Long Chain Fatty Acid	palmitate (16:0)	1.24	1.24	0.0193	0.0267	1.0105	1.2489
906	Carbohydrate	Fructose, Mannose and Galactose Metabolism	mannose	1.40	1.23	0.0197	0.0270	0.9723	1.1979
1022	Lipid	Long Chain Fatty Acid	10-heptadecenoate (17:1n7)	1.77	1.65	0.0198	0.0270	1.0032	1.6548
1023	Lipid	Long Chain Fatty Acid	stearate (18:0)	1.29	1.23	0.0202	0.0273	0.9912	1.2197
1784	Lipid	Steroid	4-androsten- 3beta,17beta-diol disulfate (2)	0.79	0.62	0.0203	0.0274	1.2465	1.0254
1664	Lipid	Mevalonate Metabolism	3-hydroxy-3-methylglutarate	0.85	0.80	0.0205	0.0275	1.1788	0.9375
54	Amino Acid	Histidine Metabolism	N-acetylhistidine	0.78	0.81	0.0212	0.0283	1.0851	0.8799
1133	Lipid	Fatty Acid, Dicarboxylate	2-hydroxyglutarate	1.71	1.61	0.0214	0.0284	0.9920	1.5942
2452	Nucleotide	Purine Metabolism, Adenine containing	adenosine	0.81	0.79	0.0215	0.0284	3.4216	2.9964
1834	Lipid	Steroid	pregnanolone/allopregnanolone sulfate	0.79	0.58	0.0216	0.0284	1.0092	0.5669
2448	Nucleotide	Purine Metabolism, Adenine containing	adenosine 3',5'-cyclic monophosphate (cAMP)	0.81	0.77	0.0217	0.0284	1.1050	0.8499
270	Amino Acid	Leucine, Isoleucine and Valine Metabolism	2-methylbutyrylcarnitine (C5)	0.89	0.86	0.0219	0.0285	1.0657	0.9210
1057	Lipid	Polyunsaturated Fatty Acid (n3 and n6)	linolenate (18:2n6)	1.42	1.38	0.0222	0.0288	1.0030	1.3855
3034	Xenobiotics	Food Component/Plant	3-allylcysteine	0.73	0.31	0.0225	0.0289	1.3827	0.4245
180	Amino Acid	Phenylalanine and Tyrosine Metabolism	5-hydroxymethyl-2-furoic acid	0.82	0.54	0.0227	0.0289	1.6842	0.9148
1519	Lipid	Lysolipid	1-linoleoyl-GPE (18:2)*	0.84	0.76	0.0228	0.0289	1.1630	0.8872
2521	Nucleotide	Pyrimidine Metabolism, Uracil containing	5-methyluridine (ribothymidine)	0.84	0.83	0.0228	0.0289	1.0719	0.8916
2704	Xenobiotics	Benzoate Metabolism	3-methyl catechol sulfate (1)	0.77	0.53	0.0229	0.0289	1.4839	0.7879
1016	Lipid	Long Chain Fatty Acid	myristoleate (14:1n5)	1.80	1.64	0.0233	0.0293	1.3317	2.1785
1268	Lipid	Fatty Acid, Monohydroxy	3-hydroxylaurate	1.57	1.36	0.0234	0.0293	0.9687	1.3179
1222	Lipid	Fatty Acid Metabolism(Acyl Glycine)	hexanoylglycine	1.38	1.45	0.0237	0.0294	0.9499	1.3790
1699	Lipid	Sterol	campesterol	0.67	0.61	0.0241	0.0298	1.1340	0.9240
284	Amino Acid	Leucine, Isoleucine and Valine Metabolism	3-hydroxyisobutyrate	0.86	0.81	0.0243	0.0299	1.1686	0.9447
1280	Lipid	Fatty Acid, Monohydroxy	16-hydroxypalmitate	1.44	1.39	0.0246	0.0300	0.9760	1.3539
1015	Lipid	Long Chain Fatty Acid	myristate (14:0)	1.64	1.51	0.0247	0.0300	1.0213	1.5461
1247	Lipid	Fatty Acid Metabolism(Acyl Carnitine)	myristoleoylcarnitine*	3.62	2.54	0.0247	0.0300	0.6640	1.6882
198	Amino Acid	Tryptophan Metabolism	indoleacetate	0.69	0.62	0.0250	0.0301	1.4667	1.1978
30	Amino Acid	Alanine and Aspartate Metabolism	asparagine	0.94	0.93	0.0258	0.0310	1.0885	1.0128
3070	Xenobiotics	Food Component/Plant	4-allylphenol sulfate	0.80	0.38	0.0263	0.0315	2.5218	0.9684
982	Energy	TCA Cycle	aconitate [cis or trans]	1.29	1.22	0.0264	0.0315	0.9360	1.1391
1009	Lipid	Medium Chain Fatty Acid	caprate (10:0)	1.65	1.48	0.0274	0.0325	1.0080	1.4871
1052	Lipid	Polyunsaturated Fatty Acid (n3 and n6)	docosapentaenoate (n3 DPA; 22:5n3)	1.45	1.37	0.0277	0.0326	0.9218	1.2634
2747	Xenobiotics	Xanthine Metabolism	7-methylxanthine	0.81	0.61	0.0290	0.0340	1.4468	0.8764
1391	Lipid	Inositol Metabolism	myo-inositol	0.87	0.88	0.0293	0.0342	1.0789	0.9455
179	Amino Acid	Phenylalanine and Tyrosine Metabolism	thyroxine	0.95	0.94	0.0296	0.0344	1.0667	1.0056
303	Amino Acid	Methionine, Cysteine, SAM and Taurine Metabolism	alpha-ketobutyrate	1.75	1.48	0.0300	0.0347	0.9656	1.4328
2749	Xenobiotics	Xanthine Metabolism	5-acetylamino-6-formylamino-3-methyluracil	0.80	0.51	0.0304	0.0350	1.2637	0.6425
174	Amino Acid	Phenylalanine and Tyrosine Metabolism	3-phenylpropionate (hydrocinnamate)	0.81	0.67	0.0326	0.0373	2.1967	1.4678
1540	Lipid	Lysolipid	1-linoleoyl-GPI (18:2)*	0.82	0.78	0.0326	0.0373	1.1479	0.8917
751	Peptide	Polypeptide	bradykinin, des-arg(9)	0.84	0.68	0.0329	0.0374	1.5225	1.0516
399	Peptide	Gamma-glutamyl Amino Acid	gamma-glutamylisoleucine*	0.87	0.81	0.0342	0.0387	1.1889	0.9599
63	Amino Acid	Histidine Metabolism	imidazole propionate	0.87	0.90	0.0344	0.0388	1.4112	1.2745
1217	Lipid	Fatty Acid Metabolism (also BCAA Metabolism)	propionylglycine	0.78	0.77	0.0349	0.0391	1.0967	0.8400
2614	dfactors and Vitamir	Tocopherol Metabolism	gamma-tocopherol	0.82	0.55	0.0359	0.0401	1.4611	0.9659
2710	Xenobiotics	Benzoate Metabolism	methyl-4-hydroxybenzoate	49.34	30.31	0.0363	0.0404	0.9339	28.3049
395	Peptide	Gamma-glutamyl Amino Acid	gamma-glutamylglutamate	0.80	0.77	0.0367	0.0406	1.2617	0.9684
1798	Lipid	Steroid	5alpha-androstan-3beta,17beta-diol disulfate	0.86	0.87	0.0375	0.0413	1.4057	1.2287
1853	Lipid	Secondary Bile Acid Metabolism	glycodeoxycholate	1.79	1.44	0.0386	0.0423	0.9031	1.3019
39	Amino Acid	Glutamate Metabolism	glutamine	1.07	1.06	0.0387	0.0423	0.9626	1.0249
1064	Lipid	Polyunsaturated Fatty Acid (n3 and n6)	docosapentaenoate (n6 DPA; 22:5n6)	0.86	0.81	0.0397	0.0432	1.2608	1.0182
1826	Lipid	Steroid	etiocholanolone glucuronide	0.88	0.93	0.0401	0.0434	1.4135	1.3203
2994	Xenobiotics	Food Component/Plant	N-(2-furoyl)glycine	0.91	0.41	0.0411	0.0443	2.3108	0.9588
249	Amino Acid	Leucine, Isoleucine and Valine Metabolism	beta-hydroxyisovaleroylcarnitine	0.88	0.88	0.0444	0.0477	1.1032	0.9744
696	Peptide	Dipeptide	threonylphenylalanine	0.81	0.73	0.0449	0.0480	1.1720	0.8526
1041	Lipid	Long Chain Fatty Acid	erucate (22:1n9)	2.39	1.38	0.0463	0.0493	1.0137	1.4032
404	Peptide	Gamma-glutamyl Amino Acid	gamma-glutamylthreonine*	0.90	0.90	0.0482	0.0511	1.0908	0.9776

[Biochemical Name] Indicates compounds that have not been officially confirmed based on a standard, but we are confident in its identity.*

Supplementary Table 3. Top-ranked compounds associated with different pathways

Amino acid metabolism including immune-regulatory compounds	
cysteine-s-sulfate	↓
tryptophan	↓
isovalerate	↓
histidine	↓
guanidinoacetate	↓
5-hydroxyindolacetate	↓
N-acetyltaurine	↑
N-acetylmethionine	↑
N-acetylserine	↑

Compounds involved in inflammatory effects	
choline phosphate	↓
guanosine	↓
dihydroorotate	↓
ergothioneine	↓
gulonic acid	↑

Genetics and epigenetics	
inosine	↓
phosphate	↓
N-succinyladenosine	↑
orotidine	↑
pseudouridine	↑

Vascular biology	
guanosine	↓
5-hydroxyindoleacetate	↓
O-sulfo-L-tyrosine	↑
allantoin	↑

Energy/glycogen metabolism	
maltose	↑
maltotriose	↑

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