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Separation and Further Characterization of Hematopoietic Cell Populations Based on Phenotypic and Biophysical Properties

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Separation and Further Characterization of Hematopoietic Cell Populations Based on Phenotypic and Biophysical Properties

Doctoral thesis

by

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Division of Hematology and Transfusion Medicine
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With the approval of the Lund University Faculty of Medicine,
this thesis will be defended on February 15, 2013, at 09:15,
in Föreläsningssal 3, University Hospital, Lund

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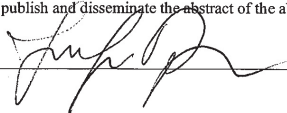
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<p>Abstract</p> <p>Hematopoietic stem cells (HSC) are multipotent and possess self-renewal capacity. Thus, they can sustain a life-long hematopoiesis and are also capable of restoring the hematopoietic system when transplanted to a patient. In allogeneic hematopoietic stem cell transplantation (HSCT), a potent graft-versus-leukemia (GvL) effect must be balanced against the concomitant risk of severe immunological response to non-malignant host cells; graft-versus-host-disease (GvHD). In case of recipient-donor tissue antigen disparity, such as human leukocyte antigen (HLA)-mismatch or ABO-incompatibility, depletion of allo-reactive or incompatible blood constituents by HSCT graft processing may be required. The aim of this work was to investigate the development of human erythroid/myeloid progenitor cells and their progeny in normal differentiation and in the chimeric state of transfusion and allogeneic HSCT. Furthermore, novel technology platforms for optimal HSCT graft engineering in the setting of recipient-donor tissue antigen disparity were investigated.</p> <p>We utilized two <i>in vitro</i> culture systems (Paper II) to obtain cells of advanced neutrophil and erythroid maturation from adult human CD34⁺ bone marrow cells, and to study the relationship between clonogenicity, gene expression and phenotype during early myeloid development. Cells were sorted by fluorescence-activated-cell-sorting (FACS) according to selected surface markers into defined populations of sequential developmental stages for further analysis. Thus the expression profiles of several genes could be directly correlated to changes in clonogenic potential and to lineage commitment, as defined by surface expression of CD15 (neutrophil differentiation) and group A antigen (erythroid differentiation).</p> <p>With regard to the established surface markers for neutrophil and erythroid lineage-affiliation, we investigated (Paper I) the origin of reappearing recipient-derived RBC in a patient with relapsed myelodysplastic syndrome (MDS) following allogeneic HSCT. The presence of the original cytogenetic 20q deletion in myeloid cell populations of different maturity, suggested an origin common to the original myeloid malignant clone and the reappearing autologous RBC, i.e. a progenitor cell with preserved granulocyte/monocyte and megakaryocyte/erythroid potential. We continued our study of chimeric post-HSCT recipients by addressing (paper V) the phenomenon of blood group A/B antigen acquisition by donor group O RBC, following transfusion or ABO-incompatible HSCT. Using a highly sensitive flow cytometry assay, donor group O RBC were found to express variable levels of acquired antigen, ranging from very small amounts in non-secretor individuals to ABO-subgroup levels in group A₁ secretors. Our findings support the major role of A/B antigen adsorption from secretor plasma, but indicate a secretor-independent mechanism for A/B-antigen acquisition.</p> <p>To improve graft processing in HLA-mismatched HSCT, we investigated (Paper III) and optimized the performance of a novel magnetic cell sorting program (Depletion 3.1, CliniMACS System), for large-scale direct depletion of T-cell from peripheral blood progenitor cell (PBPC). The optimized D3.1 program can be utilized for large-scale, time saving direct T-cell depletion with excellent recovery of CD34⁺ cells and an effective reduction of T-cell numbers. Furthermore, a novel micro-chip based acoustophoresis technique was investigated (paper IV) for removal of platelets from PBPC products and was found to efficiently deplete PBPC samples of intact platelets, whilst preserving the target leukocyte fraction, cell viability and progenitor cell colony-forming ability. Acoustophoresis is, thus, an interesting technology to improve current cell processing methods.</p> <p>In summary, this work has provided knowledge on differentiation-associated changes in human myeloid development. Surface restriction markers for erythroid (group A antigen) and neutrophil differentiation (CD15) have been established and utilized to investigate the origin of reappearing host cells post-HSCT. In a clinical context, the level of A/B-antigen found to be acquired by donor group O RBC suggests implications for plasma component selection. Moreover, current clinical cell separation methodology was improved and the novel acoustophoresis technology was demonstrated to provide efficient platelet depletion, with a potential application in future clinical graft engineering.</p>		
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Separation and Further Characterization of Hematopoietic Cell Populations Based on Phenotypic and Biophysical Properties



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Moral of the Work

IN WAR: RESOLUTION

IN DEFEAT: DEFIANCE

IN VICTORY: MAGNANIMITY

IN PEACE: GOODWILL

W.S.C

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ABBREVIATIONS

A/B-GT	A/B-glycosyltransferases
AML	Acute myeloid leukemia
BFU-E	Burst forming unit- erythroid
CFU-E	Colony forming unit-erythroid
CFU-GM	Colony forming unit-granulocyte/monocyte
CFU-MK	Colony forming unit-megakaryocyte
CLP	Common lymphoid progenitor
CMP	Common myeloid progenitor
DLI	Donor lymphocyte infusion
EKLF	Erythroid kruppel-like factor
ER	Endoplasmatic reticulum
Epo	Erythropoietin
FACS	Fluorescence activated cell sorting
Fuc	Fucose
Gal	Galactose
GalNac	<i>N</i> -acetyl galactosamine
G-CSF	Granulocyte-colony stimulating factor
GM-CSF	Granulocyte/macrophage-colony stimulating factor
GMP	Granulocyte/monocyte progenitor
GPA	Glycophorin A
GPB	Glycophorin B
GPC	Glycophorin C
GT	Glycosyltransferase

GvHD	Graft-versus-Host-Disease
GvL	Graft-versus-Leukemia
GvT	Graft –versus-Tumor
HDFN	Hemolytic disease of the fetus or newborn
HLA	Human leukocyte antigen
HSC	Hematopoietic stem cell
HSCT	Hematopoietic stem cell transplantation
HTR	Hemolytic transfusion reaction
Lu	Lutheran
MACS	Magnetic activated cell sorting
M-CSF	Monocyte-colony stimulating factor
MDS	Myelodysplastic syndrome
Meg	Megakaryocyte
MEP	Megakaryocyte/erythroid progenitor
mHag	minor histocompatibility antigen
MNC	Mononuclear cell
MPP	Multipotent progenitor
mRNA	Messenger RNA
NF-E2	Nuclear factor erythroid-derived 2
NK	Natural killer cell
PBMC	Peripheral blood mononuclear cell
PBPC	Peripheral blood progenitor cell
PR3	Proteinase 3
PRCA	Pure red cell aplasia
RBC	Red blood cell
RhAG	Rh-associated glycoprotein
rRNA	Ribosomal RNA
SCL/Tal1	Stem cell factor
SNP	Singel nucleotide polymorphism

STR-PCR	Short tandem repeat-polymerase chain reaction
Tpo	Thrombopoietin
TRAP	Thrombin receptor activator peptide
UDP	Uridine diphosphate

PAPERS INCLUDED

- I. **JH Dykes**, A Lindmark, S Lenhoff, I Winqvist, B Johansson, T Olofsson, ML Olsson. Autologous del(20q)-positive erythroid progenitor cells, re-emerging after DLI treatment of an MDS patient relapsing after allo-SCT, can provide a normal peripheral red blood cell count.
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PLoS One. 2011; 6(8):e23074
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HEMATOPOIESIS

Hematopoiesis, the continuous formation of blood cells, is a dynamic process where blood cells are generated from hematopoietic stem cells (HSC) which reside mainly in the liver during the fetal period and in the bone marrow after birth.¹ Like other stem cells, HSC have the capacity to self-renew and are also multipotent. Thus, HSC can sustain a life-long hematopoiesis and also differentiate to all hematopoietic lineages by giving rise to highly proliferative progenitors with gradually more restricted developmental potential and no self-renewing capacity. Eventually lineage-committed progenitors will mature into fully differentiated blood cells.

The hematopoietic hierarchy

Hematopoietic stem cells

Through the process of asymmetric division, a single HSC division can result in the formation of both an identical stem cell and a more differentiated cell. Signaling pathway components which are suggested to promote self-renewal include Wnt and hedgehog ligand molecules and the trans-membrane protein Notch.¹⁻⁴

In the mouse, which is a frequently used model system for human hematopoiesis, HSC are identified by their ability to reconstitute hematopoiesis upon transplantation to lethally irradiated recipients. HSC reside in the Lin⁻Sca-1^c-Kit⁺ fraction of murine bone marrow.⁵⁻⁷ This compartment, still heterogeneous in terms of self-renewal capacity and sustainability of hematopoietic reconstitution, has been further characterized by the expression of additional surface markers such as CD34^{8,9} and the SLAM family receptors.¹⁰ HSC give rise to multipotent progenitors (MPP) which retain full lineage potential but lack self-renewal capacity and, thus, can only support hematopoiesis transiently.

Human candidate HSC, with the capacity to repopulate animals in xenotransplantation models, reside predominantly in the fraction of Lin⁻CD34⁺CD38⁻CD90⁺CD45RA⁻ cells.¹¹⁻¹⁴ Recently, the integrin receptors $\alpha 6^{15}$ and $\alpha 2^{16}$ were identified as novel markers for long-term reconstituting HSC in cord

blood. A small fraction of HSC has also been found amongst Lin⁻CD34⁺CD38⁻ cells.^{1,17} The human candidate MPP population has been identified in cord blood as Lin⁻CD34⁺CD38⁻CD90⁺CD45RA⁻, given its typical MPP characteristics of multipotency and loss of self-renewal.¹³

Hematopoietic progenitors and their lineage restriction

In the classical model of hematopoiesis the myeloid and lymphoid lineages are symmetrically derived from a single MPP, representing the first step of irreversible lineage commitment.^{18,19} This model is further supported by the characterization of a murine common lymphoid progenitor (CLP)²⁰ and a common myeloid progenitor (CMP), with the latter differentiating into a granulocyte/monocyte progenitor (GMP) and a megakaryocyte/erythroid progenitor (MEP)²¹ (Figure 1). However, alternative models have proposed a common granulocyte/monocyte and lymphoid pathway.^{22,23} suggesting that CMP are derived from more primitive MPP fractions whereas lymphoid-specified MPP gradually lose myeloid differentiation potential before definite lymphoid lineage commitment.^{24,25}

Candidate populations for a human CLP have been identified in bone marrow as Lin⁻CD34⁺CD38⁺CD10⁺.²⁶ The human counterparts of the myeloid progenitors CMP, GMP and MEP all reside in the Lin⁻CD34⁺CD38⁺ bone marrow fraction and have been further defined as IL3R α ^{lo}CD45RA⁻, IL3R α ^{lo}CD45RA⁺ and IL3R α ⁻CD45RA⁻, respectively.²⁷ The isolation of these progenitors has hitherto supported the classical model of progenitor cell differentiation in human hematopoiesis. Recently, though, a multi-lymphoid progenitor (MLP) giving rise to all lymphoid cells as well as monocytes, macrophages and dendritic cells was identified as a CD90^{-low}CD45RA⁺ population in the Lin⁻CD34⁺CD38⁻ bone marrow compartment.²⁸

Mature blood cells

Progenitors of the different hematopoietic lineages will subsequently generate mature blood cells that are released into circulation. Lymphoid progenitors will give rise to B, T and NK cells, whereas the myeloid progenitors will differentiate to granulocytes, monocytes, macrophages, megakaryocytes and erythrocytes.

Erythroid progenitors are divided into early (burst forming unit- erythroid, BFU-E) and late (colony forming unit-erythroid, CFU-E) stages, based on their colony forming potential *in vitro*. CFU-E will differentiate into morphologically distinguishable precursor cells; the proerythroblast and the subsequent basophilic, polychromatic and orthochromatic erythroblasts. At the reticulocyte stage, the nucleus

is extruded and mitochondria and ribosomes are lost as the cell reaches full erythrocyte maturation within a few days.

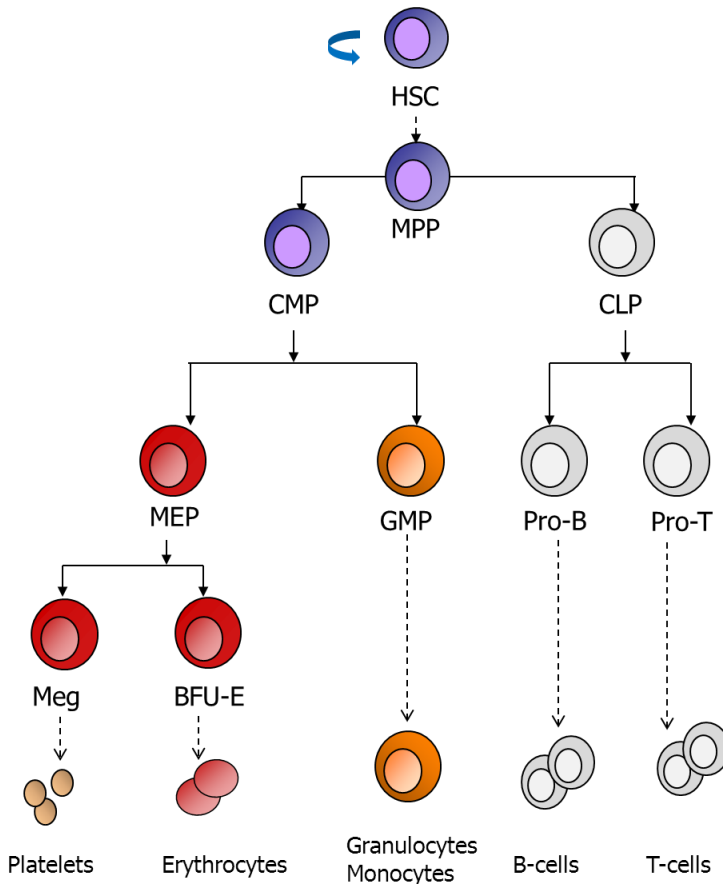


Figure 1. The classical model of hematopoietic development. HSC give rise to all mature blood cell types through a series of gradually more lineage-restricted progenitor and precursor cells. Abbreviations: HSC, hematopoietic stem cell; MPP, multipotent progenitor; CMP, common myeloid progenitor; CLP, common lymphoid progenitor; MEP, megakaryocyte/ erythrocyte progenitor; GMP, granulocyte/monocyte progenitor; Meg, megakaryocyte; BFU-E, burstforming unit-erythroid.

Hematopoietic regulation

Hematopoiesis is strictly regulated by extrinsic regulators, such as surrounding cells, adjacent matrix components and soluble factors, and intrinsic changes in expression of transcription factors.

The role of transcription factors in regulating commitment and differentiation is generally accepted, whereas two different modes of cytokine functions have been suggested. In the stochastic (or supportive) model, random cell-intrinsic events result in commitment to a certain lineage and subsequent expression of lineage-specific cytokine receptors. Thus, cytokines act on already committed cells to support survival, proliferation and complete maturation. In contrast, the instructive model proposes that cytokines direct uncommitted cells to differentiate to a specific lineage by initiating transcriptional changes.²⁹⁻³¹

Hematopoietic transcription factors

Transcription factors of multiple lineages are co-expressed in HSC and MPP at the single cell level, thus maintaining differentiation flexibility.³²⁻³³ This lineage priming is restricted in a hierarchal fashion during progenitor differentiation, suggesting that lineage commitment is associated with the up-regulation of genes related to the selected lineage and concomitant suppression of unrelated genes.^{25,34} Transcription factors interact to exhibit lineage- and stage-restricted expression patterns and several transcription factors have been attributed key roles in myeloid development.³⁵ (Figure 2).

In the initial establishment of hematopoiesis, stem cell factor leukemia (SCL/Tal1), LMO2, RUNX1 (also known as AML-1) and GATA-2 play essential roles.³⁶⁻³⁸ PU.1 is required to direct HSCs to the CMP stage and plays an important part in further granulocytic/monocytic differentiation, whereas the interaction of PU.1 with GATA-1 inhibits megakaryocytic/erythroid development.^{36,37,39} CCAAT/enhancer binding protein alpha (C/EBP α) is required for the transition of CMPs to GMPs and interacts with PU.1, which directs further lineage commitment in a dose-dependent manner, with high expression levels favoring monocyte over neutrophil development.^{35,40} Increased PU.1 activity facilitates interactions with interferon regulatory factor (IRF-8) and Egr-1, thus stimulating monopoiesis. C/EBP α and PU.1, expressed at low levels, are required for granulopoiesis, with retinoic acid receptor alpha (RAR α) and C/EBP ϵ being important in terminal neutrophil maturation.³⁹

Early erythroid development involves GATA-2 and lineage commitment is associated with the expression of GATA-1, which in co-operation with FOG-1, SCL/Tal1 and LMO2 plays a major role in further erythroid and megakaryocytic development.

KLF1 (also known as erythroid Krüppel-like factor, EKLF) and nuclear factor erythroid-derived 2 (NF-E2) are other important transcription factors. The former is erythroid-specific while the latter plays a part in late erythropoiesis and, foremost, is crucial to terminal megakaryocytic differentiation.^{38,41-45}

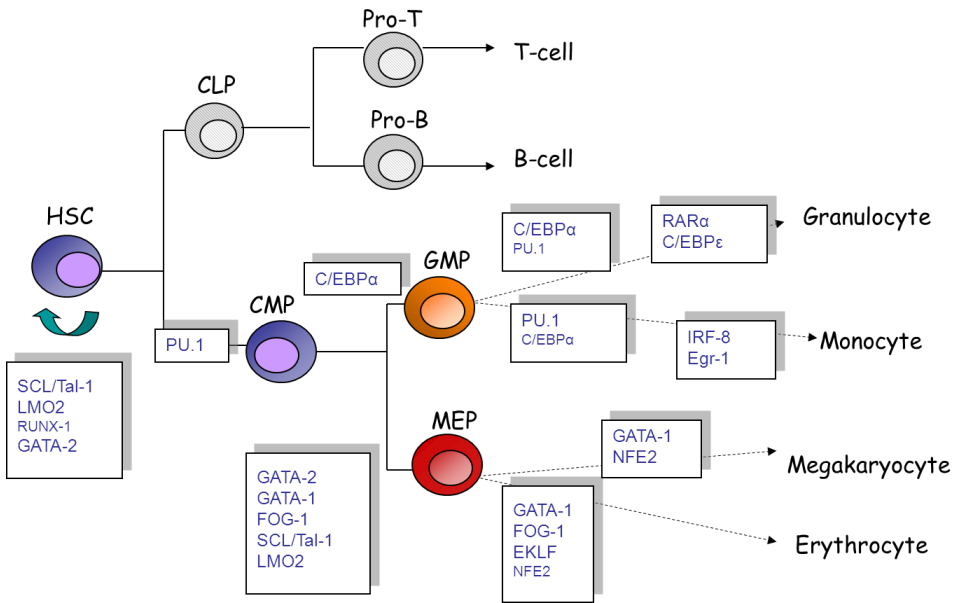


Figure 2. Transcriptional regulation of myeloid progenitors and their progeny. Selected transcription factors with important roles in early myeloid development are depicted at their suggested site of action. Low-level expression is indicated in a smaller font size.

Hematopoietic cytokines and receptors

Hematopoiesis is controlled by a number of cytokines which can be presented to target cells in soluble form, either generated in a different organ or in the local environment, or as membrane-bound factors. Each cytokine has multiple functions mediated by specific trans-membrane receptors whose cytoplasmic domains contain specialized regions initiating the various responses – survival, proliferation, differentiation and mature functionality. Cytokines exhibit synergistic effects and also overlapping effects on specific cells.^{46,47}

Cells at different developmental stages and lineages respond to different cytokines. Stem cell factor (SCF)/c-kit, FL/flt3, Thrombopoietin (Tpo) and IL-6 are important in early hematopoiesis, acting on HSC and MPP. IL-3 and granulocyte/macrophage-colony stimulating factor (GM-CSF) both act on oligo- and unipotent progenitors of the myeloid lineage, whereas granulocyte (G)-CSF, macrophage (M)-CSF, Erythropoietin (Epo), and Tpo are associated with the progenitors and more mature cells of specific lineages.^{36,46-48} The response of a cell to particular cytokines is dependent on the expression of specific receptors, which is regulated by the transcription factors associated with a certain developmental stage. Thus, PU.1 and C/EBP α are involved in the regulation of the GM-CSF, M-CSF and G-CSF receptors^{36,39} and GATA binding sites are found in regulatory elements of the Tpo receptor.⁴⁹

Microenvironment – niche

HSC depend on their microenvironment for regulation of quiescence, self-renewal and differentiation. The bone marrow niche comprise the physical association between HSC and the specialized cells that support them, as well as the signaling microenvironment of cytokines, adhesion molecules and their corresponding receptors created by HSC and niche cells. Several studies have defined endosteal and vascular compartments intertwined within the bone marrow HSC niche,⁵⁰ as well as different cell populations residing in these compartments, including osteoblasts, mesenchymal stem cells, reticular stromal cells, endothelial cells, nerve cells, macrophages and megakaryocytes.^{3,51} Recent reports have also suggested that hypoxic conditions in the bone marrow niche contributes to HSC maintenance and protection from oxidative stress.⁵² Although the complex signaling network between HSC and their niche cells is not fully elucidated, a number of cytokines critical to balancing HSC quiescence, self-renewal, and differentiation (CXCL-12, angiopoietin-1, Tpo, SCF, FL/flt3, IL-3, G-CSF, GM-CSF) are known to be provided by the niche constituents.^{3,4,51} Also, key developmental factors that are suggested promoters of HSC self-renewal are expressed in the HSC niche, such as Wnt, Hedgehog and Notch components.^{3,4}

The niche supporting erythroid differentiation, the erythroblastic island, has been well-characterized and comprises maturing erythroblasts surrounding a central macrophage. A number of adhesion molecules contribute to island integrity by mediating both erythroblast/macrophage and erythroblast/erythroblast interactions, as well as attachment to extra cellular laminin and fibronectin. Erythroid adhesion molecules $\alpha 4\beta 1$ integrin and ICAM-4 have been demonstrated to bind macrophage VCAM-1 and αV integrin, respectively.^{53,54} Erythropoiesis is responsive to hypoxia which induces an increase in synthesis of Epo in the kidney. Epo acts via the Epo receptor mainly on late progenitors (CFU-E) and pronormoblasts to stimulate

proliferation and maturation. Severe stress response (e.g. bleeding) involves cooperation with SCF, to expand the early progenitor compartment and increase the influx of cells into the Epo-responsive stages.⁵⁵ Interactions between the cellular constituents within the erythroblastic island and with extra cellular matrix components, as well as cytokine secretion by central macrophages co-operate with Epo-signaling to support survival, proliferation and terminal differentiation of erythroid cells.⁵⁴

Hematopoietic dys-regulation - MDS

Myelodysplastic syndromes (MDS) are a heterogeneous group of clonal HSC disorders characterized by dysplastic bone marrow with cytopenia, generally involving single or multiple myeloid lineages, and frequent progression to acute myeloid leukemia (AML). Allogeneic hematopoietic stem cell transplantation (HSCT) is the only option for curative treatment in MDS. Given the rare occurrence of lymphoid lineage engagement, a myeloid progenitor rather than a multipotent HSC was previously suggested as the cell of origin in MDS.⁵⁶ However, studies addressing the potential involvement of a myeloid-lymphoid HSC have challenged this concept. In cytogenetic analysis of hematopoietic progenitor cells from patients with the most common subtype of MDS, del(5q), virtually all CD34⁺CD38⁻ HSC and in some patients a majority of the CD34⁺CD19⁺ pro B-cells were shown to harbor the 5q-deletion. The involvement of multipotent HSC deficient in their ability to differentiate towards lymphocytes was suggested.⁵⁷ The resemblance between normal HSC and 5q- deleted MDS stem cells was further substantiated in comparative gene expression profiling.⁵⁸

MDS patients may display chromosomal abnormalities present as sole entities or as complex anomalies. The most frequent cytogenetic alterations include deletion of the long arm of chromosome 5 [del(5q)] or chromosome 20 [del(20q)], loss of chromosome 7 (monosomy 7) or gain of chromosome 8 (trisomy 8).⁵⁹ Analysis of CD34⁺ cells from MDS patients, have revealed altered gene expression profiles and dys-regulation of related signaling pathways relevant for HSC self-renewal, such as thrombopoietin signaling and Wnt pathways. Subgroups of advanced MDS are characterized by dys-regulation of DNA damage response pathways, similar to what has been observed in AML.⁶⁰ Ribosomal protein deficiencies, which are often responsible for the inefficient erythropoiesis of some inherited bone marrow failure syndromes, e.g. in Diamond-Blackfan anemia, have also been identified in del(5q) MDS.⁶¹ As part of the normal ribosome biogenesis, ribosomal proteins associate with immature ribosomal RNA (rRNA) to facilitate its processing, nuclear export and maturation, which is central to the regulation of overall protein synthesis.

BLOOD GROUPS

Nomenclature

Blood group antigens are per definition expressed on the surface of red blood cells (RBC) and are capable of inducing the production of human alloantibodies, upon presentation to a foreign immune system. In blood group nomenclature, antigens encoded by a single gene or a cluster of closely linked homologous genes are assigned to the same blood group system. Currently, 33 blood group systems, including 297 antigens have been identified but a total 339 antigens have been acknowledged to date by the International Society of Blood Transfusion Working Party on Red Cell Immunogenetics and Blood Group Terminology (www.isbtweb.org/working-parties/red-cell-immunogenetics-and-blood-group-terminology). The antigens which do not fit the criteria required for blood group system status are categorized in collections, when serologically, biochemically and genetically related, or, otherwise, in series of low prevalence (700 series) or high prevalence (901 series) antigens.

Blood group polymorphism

The inherited differences in RBC surface structures from one person to another are recognized as blood group antigens by human antibodies, “naturally“ occurring as a response to blood-group-active microbes in the normal intestinal flora⁶² or produced as a result of alloimmunization by foreign red cells, introduced by transfusion, transplantation or pregnancy.⁶³ Correspondingly, blood group antibodies may cause hemolytic transfusion reactions (HTR), transplant rejection and hemolytic disease of the fetus or new-born (HDFN).

The allelic variants responsible for the blood group antigens within each system are most often defined by one or more single nucleotide polymorphisms (SNP), encoding amino acid substitutions in the translated gene product. SNP that result in single amino acid changes of a protein are known as missense mutations, and the variant blood group antigens that arise from a SNP at a defined position are referred to as antithetical. Nonense SNP are those that introduce a premature stop codon in the

coding sequence and often give rise to a non-functional gene product, such as for the common *O* alleles of the *ABO* gene. SNP that occur in introns or regulatory regions can also alter blood group expression. Other molecular mechanisms that generate blood group diversity include gene deletion, which accounts for the common RhD negative phenotype; gene duplication and hybrid allele formation.^{64,65}

Structure and function

Blood group antigens are determinants of protein or carbohydrate structures exposed on the extracellular surface of the RBC membrane. Protein-based blood group antigens represent the primary products of blood group genes. In the case of carbohydrate-based antigens, their corresponding genes encode glycosyltransferases, which in turn catalyze the addition of antigen-specific sugars onto precursor oligosaccharides carried by glycoproteins or glycolipids on the cell surface. Many blood group systems are not restricted to the surface of RBC but are found on various cells and tissues and are, thus, denoted histo-blood groups.

Various known or putative functions have been assigned to the blood group active RBC surface proteins, such as membrane transporters, enzymes, complement regulators, adhesion molecules and cell structural components.⁶⁶ Blood group expressing carbohydrates are important contributors to the red cell glycocalyx, the sugar matrix that provides protection against mechanical damage and pathogen invasion. Blood group active molecules may also serve important functions in cells and tissues other than the RBC.

Although the blood group antigen polymorphisms generally do not affect carrier-molecule function, the consequences of completely lacking a blood group molecule (null phenotype) may range from none or mild, to severe RBC dysfunction or multi organ pathology in rare cases.^{66,67} Notably, the null phenotype corresponding to one of the most abundant RBC protein, the anion exchange protein band 3, is associated with severe hereditary spherocytosis and growth retardation, reflecting its essential role in maintaining cellular integrity and respiratory function of the mature RBC.^{68,69} For less abundant blood group molecules, the benign nature of the corresponding null phenotypes suggests overlapping functionality between molecules in the RBC membrane, or that the proteins lacking are not of importance to normal RBC function. Maturing human erythroid progenitor cells have also been found to lack components of the quality control processes for normal glycoprotein biosynthesis found in other cell types.⁷⁰ In later stages of erythropoiesis, a loss or decrease of selected endoplasmatic reticulum (ER) chaperone proteins associated with glycoprotein folding and quality control was observed, and suggested to facilitate the rapid synthesis of large quantities of essential membrane proteins like band 3 and

glycophorin A (GPA), but also to allow non-essential proteins, otherwise retained in the ER, to reach the RBC surface.

Blood group expression in erythropoiesis

Different research groups have mapped the appearance of many cell surface markers in erythropoiesis, using *in vitro* hematopoietic cell liquid culture systems supporting the proliferation and maturation of human erythroid progenitors.⁷¹⁻⁷⁴ Using different stem cell sources, i.e. bone marrow, cord blood or peripheral blood, CD34+ selected or not, as well as slightly varying culturing techniques, principally similar results are presented by the different investigators, concerning the time course expression of blood group antigens. The temporal order of appearance suggests complex interaction or possible roles for some of these molecules during erythroid differentiation.

The major blood group antigen-carrying proteins on the RBC surface ($> 200 \times 10^5$ copies per RBC) function to facilitate RBC transport of O_2 and CO_2 in the blood and to maintain the shape and deformability of RBC, by linking the membrane to the cytoskeleton. The sequential appearance of the Rh-associated glycoprotein (RhAG), GPA, band 3, the Rh polypeptides (RhD and RhCcEe) and glycophorin B (GPB) on the surface of terminally differentiating erythroid precursors *in vitro*, suggests that the individual proteins interact to facilitate trafficking through the ER and Golgi, and subsequent assembly of the band 3 macro-complex in the RBC membrane.⁷⁵ The additional linkage of the RBC membrane to the cytoskeleton by glycophorin C (GPC)⁶⁷ motivates its early expression on the cell surface.

The developmental appearance of some blood group active glycoproteins belonging to the Ig superfamily of adhesion molecules coincides with important hallmarks of erythropoiesis. The LW glycoprotein (ICAM-4) is detected *in vitro* at the late progenitor to proerythroblast stages. Given its function as a ligand for $\alpha 4\beta_1$ and αV integrin binding, LW has been demonstrated to play a role in the formation of erythroblastic islands between bone marrow macrophages and cells differentiating through the proerythroblast to immature reticulocyte stages.^{53,76} The Lutheran (Lu) glycoproteins which appears at the orthochromatic erythroblast stage are receptors for the $\alpha 5$ chain of laminin, found in basement membranes of bone marrow sinusoidal endothelium.^{77,78} A postulated role has been appointed to the Lu glycoproteins in facilitating migration of maturing erythrocytes across the sinusoidal endothelium, to the peripheral blood.

The inefficient erythropoiesis associated with some clinical conditions involving blood group antibodies may be explained by the temporal expression of the corresponding antigens. Blood group A antigens are found early during *in vitro*

erythroid maturation and are thus potential targets for immune mediated impairment of erythroid development.^{79,80} Notably, when HSC are transplanted from a blood group A donor to a blood group O recipient, a delayed recovery of donor derived erythropoiesis may be associated with the persistence of high titer anti-A recipient antibodies and the absence of erythroid precursors in the bone marrow; pure red cell aplasia (PRCA).⁸¹ The appearance of Kell glycoproteins already at the erythroid progenitor level is consistent with the clinical features of fetal anemia related to maternal Kell alloimmunization. A severe anemia with inappropriately low levels of bilirubin in amniotic fluid suggests a suppression of fetal erythropoiesis, through an inhibitory effect of anti-Kell antibodies on erythroid progenitor growth,⁸² or due to the immune destruction of sensitized progenitors by macrophages in the fetal liver.⁸³

Carbohydrate blood groups

Several of the ABO, H, LE, I, P1PK, GLOB and FORS carbohydrate blood group system antigens and their corresponding antibodies are clinically important since they are known to be involved in conditions such as HTR, hyperacute transplant rejection HDFN, and/or recurrent spontaneous abortion. Furthermore, carbohydrate antigens also act as receptors for pathogens and related toxins and, thus, may have an influence on susceptibility to and protection against infectious diseases.^{84,85}

Glycosylation

Glycosylation is a very common modification of proteins and lipids, generating branched oligosaccharide structures (glycans) of great diversity given the in numerous possible combinations of different sugars, glycosidase linkage (α or β) and modifying molecules (phosphate, acetat, sulfat etc.). In humans more than 200 glycosyltransferases (GT) are known. They are located in the Golgi compartment, where they catalyze the sequential addition of one sugar to another on a glycan acceptor, generally linked to protein or lipid. The specificity of each GT is defined by its donor and acceptor sugar, and also by the glycosidic linkage formed. The transfer of each sugar generates a specific acceptor for the next GT, which must act at a particular stage in the glycosylation pathway. The sugar transferred by each GT emanates from a high energy nucleotide donor sugar, synthesized in the cytoplasm and imported into the Golgi via membrane transporters. The Golgi GT are type II membrane proteins with a short amino-terminal cytoplasmic tail, a trans-membrane domain, a stem region and a globular catalytic domain in the Golgi lumen.^{86,87} Some glycosyltransferases can be solubilized through protease-mediated cleavage at the stem region and are present in plasma, possibly to allow them to act at the cell surface or in

the extracellular compartment. Recently, nucleotide sugar transporters of the Golgi were found to facilitate nucleotide sugar release from the cell.⁸⁸

Blood group active glycoconjugates

The basic core structures of blood group active glycoconjugates – glycoproteins and glycosphingolipids - are formed in the ER. Proteins are glycosylated either at asparagine sites by the linkage of *N*-acetylglucosamine (N-linked glycan), or at serine or threonine sites by attachment of *N*-acetylgalactosamine (O-linked glycan). Lipids are glycosylated by linkage of glucose to ceramide, and further classified as (neo)lacto-, globo- and ganglio-series. The subsequent synthesis of glycans is accomplished in the Golgi. Common to many of the glycoproteins and lacto-series glycolipids is an inner core based on a poly *N*-acetylglucosamine structure, i.e. they are extended by repeating Gal β 1 \rightarrow 4GlcNAc β 1 disaccharides, also known as type 2 chains. The extension of glycans is halted by terminal glycosylation of variant oligosaccharides, known as peripheral core structures. Among these, type 1-4 chains commonly serve as precursors for some blood group related glycosyltransferases.⁸⁹

The type 1-4 precursor chains, which are all used as acceptors for the enzymatic synthesis of ABH antigens, differ in glycosidic linkage, in nature of the glycoconjugate (glycolipid or glycoprotein, N-linked or O-linked) and in tissue distribution.⁹⁰ (Table 1). The principal histological distribution of type 1-4 based ABH antigens reflects the embryologic origin - ectoderm, mesoderm or endoderm - as well as the differentiation pattern of tissues and cells, and is determined by the ability of the respective enzymes to use a particular precursor type as acceptor substrate for the ABH-defining sugars, as further discussed below.^{91,92}

Antigens of different blood group systems may be concomitantly expressed on the same glycans or alternatively expressed on the same peripheral precursor. Blood group I/i antigens are inner core constituents of glycoproteins and lacto-series glycolipids that may co-express peripheral ABH and Lewis antigens. In normal development i-active linear repeats of *N*-acetylglucosamine, which are prevalent on fetal and differentiating cells adult cells are converted to I antigens by enzymatic branching of i-active inner core glycans.⁹⁰ Paragloboside, a neolacto-series glycolipid, may serve as precursor for the either the expression of P1 or type 2 ABH antigens.⁹³ Also glycolipids of the globoseries which harbor the inner core P^k and P (globoside) antigens may be extended to express type 4 H and, in the presence of an *A* or *B* allele, subsequent type 4 (globo) A or B antigen.⁹⁴ An exception to the concept of A and B antigens representing terminal glycosylation is the elongation of A type 2 antigen to A type 3 (repetitive).⁹⁵

Table 1 Peripheral core chains and their tissue distribution

Peripheral core type	Structure	Distribution
Type 1	Gal β 1 \rightarrow 3 GlcNAc β 1 \rightarrow R	Endodermal tissue, secretions, plasma
Type 2	Gal β 1 \rightarrow 4 GlcNAc β 1 \rightarrow R	Ectodermal and mesodermal tissue, e.g RBC
Type 3	Gal β 1 \rightarrow 3 GalNAc α 1 \rightarrow R	Varios tissues (O-linked mucin-type) Repetitive-H/A on RBC
Type 4	Gal β 1 \rightarrow 3 GalNAc β 1 \rightarrow R	Glycolipids in kidneys and on RBC

THE ABO SYSTEM

In clinical transfusion medicine and transplantation, ABO is considered the most important blood group system, originally recognized by Landsteiner in 1900.⁹⁶ The naturally occurring antibodies to lacking ABO antigens may cause immediate and fatal HTR and organ transplant rejection. Expression of ABO blood group antigens is subjected to change during differentiation and maturation of normal hematopoietic cells. In addition, changes of ABO antigen expression have been documented in such differing conditions as pregnancy⁹⁷ and malignancy.⁹⁸ Since the cloning in 1990 of complementary DNA corresponding to messenger RNA (mRNA) transcribed at the *ABO* locus,⁹⁹⁻¹⁰¹ polymorphisms and phenotype-genotype correlation have been reported by several investigators.¹⁰² To date, more than 200 different *ABO* alleles have been described, many of which result in weak A/B antigen expression, presenting problems in routine blood grouping.¹⁰²

ABO polymorphism

Four different antigens; A, B, A₁ and A₂B, each defined by the corresponding antibody specificities, have been assigned to the ABO blood group system (ISBT 001). The A and B antigens are inherited in a co-dominant fashion, i.e. the phenotype expressed on the RBC surface will correspond to the alleles present at the *ABO* locus.⁸⁴ Variations in A antigen expression has led to the subdivision of the A blood group into the major subgroups A₁ and the weaker A₂, and the identification of four major alleles: *A*¹, *A*², *B* and *O*. The common ABO phenotypes; A₁, A₂, B, A₁B, A₂B, and O are defined by the antigens found on the RBC and the presence of the anticipated antibodies in serum. (Table 2).

The gene encoding the blood group A and B glycosyltransferases is located on the long arm of chromosome 9q34 and consists of seven exons.^{103,104} The common *A* sequence; the *A*¹ allele, is referred to as the consensus sequence, and is used as a reference for all other *ABO* alleles. The weaker A₂ phenotype is the result of an A transferase with lower activity and (most likely) altered acceptor preference. It is extended with 21 amino acids, owing to a nucleotide deletion in the *A*² allele which disrupts the stop codon and elongates the open reading frame.¹⁰⁵ *A* and *B* alleles are

distinguished by 7 nucleotides in the coding sequence, which result in 4 amino acid differences between the A and B enzymes. The common *O*¹ allele and the variant *O*^{1'} differ from the *A*¹ sequence by a single nucleotide deletion at position 261, introducing a premature stop codon and, thus, a non-functional enzyme.¹⁰¹ The latter of the two contains an additional number of nucleotide substitutions in comparison to the consensus sequence.¹⁰⁶ Another null allele of significant frequency, *O*² lacks the 261 nucleotide deletion, but has a critical SNP at position 802, resulting in an amino acid substitution which presumably abolishes the enzymatic activity of the A₁ transferase.^{107,108}

Table 2. Phenotypes and antibodies of the ABO system

Phenotype	ABO antigens on RBC	ABO antibodies in serum
A ₁	A	Anti-B
A ₂	A	Anti-B, Anti-A1*
B	B	Anti-A
A ₁ B	A, B	-
A ₂ B	A, B	Anti-A1*
O	H	Anti-A , Anti-B, Anti-A,B

* Not always present

ABO biosynthesis

The blood group A and B determinants constitute the terminal trisaccharides of variant carrier glycans, presented as part of glycoproteins or glycolipids on RBC as well as in tissues and body fluids. The *A* and *B* allelic variants of the *ABO* gene encode two different glycosyltransferases which catalyse the transfer of either *N*-acetyl-D-galactosamine (GalNAc) or D-galactose (Gal) from the uridine diphosphate (UDP) donor to the precursor structure, H antigen, forming A or B antigens, respectively. The distinction between the A and B blood group is, thus, dependent only on the nature of the immuno-dominant terminal monosaccharide. The gene product of the *O* allele is non-functional, leaving the immunodominant L-fucose (Fuc) of the H determinant unaltered. The precursor H structure is in turn synthesized by either of two 2- α -L-fucosyltransferases, encoded by the *H* gene (*FUT1*)

or the closely linked secretor gene (*FUT2*), depending on tissue localization.¹⁰⁹(Figure 3).

In hematopoietic tissue, the *FUT1* gene encodes the enzyme that synthesizes H antigen preferentially on type 2 precursor chains. In epithelial cells, the *FUT2* gene governs the synthesis of H antigen, preferentially on type 1, 2 and 3 chains,^{90,110} and determines the presence of type 1H antigen, and thereby also type 1 A and B antigens, in secretion and plasma of approximately 80% of people, so-called secretor individuals.

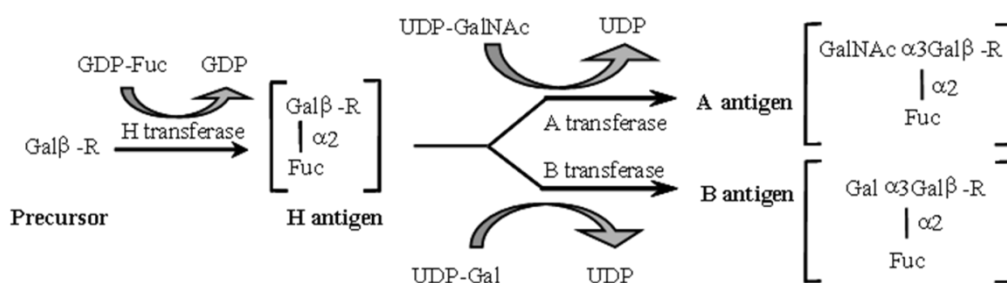


Figure 3. Schematic depiction of the biosynthesis pathways for conversion of H determinant to A or B determinant. Published with kind permission from Dr. Bahram Hosseini-Maaf.

ABO expression on mature red blood cells

ABH antigens found on RBC as glycoproteins (approximately 90%) and glycolipids (approximately 10%) are predominantly based on type 2 chains. In secretor individuals, also type 1 ABH antigens may be found on RBC due to the adsorption of glycolipids from plasma.¹¹¹⁻¹¹³ Moreover, significant quantities of A type 3 (repetitive A) and, to a lesser extent, A type 4 (globo-A) are found on RBC glycolipids, irrespectively of secretor status.¹¹⁴ Earlier reports on the relative inability of the A₂-enzyme to use type 3 and 4 precursor structures suggested a basis for the qualitative differences between the A₁ and A₂ RBC phenotypes.⁹⁵ Although in a recent study, type 3 A was found to be equally expressed on A₁ and A₂ RBC and the difference in A₁ versus A₂ phenotype was restricted to the absence of type 4 A glycolipids in the latter.¹¹⁵

ABO, Hh and Lewis systems

The presence of ABH antigens in plasma is governed by the complex interaction between the *ABO* gene, the secretor gene (*FUT2*) and the Lewis gene (*FUT3*), and is influenced by allelic variants at the three loci.^{110,116}

Lewis antigens are considered blood group antigens, although they are not synthesized by red cells but absorbed as glycolipids from plasma. The Lewis gene (*FUT3*) encodes a 4- α -L-fucosyltransferase that catalyzes the addition of an L-fucose residue to H type 1 antigen in secretions to produce Le^b antigen (difucosylated) or, when no H antigen is present (non-secretors), to the precursor of H type 1 to produce Le^a antigen (monofucosylated). Consequently, the acquired RBC phenotype of most secretors is Le(a-b+) and of most non-secretors Le (a+b-).

The enzymes encoded by the *ABO* and Lewis genes, compete for the common H type 1 acceptor substrate to produce A /B antigens or difucosylated Le^b antigen. Once converted to Le^b antigen, the type 1 precursor is no longer a substrate for the A- or B-glycosyltransferases (Figure 4). The highest levels of soluble A and B antigens in plasma are, thus, found in secretor individuals who lack the Lewis gene, Le(a-b-) phenotype. In the presence of a functioning Lewis gene, the enzyme produced by the *A*¹ allele of the ABO-locus is more efficient than its *A*² allelic variant, in competing with the Lewis enzyme for type 1 H acceptor substrate. Accordingly, in secretors of the Le(a-b+) phenotype levels of soluble A antigen are higher in A₁ than A₂ individuals,^{111,117,118} reflecting the quantitative difference in A₁ and A₂ glycosyltransferase activity at the site of A type 1 antigen synthesis.^{119,120} Moreover, A or B antigens of type 1 may serve as substrates for conversion by the Lewis gene fucosyltransferase, and indeed the compound ALe^b structure appears to be the predominant form of adsorbed A antigen found on RBC, as unraveled by the use of monoclonal antibodies specific for ALe^b (difucosylated) and A type 1 (monofucosylated), respectively.^{121,122}

Also in non-secretor plasma, ABH antigens are found, albeit in very small quantities, on type 2 chains linked to glycoproteins,¹²³⁻¹²⁵ and, predominantly to glycolipids, and are presumed to emanate from endothelial or hematopoietic cells.^{111,117,126,127}

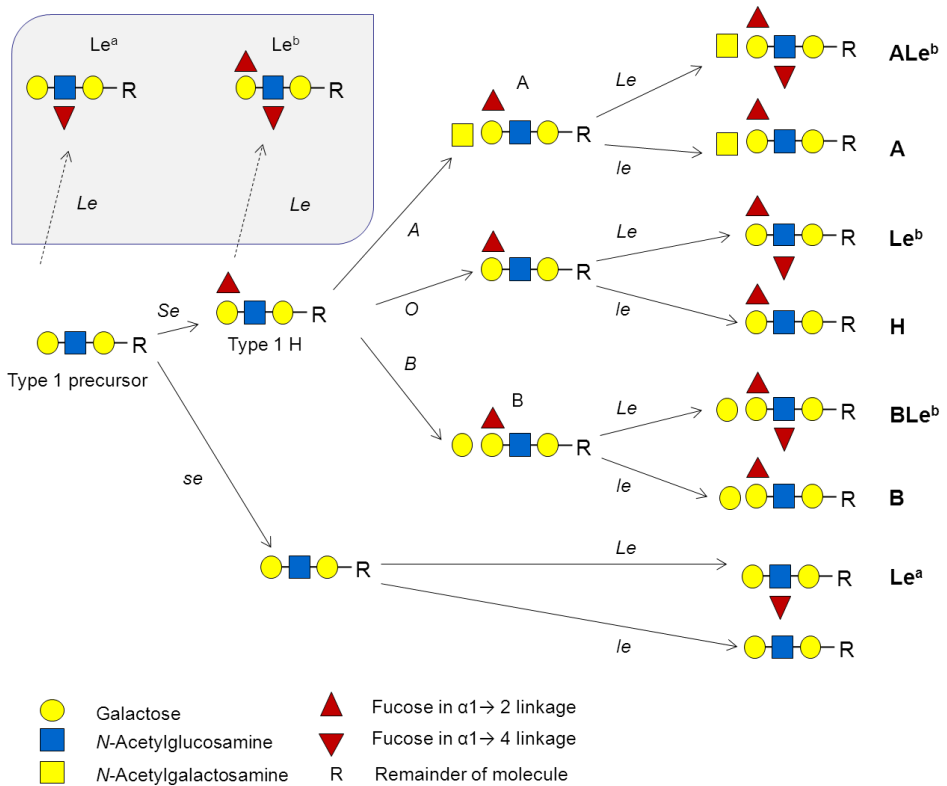


Figure 4. Schematic description of the biosynthetic pathways for ABH and Lewis antigens derived from type 1 chains. Dashed lines show how Le^a and Le^b , produced from the type 1 precursor and H structures, respectively, are not substrates for the Se or A/B glycosyltransferases. Abbreviations: Se, secretor gene present; se, secretor gene absent; Le, Lewis gene present; le, Lewis gene absent.

ABO expression in hematopoiesis

Earlier studies on the developmental expression of ABO blood group antigens in hematopoiesis present conflicting data. A antigen expression on the earliest committed erythroid and myeloid progenitors, BFU-E and CFU-GM in colony assays has been reported.^{128,129} Others have found blood group A activity on cells of erythroid series only, first detectable at the basophilic normoblast stage.^{74,130} Following major ABO-incompatible HSCT, persistent host ABO antibodies have been shown to inhibit selectively the growth of donor-derived later stage erythroid progenitors, CFU-E, *in vitro*, with no effect on BFU-E and CFU-GM.⁸⁰

The expression pattern of ABH antigens on maturing RBC is coupled to the increased branching of carrier glycans during erythroid differentiation, i.e. the conversion of i to I antigen.¹³¹ A possible role for transcription factor C/EBP α in regulation of RBC glycan branching was recently suggested.¹³² In clinical conditions associated with stress hematopoiesis, such as thalassemia, sickle cell disease and leukemia, RBC with immature glycosylation are found in circulation.¹³³ As demonstrated in paper II, the *ABO* gene is transcribed early during *in vitro* erythroid differentiation but rapidly down-regulated in later stages of erythroid maturation. These findings were later substantiated, demonstrating that *A*¹ and *A*² alleles are expressed at a higher level than *B* or *O* alleles in early erythropoiesis. In contrast, *A*¹ and *A*² transcripts could not be isolated from peripheral blood samples whereas *B* or *O* transcripts were readily detected.¹³⁴ The regulatory mechanism of the *ABO* gene in hematopoietic cells is not clear. Elements of suggested importance for hematopoietic transcription have been identified, including a binding site for the transcription factor Sp1 in the *ABO* promoter,¹³⁵ and an enhancer region located upstream of exon 1 containing a CBF/NF-Y binding site.¹³⁶ In patients with myeloid malignancy, a decrease in RBC expression of A or B antigens is a well-documented phenomenon.¹³⁷ This is generally associated with a severe reduction in RBC A- or B- transferase activity, indicating a deficiency affecting the *ABO* locus rather than an alteration at the cell surface or of precursor structures.^{138,139} Weakened A or B antigen expression may precede diagnosis of malignancy, and has also been shown to correlate with the course of the disease; i.e. return to normal upon remission.^{138,139}

In mature hematopoietic cells, expression of endogenously synthesized type 2 ABH antigens is restricted to RBC and platelets. Like RBC, platelets and also lymphocytes can adsorb type 1 ABH antigens from secretor plasma.^{140,141} Granulocytes and monocytes, however, do not express ABH antigens.^{142,143}

HEMATOPOIETIC STEM CELL TRANSPLANTATION

In 1959, Donald Thomas performed the first human bone marrow transplant, providing a proof of concept that infusing bone marrow cells could provide hematological reconstitution in lethally irradiated patients with acute leukemia.¹⁴⁴ Today, HSCT is a well-established therapy for hematological malignancies, selected solid tumors and other non-malignant disorders, using stem cells from bone marrow, peripheral blood or cord blood from patients, family donors or unrelated voluntary donors.¹⁴⁵

Autologous HSCT, using the patient's own cells, is employed as a supportive strategy to restore hematopoietic function, following administration of hematotoxic high dose radio/chemotherapy for malignant diseases such as multiple myeloma and relapsed aggressive lymphomas.¹⁴⁶ Allogeneic HSCT, using cells from a healthy donor, combines hematopoietic regeneration with a potent immunological anti-malignancy effect. The transplanted stem cells build up not only a new hematopoietic system but also a new immune system that is capable of mediating a transplant-versus-malignancy effect (graft-versus-tumor, GvT, or graft-versus-leukemia, GvL).¹⁴⁷ Standard indications for allogeneic HSCT are therefore mainly high-risk and relapsed acute leukemia.¹⁴⁶ However, this reaction can also develop into a potentially lethal condition involving a response against non-malignant recipient cells; graft-versus-host disease, GvHD.¹⁴⁸

Stem cell source

Traditionally, bone marrow cells were harvested under general anesthesia from the pelvic posterior iliac crest and used for transplantation. Currently, mobilized peripheral blood progenitor cells (PBPC) are used for the majority of HSCT procedures.¹⁴⁵ PBPC can be induced to exit the bone marrow by the administration of hematopoietic growth factors, such as G-CSF,^{149,150} and mobilized into the peripheral blood in sufficient numbers for collection by standard apheresis technology.¹⁵¹ In autologous donors, chemotherapy is often administered as part of the mobilization

treatment. Surface expression of CD34 includes the majority of cells with hematopoietic reconstitution potential and is, thus, used as a marker for enumeration of collected PBPC. A minimum CD34⁺ cell dose of 2-5x 10⁶/kg recipient body weight is generally required for transplantation. Cord blood cells, collected and cryopreserved at birth, are used as an alternative source of stem cells in allogeneic HSCT.¹⁴⁵

Donor selection

In allogeneic HSCT, the occurrence of severe GvHD is related to the degree of donor-recipient human leukocyte antigen (HLA) disparity,^{152,153} which is why the match between donor and recipient HLA molecules is the most important determinant of patient outcome.

The primary role of HLA molecules in adaptive immunity is to present peptides to T-cells, enabling them to recognize and eliminate foreign particles but prevent recognition of “self” as foreign. The HLA system exhibits extensive polymorphism, supposedly driven by evolutionary pressure to keep pace with the diversity of pathogens, and the number of HLA Class I and II alleles named to date exceeds 8000 (www.ebi.ac.uk/ipd/imgt/hla/stats.html). HLA molecules are expressed on the cell surface. Class I molecules, which are found on most nucleated cells and platelets, interact with cytotoxic T-cells (CD8+). Class II molecules are generally restricted to certain cells of the immune system, e.g. B-cells and dendritic cells, where they present peptides to helper T-cells (CD4+).¹⁵⁴

An HLA-identical sibling donor, as determined by tissue typing, is considered first choice in allogeneic HSCT. On the short arm of chromosome 6, HLA Class I genes (*HLA-A*, *-B* and *-C*) and Class II genes (*HLA-DR*, *-DQ* and *-DP*) are closely linked and therefore generally inherited together as a cluster; a haplotype.¹⁵⁵ A child will inherit one HLA-haplotype (i.e. six *HLA* alleles) from each parent and the degree of HLA-match between a parent and a child is, thus, haploidentical (Figure 5). The inheritance of parental HLA-haplotypes within a family, can give rise to four possible HLA-types among siblings. Consequently, the probability of two siblings being HLA-identical is 25%.

When a suitable sibling donor is not available, the search for a HLA-matched unrelated registry donor is generally initiated. Currently, 20 million potentially suitable adult donors are registered in Bone Marrow Donor Worldwide (www.bmdw). High resolution DNA-based typing methods are employed to resolve the patient and donor tissue types to allele level, and provide the basis for registry donor selection.¹⁵⁶ A donor who is matched, at high resolution, for the *HLA-A*, *-B*, -

C, *-DRB1* and *DQB1* loci, i.e. a 10/10 *HLA*-allele match, is generally considered as the gold standard.¹⁵⁷ The probability of finding a suitably matched unrelated donor is 70-85% for Caucasian patients but only 10% for ethnic minorities.¹⁵⁸

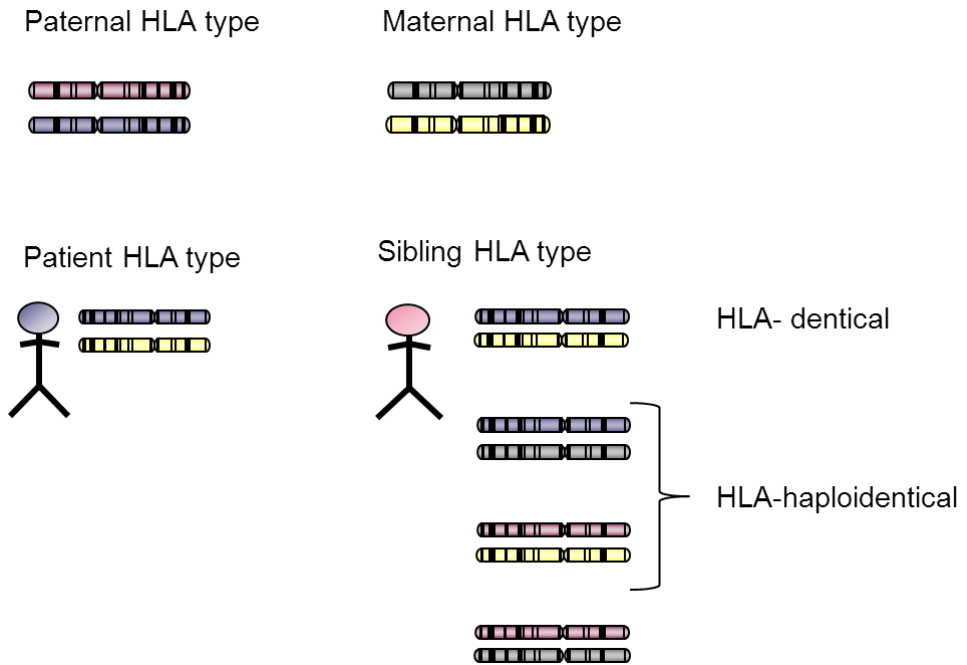


Figure 5. Inheritance of HLA haplotypes within a family.

The patient has inherited one HLA haplotype (located on chromosome 6) from his father (blue) and one from his mother (yellow) and is thus HLA-haploidentical with each of his parents. The inheritance of parental HLA haplotypes within a family can give rise to four possible HLA types among siblings. Consequently, the probability of HLA-identity between the patient and a sibling is 25 % whereas the probability of HLA-haploidentity is 50%.

HLA-haploidentical HSCT

For patients who do not have a suitable sibling or unrelated donor, or when the need for an HSCT is urgent due to rapid progression of the patient's disease, there are two alternative stem cell sources: a HLA-haploidentical family donor^{159,160} or cryopreserved unrelated donor cord blood from a cord blood bank.¹⁶¹ Common to these alternative stem cell donor sources is an immediate availability but also the problem of delayed immune reconstitution, leading to a high rate of infectious complications following transplantation. The choice of cord blood *versus* haploidentical family donor is currently under debate,^{162,163} however, the reason for selecting one or the other approach is often dependent on the expertise and resources of the transplantation center. With reference to the scope of this thesis, cord blood HSCT will not be further discussed.

Cell procurement and graft processing

The haploidentical donor alternative may imply a selection of candidate family donors readily available for sequential collections of mobilized PBPC, to provide the "mega dose" of stem cells required for transplantation across the HLA barrier.¹⁶⁴

Given the high frequency of T cells that recognize major Class I and II HLA donor-recipient disparities in haploidentical HSCT, measures are required to prevent the occurrence of alloreactive responses, i.e. graft rejection (host- versus-graft response) or severe GvHD. Combining high numbers of PBPC with an extensive depletion of donor T-cells from the transplant (graft), has proven a successful strategy to prevent graft rejection as well as GvHD after haploidentical HSCT in children^{160,165,166} and adult patients.^{167,168}

Utilizing large scale, magnetically activated cell separation (MACS) technology, T-cells can be efficiently depleted from PBSC products, in accordance with Good Manufacturing Practice standards. Positive selection of CD34⁺ cells has been the standard method for obtaining efficiently T-cell-depleted grafts containing high doses of purified CD34⁺ cells ($\geq 10 \times 10^6/\text{kg}$ recipient weight).^{169,170} The positive selection technique however, excludes a variety of immune-competent cells that may play a role in improving the outcome of HSCT. The extensive depletion of all lymphoid cells results in a considerably delayed immune recovery leading to a high rate of severe infectious complications.^{160,168,171} Moreover, increasing knowledge on the role of alloreactive natural killer (NK) cells in exerting anti-leukemic¹⁷² and engraftment-facilitating effects,¹⁷³ suggests the advantage of a direct T-cell depletion strategy, preserving potentially important cells in the graft.

In recent years, the approach of direct depletion of CD3⁺ T-cells from PBPC has been adopted in haploidentical graft engineering.¹⁷⁴⁻¹⁷⁶ In contrast to the positive selection method, large numbers of monocytes, NK cells, antigen presenting cells and myeloid cells are retained in the graft, contributing to a comparatively enhanced immune reconstitution after transplantation.¹⁷⁷⁻¹⁷⁹ The latest development in the effort to further increase T-cell depletion, while maintaining anti-infectious and anti-leukemic properties of the grafts was introduced just recently, providing a method for large-scale highly efficient depletion of GvHD-inducing alpha/beta (α/β) T-cells while retaining potentially beneficial gamma/delta (γ/δ) T-cells in the graft.^{180,181}

Post-transplant cellular therapy

Post-transplant monitoring and prevention of infections and disease recurrence are crucial.¹⁸²⁻¹⁴⁷ Donor lymphocyte infusions (DLI), which are frequently used as a “tool” to boost anti-viral immunity and to enhance the graft-versus-leukemia effect post-transplant, harbor a significant risk of inducing severe GvHD in the haploidentical HSCT setting.

Clinical grade, MACS technology protocols for the selection or depletion of specific donor-derived lymphocyte subsets, have been applied in the recent development of strategies aimed to accelerate immune recovery, and thereby prevent severe infection and disease recurrence. Virus-specific donor T cells have been isolated and used in the treatment of infections with cytomegalovirus,^{183,184} adenovirus¹⁸⁵ and Epstein-Barr virus.¹⁸⁶ Infusion of donor lymphocytes magnetically enriched for CD56⁺ NK cells¹⁸⁷ or depleted of cytotoxic CD8⁺ T cells,¹⁸⁸ have been shown to promote immune recovery following HSCT with limited incidence of GvHD.

ABO-incompatible HSCT

In the case of several well HLA-matched potential donors, the choice of an ABO-matched donor is recommended.¹⁸⁹ However, 40-50% of allogeneic HSCT are performed across the ABO blood group barrier. Generally, an ABO-incompatible donor is considered an acceptable alternative, provided that the risks of early and delayed complications are appropriately addressed by means of state-of-the art graft processing and adequate blood component support.^{190,191}

Cell procurement and graft processing

The immediate risk of an ABO-incompatible HSCT is the necessity of infusing incompatible RBC and/or plasma with the stem cell graft, potentially resulting in acute hemolysis.¹⁹² Although it is not possible to define a maximum volume of ABO-incompatible RBC that can be safely infused, a limit of 20 ml RBC in the graft is generally recommended.^{190,191,193} Usually, the RBC contents of a bone marrow harvest exceed a volume of 200 ml, which is why the graft should be depleted of ABO-incompatible RBC using either of the available techniques for; RBC sedimentation, enrichment of buffy coat cells (i.e. mononuclear cells (MNC)) and granulocytes, or purification of MNC .¹⁹⁴ Mobilized apheresis products (PBPC) typically contain less than 20 ml of packed RBC and are usually infused without prior RBC depletion. Reduction of ABO-incompatible plasma, by centrifugation and plasma extraction, is recommended if the donor has high-titer ABO antibodies.¹⁹²

Delayed complications

Delayed complications related to the RBC-incompatibility are well-recognized. In minor ABO-incompatible transplantation (e.g. a group O donor to a group A recipient) delayed hemolysis may occur 7-14 days post-transplant, mediated via “passenger” B-lymphocytes in the graft producing ABO antibodies directed against the recipient RBC.^{192,195} Clinical signs of hemolysis are often mild and the course of donor-derived ABO antibody titers is transient. In rare cases, however, donor-derived ABO antibodies may induce destruction also of transfused group O RBC, possibly mediated through the adsorption either of soluble A/B antigens, A/B antigen/antibody immune-complexes or complement components onto the RBC surface.¹⁹⁵⁻¹⁹⁷

In the setting of major ABO incompatibility (e.g. a group A donor to a group O recipient) delayed RBC recovery may occur, resulting from persistent recipient B-lymphocytes producing ABO antibodies directed against donor-derived erythroid precursors.^{193,198} In the presence of normal myeloid, lymphoid and megakaryocytic engraftment, PRCA is defined as reticulocytopenia (<1%), with absent erythroid precursors in the bone marrow.¹⁹⁰ High-titer anti-donor ABO-antibodies may persist for months, resulting in long-term transfusion requirements.

Blood group monitoring and transfusion support

Change from recipient to donor RBC ABO type following HSCT is generally determined by standard serological forward and reverse ABO typing, i.e. detection only of donor ABO-type RBC in the recipient’s sample and lack of ABO antibodies

directed against donor RBC.¹⁹⁹ Given the maximum time of RBC survival in circulation (120 days), conversion to donor blood group can usually be determined no sooner than 4 months post HSCT.

Current guidelines for transfusion support in ABO-incompatible HSCT, prescribe components which are compatible with both the recipient and the donor ABO types, to avoid hemolysis of the transfused RBC, the residual recipient RBC and the engrafting donor-derived RBC. In adherence with these standards, erythrocytes are selected for transfusion based on their RBC surface expression of ABH antigens, whereas plasma and platelets are primarily selected according to their contents of ABO antibodies. In the setting of an HSCT where blood group A donor cells are transplanted to a blood group O recipient (major ABO-incompatibility), or vice versa (minor ABO-incompatibility), the recipient is recommended to receive packed RBC units of blood group O, and plasma or platelet units of blood group A or AB for transfusion. From the time of conversion to donor RBC ABO group, the recipient is recommended to receive blood components of donor ABO type.^{189,200}

ABO expression following HSCT

Following a minor ABO-incompatible HSCT, with a group O donor to a group A recipient, the stably engrafted recipient is a complete chimera of donor group O, expressed on RBC and platelets,^{140,201} and recipient-group A antigens, ubiquitously distributed in tissues and body fluids (in secretors).^{92,124,202} In addition, donor-derived group O blood cells may exhibit low levels of recipient-group A antigens,²⁰³⁻²⁰⁵ expressed on glycolipids adsorbed from plasma.¹¹¹⁻¹¹³ Also, normal A glycosyltransferase activity may be found in the recipient's plasma, as soluble A/B-enzymes in sera originate predominantly from non-hematopoietic tissue.^{119,206} The recipient of a major ABO-incompatible HSCT (e.g. a group A donor to a group O recipient), will exhibit a corresponding chimeric ABO status of hematopoietic (donor) versus non-hematopoietic (recipient) tissues.

The possible role of red cell antigen disparity in eliciting GvHD responses has been debated.^{191,207} *In vitro* studies have shown that polymorphic peptides derived from A/B glycosyltransferases are allogeneic and may, when presented by HLA molecules, act as minor histocompatibility antigens (mHags) in generating specific T-cell responses.²⁰⁸ Also, in recipients with manifest GvHD following minor ABO-incompatible HSCT, inhibiting antibodies directed against recipient-derived A/B glycosyltransferases have been detected.^{209,210} However, no deleterious effects from the use ABO-incompatible grafts on transplant outcomes, in terms of graft rejection or incidence of acute or chronic GvHD have been consistently demonstrated.¹⁹¹ In minor ABO-incompatible HSCT, the transient course of donor-derived ABO

antibody titers and apparent escape of endothelial cells from GvHD, has been ascribed to antibody adsorption, induction of donor lymphocyte tolerance or endothelial cell accommodation.^{207,211}

In contrast, repeat transfusion of minor ABO-incompatible donor -type plasma and platelet components have been shown to cause immune-complex formation, multi-organ dysfunction and impaired survival.^{212,213} In the setting of a minor ABO-incompatible HSCT, with a group O donor to a group A recipient, the switch to donor-group transfusion support upon complete donor engraftment will, indeed, expose the recipient to antibodies directed against blood group A epitopes, expressed on vascular endothelium, carried by soluble structures in plasma and, possibly, present on circulating group O HSC-donor-derived blood cells.

Monitoring of post-HSCT chimerism

Monitoring of post HSCT chimerism is an important tool in assessment of donor hematopoietic engraftment and may provide a timely alert of impending relapse as indicated by persistent or increasing levels of 'mixed chimerism', i.e. an increase in the ratio of recipient- versus donor-derived hematopoietic cells.^{214,215}

In previous years, RBC phenotyping by standard agglutination technique was frequently used as a method for monitoring of post-HSCT chimerism.²¹⁶⁻²¹⁸ This method depends on the identification of recipient/donor-discriminative RBC blood group antigen markers, and requires adherence to a strict post-transplant policy of only transfusing RBC concentrates that are negative for the selected markers. The sensitivity limit of RBC agglutination assays is generally in the range of 1-5%, and is further obscured by residual host RBC circulating for 3-4 months following HSCT. Moreover, attempts to quantify the ratio of recipient- versus donor-derived cells are impaired by the presence of transfused RBC. Adopting more sensitive techniques, such as flow cytometry, or including reticulocytes in the analysis may improve sensitivity.²¹⁶ Notwithstanding, although several studies have demonstrated that reappearance of recipient RBC may herald relapse in hematological malignancies,²¹⁶⁻²¹⁸ RBC phenotyping may not be optimal for detecting a clonal expansion of progenitors not involving the erythroid lineage.²¹⁷ In ABO-/RhD-incompatible HSCT, however, pre-transfusion blood typing of the stably engrafted recipient is important, as detection of a RBC mixed chimerism implies selection of blood components compatible with both recipient and donor ABO/RhD-blood groups.¹⁹⁹

In the last decade, analysis of polymorphic DNA fragments, i.e. short tandem repeat-polymerase chain reaction (STR-PCR) coupled with fluorescence detection of recipient/donor alleles using capillary electrophoresis, has replaced RBC phenotyping

as the standard method for monitoring of chimerism.²¹⁹ A detection limit of 1 % recipient-derived cells in peripheral blood may be increased by investigating enriched leukocyte fractions, e.g. T-cells, B-cells or myeloid cells. Targeting the original phenotype of the leukemic clone may further enhance sensitivity.²²⁰ Notably though, persistent or increasing levels of mixed chimerism in the early post-transplant period has been demonstrated to predominantly represent an increase in normal recipient cells which, however, reliably predicted relapse.²¹⁴ Moreover, the time-course of mixed chimerism patterns following HSCT is influenced by the intensity of the pre-transplant treatment (myeloablative or reduced-intensity conditioning) and the composition of the graft (T-cell depleted or not).²²¹

CELL SEPARATION

Cell separation technologies are essential “tools” in the collection and processing of hematopoietic progenitor cells for clinical transplantation or research applications. Separation based on biophysical properties such as cell size and density is generally employed for primary extraction of leukocytes from whole blood or bone marrow, followed by selective affinity methods for isolation or depletion of specific cell populations.

The performance of cell separation techniques is characterized by target cell recovery and purity, but should also include low mechanical stress and minimal interference such that separated cells maintain viability and biological function. Large scale separation, such as in HSCT graft processing, additionally demands high capacity and throughput. Processing of cells for clinical applications must be carried out in closed systems or, otherwise, under controlled environmental conditions in conformity with Good Manufacturing Practice standards.²²²

Separation by size and density

The sedimentation behavior of blood cells is determined by their size as well as their density relative to the surrounding media. Fractionation of blood cells by centrifugal force will separate RBC (bottom layer) from plasma (top layer), while leukocytes and platelets (the buffy coat) are packed on top of the RBC layer. By reducing time or speed of centrifugation cells will be less densely packed, such that platelets may be retained in the plasma layer and MNC (lymphocytes and monocytes) will accumulate on top of the denser granulocytes.²²³

Cell collection by apheresis

Collection of PBPC following mobilization treatment is performed by apheresis technology (Greek *aphaeresis*, to take away) which, most often, utilizes continuous flow centrifugation of anticoagulated whole blood targeted to collect the mononuclear cell fraction, while the remaining blood constituents are returned to the donor.¹⁵¹

With a reported PBPC collection efficiency of about 50 % using different apheresis devices,²²⁴⁻²²⁸ sequential apheresis procedures of 4-5 hours, on 1-3 consecutive days are often required to reach the target CD34⁺ cell dose. With a PBPC purity of only 1-3 % in the final apheresis product,^{150,224,229} the PBPC graft will comprise a variety of leukocyte populations of importance to HSCT outcome. Thus in HLA-mismatched HSCT, further purification of the PBPC product is often required. Generally, the RBC contamination of collected cells is low. In contrast, the unavoidable collection of platelets into the PBPC product may cause clinically significant depletion of donor platelet levels,^{226,230,231} and necessitate further processing of the PBPC product for platelet removal.

Cell processing by centrifugation

Centrifugation of whole blood, bone marrow or PBPC products is generally employed for volume reduction or for depletion of cellular constituents, which may interfere with further processing (e.g. platelets) or be harmful to the recipient (e.g. ABO-incompatible RBC). In small scale separation, manual centrifugation including a density gradient medium for MNC purification is often applied.²³² In HSCT graft processing, PBPC products may be subjected to platelet depletion by repeated low speed centrifugation and plasma extraction,²³³⁻²³⁵ whereas bone marrow harvests (often 1-2 L) are separated by manual or automated centrifugation for buffy coat enrichment¹⁹⁴ or by apheresis separation of MNC (75-90% recovery of MNC).^{236,237}

Magnetic-Activated Cell Sorting (MACS)

In MACS, cells in suspension are incubated with antibodies conjugated to magnetic beads and subjected to a magnetic field in which labeled cells are retained, while non-labeled cells flow through the separation column. In positive selection, labeled cells equal the target population (e.g. CD34⁺ PBPC) whereas in direct depletion strategies, unwanted cells are labeled (e.g. CD3⁺ T-cells) and thus eliminated from the target fraction.^{232,238} Specific hematopoietic cell types can be purified from whole blood, bone marrow or PBPC products, using magnetic beads coupled to target-specific antibody (direct labeling), or beads that are coupled to a secondary antibody with affinity for the primary target antibody (indirect labeling).^{232,238} Using biodegradable magnetic microbeads (MACS beads, approximately 50 nm in size),²³⁹ removal of the magnetic label prior to further research or clinical application is not needed.

Positive selection of CD34⁺ cells for purification of hematopoietic progenitors (approximately 1-5% of cells in bone marrow or PBPC samples) is frequently employed in small-scale research applications, as well as in clinical transplantation

using large-scale sterile systems for automated separation. A target cell purity and viability of > 95% and a recovery of > 60% have been demonstrated.^{169,170,240} In small-scale applications, sequential separations are often adopted to optimize purity. As indirect depletion of non-target cells is highly effective ($> \log_{10} 4.5$, i.e. > 99%), positive selection of CD34⁺ cells has been the standard method for T-cell-depletion in HSCT.^{169,170} In recent years, efforts to enhance post-transplant immune-recovery have urged the development of a selective T-cell depletion technique, preserving immune-competent cells in the target fraction. Strategies for clinical-scale T-cell depletion by direct labeling of CD3⁺ cells balance high cell capacity against effective depletion of T-cells ($\log_{10} 3-4$) and satisfactory recovery of target cells (> 60%), in a reasonable amount of time.^{175,176,179,241} The automated procedure is based on processing of cells in adequately sized fractions (staged loading). A decrease in number of sequential loading stages will enhance target cell recovery and shorten throughput time at the expense of a reduction in T-cell depletion efficiency, as demonstrated in paper III.

In flow cytometric analysis following MACS separation, it is important to select detection antibodies such that the analysis of the target fraction is not compromised by interference from the microbead-conjugated antibody. MACS microbeads are usually not removed following separation, therefore antibodies for selection/depletion and subsequent flow cytometric analysis should be selected to recognize different epitopes.^{174,242,243} Moreover, flow cytometric analysis of the depleted target fraction must be optimized for detection and enumeration of very small numbers of residual non-target cells.^{241,242}

Fluorescence-Activated Cell Sorting (FACS)

Development of (FACS) technology, which utilizes fluorochrome-conjugated target-specific monoclonal antibodies, has enabled the purification of rare cell populations based on their surface marker profile and scatter profile. A flow cytometer will measure size, granularity and relative fluorescence of single cells as they intersect a laser beam at a high velocity in flowing suspension (sheath fluid). Downstream from the analysis point, target cells can be charged and diverted in an electric field for subsequent collection, while uncharged cells are disposed of.²⁴⁴ With the availability of multi-parameter FACS sorting, a heterogeneous collection of CD34⁺ cells isolated by MACS can be further purified into progenitors of different maturity and lineage affiliation depending on the presence or absence of multiple cell surface antigens. In the study of hematopoietic development, cells can be sorted by FACS into populations of sequential differentiation stages for characterization in functional, cytogenetic and molecular assays (paper I and II). In sorting of minor cell

populations, where even a small number of contaminating cells will impact dramatically on the results of downstream analysis, high performance in purity is important and can be further enhanced (>99%) by re-sorting. However, when sorting is based on phenotypic differentiation patterns which describe a continuum rather than a clear-cut presence or absence of a surface marker, complete purity may not be obtainable.

Acoustophoresis

Acoustic forces can be utilized to separate cells by their specific pattern of migration (-phoresis) within an acoustic field. The use of acoustic forces in microfluidic systems, i.e. acoustophoresis, has developed in the last decade as a non-contact, gentle method for continuous flow cell separation, with a number of potential clinical applications.^{245,246}

When exposed to an ultrasonic standing wave field, suspended cells are affected by an acoustic radiation force which will move the cells towards either the pressure node or the pressure antinode of the standing wave. The direction of migration is dependent on the inherent acoustophysical properties of the cell, such as density and compressibility, relative to the properties of the surrounding media, and is defined by the acoustic contrast factor. The polarity of the contrast factor (+/-) determines the migration to the pressure node or antinode. In an aqueous milieu, most cells will move to the pressure node, whereas lipid droplets or air bubbles will move to the antinode. The magnitude of the acoustic force is also dependent on the size of the cell.²⁴⁷

By applying an acoustic standing wave field on to a continuously flowing cell suspension within a micro channel, the acoustic field will act as a filter to produce a band of cells at the position of the pressure node. The laminar flow properties of the micro channel will keep the position of the cells in the band also when they have passed through the sound field, thus, enabling separation of the cells from the surrounding medium further down the channel.²⁴⁸ With the use of a standing wave of half a wavelength, the cells will focus in a band at the position of the pressure node in the center of the channel (Figure 9). By designing a trifurcation outlet at the end of the micro channel, a concentrated cell suspension can be collected from the central outlet and the excess fluid can be diverted into the lateral outlets.²⁴⁹ (Figure 10A).

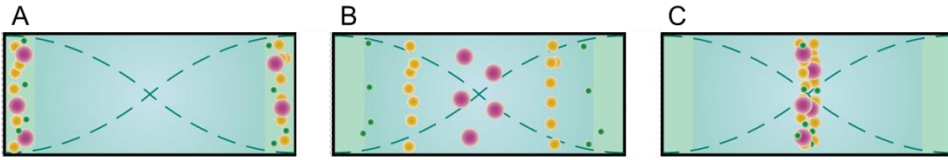


Figure 9 A) Cells and particles are affected by acoustic radiation forces when exposed to an acoustic standing wave. B) The force is proportional to the size of the particle, such that larger particles will reach the pressure node faster than smaller particles. C) Given enough time in the force field all particles will eventually focus at the pressure node. Figure kindly provided by Dr. Andreas Lenshof and published with permission from © Sage Publications.

A similar micro-chip set up can be utilized to separate particle types which in a given medium display acoustic contrast factors of different polarity and, thus, will migrate in opposite directions when exposed to acoustic forces (Figure 10B). This approach has been utilized to separate erythrocytes from lipid particles²⁴⁹ and was further developed and scaled up to remove lipid microemboli from whole blood recovered during cardiothoracic surgery.^{250,251}

Acoustic separation by cell size can be accomplished by adding a trifurcation inlet to the chip design, thus allowing the cell suspension to enter the acoustic field along the lateral walls of the separation channel and clean buffer to be infused via the central inlet²⁵² (Figure 10 C). As the magnitude of the acoustic force on a cell is proportional to the size of the cell, larger cells will migrate faster to the pressure node. By controlling the magnitude of the employed force field and the flow rate through the separation channel, a size dependent cell fractionation can be accomplished. This approach was utilized to deplete PBPC samples of platelets as described in paper IV. Recently, size dependent fractionation was adopted for separation of circulating tumor cells from other peripheral blood cells²⁵³ and for isolation of cell cycle synchronized mammalian cells,²⁵⁴ with a high recovery of viable cells. Modification of the chip design with multiple outlets for separation of complex cell or particle suspensions, or adjustment of buffer density for efficient separation of cells which would otherwise behave similarly in an acoustic field, such as RBC and platelets, have been demonstrated.²⁵⁵

Combining acoustophoresis with the use of targeted microbeads has further broadened the field of application. Very small particles ($< 1\mu\text{m}$) which are not affected by acoustic forces, such as peptides or viruses, can be extracted by the use of affinity microbeads which bind selectively to the particle of interest.²⁵⁶ The acoustic properties of the microbeads enables sample purification by transfer of the beads from a laterally flowing sample suspension into a parallel flow of clean buffer in the center of the acoustophoresis separation channel.²⁵⁷ The use of targeted microbeads may also allow separation of cells with similar acoustophysical properties, such as lymphocyte

populations (T-cells, B-cells, NK-cells etc.) Recently, a highly discriminative separation of $5\mu\text{m}$ and $7\mu\text{m}$ microbeads was demonstrated, which suggests the possibility of microbead mediated size dependent separation of multiple cell populations.²⁵³

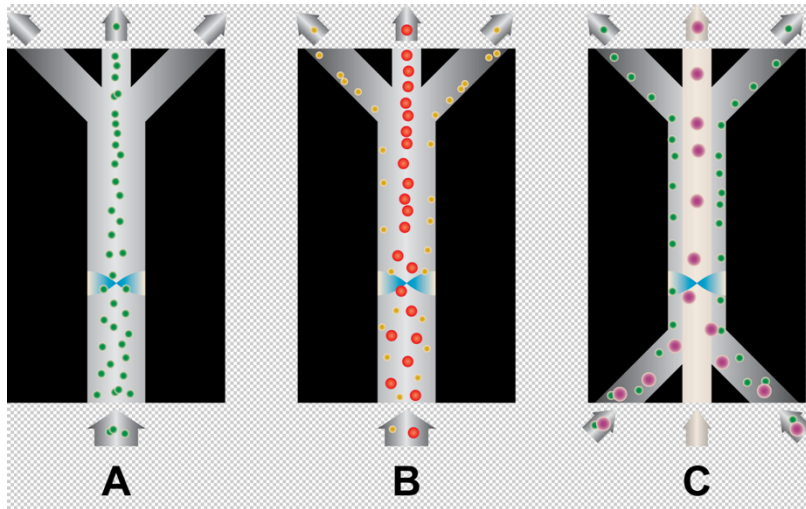


Figure 10 A) Concentration of particles by diverting excess fluid through the lateral outlets B) Separation by acoustic contrast factor. Particles with a negative contrast factor move to the lateral pressure antinodes, whereas particles with a positive contrast factor focus at the central pressure node. C) Size dependent fractionation. By tuning the flow rate such that smaller particles do not have the time to migrate to the pressure node, larger particles can be isolated from the central outlet. Figure kindly provided by Dr. Andreas Lenshof and published with permission from © Sage Publications.

PRESENT INVESTIGATION

Human myeloid progenitors differentiating *in vitro*

Paper II

Clonogenicity, gene expression and phenotype during neutrophil versus erythroid differentiation of cytokine stimulated CD34⁺ human marrow cells *in vitro*.

Aim

To characterize clonogenicity, immunophenotypes and gene expression profiles for *in vitro* neutrophil and erythroid differentiation, and to identify suitable markers for neutrophil and erythroid commitment.

Questions addressed

What surface markers can be used to identify cells of sequential neutrophil and erythroid differentiation stages, respectively?

Two *in vitro* hematopoietic cell culture systems were established, supporting the proliferation and maturation of CD34⁺ human bone marrow cells along the neutrophil or erythroid lineages, by selection of appropriate combinations of cytokines. Terminal differentiation was verified by morphological studies and flow cytometric detection of surface antigens of established neutrophil and erythroid maturation. Two surface marker combinations, CD15/CD33 and blood group A antigen/CD117, were found to visualize early to late neutrophil and erythroid differentiation, respectively, providing defined subpopulations of different developmental stages, which could be isolated for further analysis.

How do clonogenic capacity and gene expression profile correlate to phenotype during the early stages of neutrophil and erythroid differentiation?

According to the established surface marker combinations, cell populations representing sequential erythroid and neutrophil differentiation stages were sorted by FACS to clonogenic assays (methylcellulose) and to gene expression analysis by TaqMan real-time (RT-) PCR.

In clonogenic assays, CD15 and group A antigen were found to be exclusively expressed on cells committed to the granulocyte/monocyte and erythroid lineages, respectively, thus confirming their applicability as lineage-restriction markers. Gene expression analysis of transcription factors and growth factors implicated in early (GATA-2, SCL), neutrophil (CEBP/ α , PU.1, CEBP/ ϵ , the G-CSF receptor) and erythroid (GATA-1, NF-E2, Epo receptor) development, as well as molecules appearing in mature cells (GPC, GPA, Kell, ABO, β -globin, proteinase 3 (PR3), lactoferrin) was used to create a map of differentiation-associated changes. Additional surface markers were analyzed to complete the map of neutrophil versus erythroid differentiation.

How does the blood group A antigen compare to other candidate markers of erythroid-restricted commitment?

The blood group proteins Kell and GPC were found to appear in early erythroid culture but were also expressed in early and continued neutrophil differentiation, whereas GPA was exclusively expressed in erythroid maturation. The group A antigen appeared in erythroid culture concomitantly with Kell and GPC but earlier than GPA, and was confirmed in clonogenic assays to define erythroid lineage restriction.

Summary

These findings provide information on suitable surface antigens (e.g CD15 and group antigen A) and genes (e.g lactoferrin and β -globin) to use as lineage markers for neutrophil and erythroid development of human cells, and allow a direct correlation of expression patterns to lineage affiliation in future investigations of additional targets.

Human erythroid progeny emerging *in vivo* following allogeneic HSCT

Paper I

Autologous del(20q)-positive erythroid progenitor cells, re-emerging after DLI treatment of an MDS patient relapsing after allo-SCT, can provide a normal peripheral red blood cell count

Aim

To investigate a possibly common origin to the reappearing autologous erythropoiesis in a patient with myelodysplastic syndrome (MDS) relapsing after allogeneic HSCT, and the original myeloid malignant cell clone.

Questions addressed

Is the del(20q) present in reappearing autologous erythropoiesis following allogeneic HSCT?

In a patient with cytogenetic relapse of del(20q)-positive MDS following blood group RhD-mismatched allogeneic HSCT, a reappearing autologous erythropoiesis, as determined by RBC RhD-phenotyping, was found to provide a normal peripheral RBC count.

To investigate the origin of the autologous RBC, bone marrow cells of different erythroid maturity were FACS sorted according to previously established (paper II) surface markers, and examined by interphase fluorescence in situ hybridization (FISH) analysis using a probe mapping to 20q12. Virtually all erythroid progenitors (group A antigen⁺/CD34⁺) were found to harbor the del(20q) as did the vast majority of maturing erythroid cells (CD71^{hi}/GPA⁺), suggesting an origin common to functional autologous RBC and the original malignant cell clone.

How is the del(20q) distributed in myeloid versus lymphoid lineage progeny?

At the time of investigation, the patient presented with a predominantly recipient-derived myelopoiesis and a donor-derived lymphopoiesis as indicated by STR-PCR chimerism analysis. Peripheral white blood cell and platelet counts were normal.

FISH analysis of FACS sorted bone marrow cells revealed that the del(20q) was present in almost all non-erythroid/immature progenitors (A⁺/CD34⁺) and in the absolute majority of maturing granulocyte/monocyte cell populations (CD15^{low}/CD33^{high} and CD15^{high}/CD33^{low}). However, mature T-cells (CD3⁺) and B-cells (CD19⁺) were del(20q)-negative.

Summary

These findings suggest an origin common to the original myeloid malignant clone and the reappearing autologous RBC, i.e. a progenitor cell with preserved granulocyte/monocyte and megakaryocyte/erythroid potential. The finding of an apparently unaffected lymphopoiesis does, however, not rule out a more immature lymphoid-myeloid progenitor/HSC as the primary target of the initial chromosomal aberration.

Paper V

Semi-quantification of A and B antigen levels acquired by donor-derived erythrocytes following transfusion or minor ABO-incompatible hematopoietic stem cell transplantation

Aim

To investigate the phenomenon of weak blood group A or B antigen expression by donor-derived group O red blood cells, following transfusion or minor ABO-incompatible HSCT.

Questions addressed

To what extent can group A/B antigens be acquired *in vivo* by donor-derived group O RBC?

Blood samples from patients originally typed as A, B or AB and recently transplanted or transfused with cells from group O donors were analyzed, utilizing a highly sensitive flow cytometry protocol developed in our laboratory for the semi-quantification of low RBC A/B antigen levels. Donor-derived group O RBC were found to express variable levels of acquired antigen, ranging from very small amounts in non-secretor individuals to almost subgroup A_x-like profiles in group A secretors.

Analyses of repeat blood samples from group A secretor individuals transfused with a variable number of group O RBC units at different time intervals, showed a delayed onset of detectable A antigen on donor-derived RBC and a subsequent increase in antigen levels over time.

What patient-specific characteristics may influence the level of group A/B antigens acquired *in vivo* by group O RBC?

To investigate the influence of the patients' ABO type and secretor status, established methods were used for ABO phenotyping and *FUT-2* genotyping, respectively. Genomic typing of the *ABO* gene was performed in selected patients. In group A patients, levels of A antigen acquired by donor-derived group O RBC following transfusion or HSCT were greater in secretors than in non-secretors, and comparatively low in secretor individuals who typed as group A₂ or AB. In group B

secretors, low levels of B antigen were detectable on donor-derived group O RBC following transfusion but not after HSCT.

What are the possible sources of group A/B antigen available for adsorption onto group O RBC?

To investigate, in a controlled environment, the adsorption of A/B antigens from plasma and the possible direct exchange of A/B antigens between RBC, two different model systems were generated for the *in vitro* incubation of group O RBC with 1) group A or B, secretor or non-secretor donor plasma and 2) group A or B, secretor or non-secretor donor RBC in the absence of plasma.

Group O RBC were found to acquire A antigens rapidly from donor RBC and increasingly over time from donor plasma, at higher levels when using group A, secretor compared to non-secretor donors. In contrast, B antigens were acquired by group O RBC from group B donor RBC but not from group B donor plasma at detectable levels.

Can group A/B antigens be acquired through the conversion of H substance on the RBC surface by enzymatic action of ABO glycosyltransferases in plasma?

An *in vitro* system was generated for the detection of ABO glycosyltransferase activity in plasma. When incubated with group A₁, A₂ or B non-secretor plasma in the presence of the appropriate sugar substrate - UDP-GalNAc for A glycosyltransferase and UDP-Gal for B glycosyltransferase group O RBC were successfully converted to resemble group A and B RBC, respectively. An observed decrease in detectable H substance on group O RBC after *in vitro* incubation with group A₁ plasma, without the addition of donor sugar, points to a low level active conversion of H substrate to A antigen.

Summary

Conversion of donor-derived group O RBC to ABO subgroup-like RBC following transfusion or HSCT was demonstrated. Our findings indicate type 1 A antigen-specific glycolipids found in secretor plasma to be the major source of A antigen available for adsorption onto RBC, but suggest also a secretor-independent mechanism for A/B-antigen acquisition.

Separation of PBPC products using novel technology platforms

Paper III

Rapid and effective CD3 T-cell depletion with a magnetic cell sorting program to produce peripheral blood progenitor cell products for haploidentical transplantation in children and adults.

Aim

To investigate the performance of a novel magnetic cell sorting program (Depletion 3.1, CliniMACS System), for large-scale direct depletion of T-cell from PBPC products intended for haploidentical transplantation.

Questions addressed

How does the novel Depletion 3.1 (D3.1) program compare to the standard D2.1 program, in terms of direct T-cell depletion efficiency and CD34⁺ cell recovery?

PBPC products were collected following mobilization treatment from HLA-haploidentical family donors. The PBPC products were incubated with anti-CD3 antibody conjugated to magnetic micro beads and processed with the CliniMACS device. T-cells were depleted using either the D2.1 or the D3.1 program, with a median processing time per 10^{10} total cells of 0.9 hours and 0.35 hours, respectively. The median \log_{10} T-cell depletion rate was significantly better with the D2.1 (log 3.6) compared to the D3.1 (log 2.3), whereas the D3.1 was superior to the D2.1 in median recovery of CD34⁺ cells (90% versus 78%).

Can T-cell depletion efficiency be improved by preventing non-specific binding of the anti-CD3 microbeads?

The original protocol was modified by addition of human immunoglobulin (IgG) prior to anti-CD3 microbead incubation. Introducing of a blocking step to prevent nonspecific binding of non-T-cells to the anti-CD3 microbeads, significantly

improved the performance of both depletion programs, with median \log_{10} T-cell depletion rates of $\log 4.5$ (D2.1) and $\log 3.4$ (D3.1).

Is the approach of direct T-cells depletion sufficient to produce grafts suitable for haploidentical transplantation in children and adults?

PBPC grafts were produced with a target cell dose of $\geq 5 \times 10^6$ CD34+ and $\leq 1 \times 10^5$ CD3+ cells/kg recipient body weight. Grafts were composed primarily of directly T-cell depleted cells (D2.1 or D3.1), however, the addition of positively CD34+ selected cells were required in cases where the primary grafts a) contained insufficient numbers of CD34+ cells, or b) could be transplanted only in part due to excessive numbers of residual T-cells. The relative number of grafts that were exclusively composed of directly T-cell depleted PBPC products, increased by 58%, following the introduction of an IgG blocking step.

Grafts were transplanted with sustained primary engraftment in 9/11 patients. The incidence of acute GvHD was low and no severe chronic GVHD was observed. Immune reconstitution was rapid and compared favorably to previous reports on the use of directly T-cell-depleted grafts.^{176,177}

Summary

The novel D3.1 program can be utilized for large-scale, time-saving direct T-cell depletion with excellent recovery of CD34+ cells and, with the addition of an IgG blocking step, an effective reduction of T-cell numbers. Combining direct T-cell depletion with standard CD34+ selection if required enables the composition of suitable grafts for haploidentical transplantation in children and adults.

Paper IV

Efficient removal of platelets from peripheral blood progenitor cell products using a novel micro-chip based acoustophoretic platform

Aim

To investigate a novel micro-chip based acoustophoresis technique for removal of platelets from PBPC products.

Questions addressed

To what level of efficiency can the acoustophoresis technique be utilized to deplete PBPC samples of platelets?

Samples were obtained from PBPC products collected after standard mobilization treatment of patients and healthy donors, and sorted on an acoustophoresis chip. A size dependent fractionation of leukocytes versus platelets was accomplished by applying an acoustic standing wave field on to the continuously flowing PBPC suspension. Thereby, leukocytes could be almost exclusively separated to the target fraction (98%), whereas platelets were efficiently depleted (89%).

Does the acoustophoresis sorting technique have an impact on the composition and functionality of the PBPC product target cells?

The distribution of leukocyte populations in the PBPC samples and the sorted cell fractions were assessed by cell morphology (cytospin slides stained with May-Grünwald solution) and by flow cytometry analysis. The cellular compositions of the PBPC samples were conserved in the corresponding target fractions, with a comparable relative number of lymphocytes CD3⁺ cells CD4⁺ cells, CD8⁺ cells, CD14⁺ cells, CD56⁺ cells, CD19⁺ cells and CD34⁺ cells. The very few leukocytes lost to the waste fraction were mainly lymphocytes, which are smaller and less acoustically affected than the larger granulocytes/monocytes. The acoustophoresis procedure did not affect the pre-sorting cell viability (98% propidium iodide negativity). Furthermore, results from hematopoietic progenitor cell assays (methylcellulose culture) indicated a preserved colony-forming ability post-sorting.

What impact does the acoustophoresis sorting technique have on the separated platelet fraction in terms of platelet activation and functionality?

Evaluation of platelet activation by P-selectin (CD62P) expression revealed a significant increase in the median number of activated platelets (CD62P+) in the target (19%) and depleted fractions (20%) compared to the PBPC input samples (9%), however, not to the extent observed in buffy coat platelet concentrates (48%) intended for clinical transfusion. The acoustophoresis procedure did not increase the formation of platelet-leukocyte aggregates. Acoustophoresis-separated platelets showed a preserved activation capacity as indicated by an adequate response to the stimulation with Thrombin Receptor Activator Peptide (TRAP).

Summary

The acoustophoresis technique as described in this study can be utilized to efficiently deplete PBPC samples of intact platelets, whilst preserving the target leukocyte fraction, cell viability and progenitor cell colony-forming ability. Acoustophoresis is, thus, an interesting technology to improve current cell processing methods.

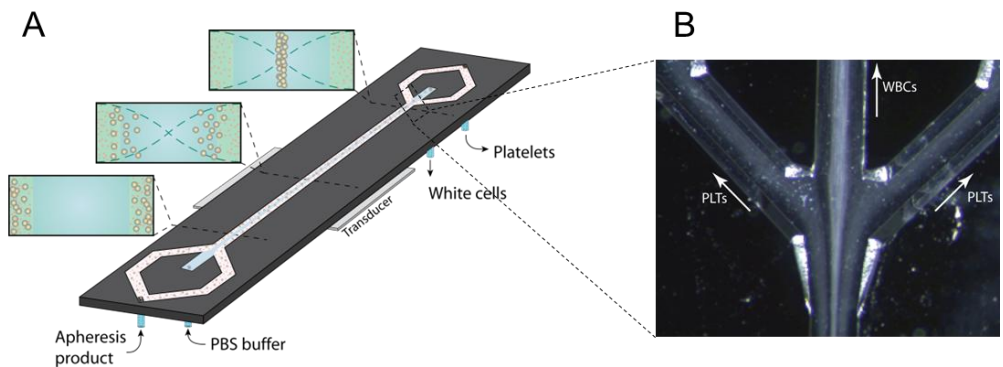


Figure 11. Acoustophoresis chip design

A) A size dependent fractionation of leukocytes versus platelets was accomplished by tuning the acoustic power such that the larger leukocytes (5-20 μm in diameter) were focused into the center of the micro-channel, while the smaller platelets (2-4 μm in diameter) which are less acoustically affected remained at the lateral walls. B) The acoustic force was tuned by visual inspection of the outlet trifurcation and set when leukocytes were optimally focused into the central outlet (collected as target fraction) and platelets were distributed to the lateral branches (collected as waste fraction).

GENERAL DISCUSSION

Myeloid progenitor differentiation

Hematopoietic stem cells develop into progenitor cells of restricted lineage commitment through a process governed by transcription factors and stimulated by growth factors to sustain survival, proliferation and complete maturation.³⁶ However, most of our current knowledge on hematopoietic differentiation emanates from studies of cell lines and mouse models. Therefore, characterizing differentiation-associated changes in the gene expression patterns of factors critical to hematopoietic development, in phenotypically defined normal human cells of known clonogenic potential, is important to better understand the determining features of hematopoietic differentiation.

Mapping neutrophil versus erythroid differentiation

We utilized two *in vitro* culture systems to obtain cells of advanced neutrophil and erythroid maturation from adult human CD34⁺ bone marrow cells, and to study the relationship between clonogenicity, gene expression and phenotype during early neutrophil and erythroid development. Importantly, the heterogeneous cells in culture were sorted according to selected surface markers into defined populations of sequential developmental stages for further analysis. Thus the expression profiles of several genes could be directly correlated to changes in clonogenic potential and to lineage commitment, as defined by surface expression of CD15 (neutrophil differentiation) and group A antigen (erythroid differentiation).

Most of the transcription factors investigated displayed a low level expression in the earliest stages of culture, supporting the concept of lineage priming, i.e. the co-expression of multiple transcription factors in early progenitor cells (Hu 1997, Akashi 2003) GATA-1 and SCL were markedly up-regulated in erythroid differentiation which agrees well with their postulated roles in erythropoiesis,^{38,41-43} whereas PU.1 and CEBP/α were highly expressed during neutrophil development, as expected.^{39,40} Supposedly targeted by these neutrophil/monocyte transcription factors,^{36,39} the G-CSF receptor exhibited a concomitant up-regulation in neutrophil differentiation. The Epo receptor was up-regulated with erythroid commitment, however, retained

low levels of expression also in CD15⁺ cells, possibly indicating a role in neutrophil development. Unexpectedly, a sustained expression of NF-E2 was found in neutrophil differentiation, suggesting a role additional to its involvement in late erythroid and megakaryocytic maturation.⁴⁵

In search of an early erythro-specific marker

Different markers have been used for characterization of erythroid differentiation and some of them have been claimed to be erythroid-specific. GPA is indeed selective for erythroid development but appears late in maturation, with a gene expression pattern similar to β -globin. As described by others, we found an early expression of blood group molecules Kell glycoprotein and GPC in erythroid maturation^{71,72,258} but also in neutrophil maturation. The presumed erythroid specificity of the Kell glycoprotein has previously been contradicted by detection of its presence in testis, brain and lymphoid tissue.^{259,260} Also, the expression of Kell in non-erythroid progenitors has been indirectly demonstrated by an inhibitory effect of anti-Kell antibodies on megakaryocyte (CFU-MK) and myeloid (CFU-GM) progenitor proliferation *in vitro*,^{261,262} suggesting that Kell blood group antibodies may account for neonatal thrombocytopenia and unexplained neutropenia. Although GPC is not restricted to erythroid cells, it has previously been proposed as a discriminative marker based on an erythro-specific sialic acid epitope.^{263,264} We found a sustained surface expression of GPC during neutrophil differentiation, using either sialic-acid dependent or independent antibodies, suggesting that GPC is not suitable as a marker for erythroid-lineage restriction.

In our hands, the group A antigen was found to be the most reliable early erythro-specific marker. We found an early expression already on a fraction of freshly isolated CD34⁺ bone marrow cells and an increase during erythroid culture, before the appearance of GPA.^{72,74} A marked up-regulation of the *ABO* gene transcription paralleled the appearance of surface group A antigen and concurrent erythroid commitment. While gene expression declined rapidly in later erythroid stages, group A antigen surface expression remained high. These findings were later supported by other members of our research group, demonstrating that *A*^I allele transcripts are present in bone marrow but not in peripheral blood.¹³⁴ In neutrophil culture, *ABO* gene transcription was detected only in CD15-negative populations, harboring early erythroid progenitors.

The identification of group antigen A as a suitable erythro-specific marker provides an important tool for further investigation of cells differentiating along the erythroid pathway.

The post-HSCT chimera - mixed or complete?

The goal of allogeneic HSCT in treatment of hematologic malignancies is to establish a state of 'complete donor chimerism', describing the co-existence of a completely donor-derived hematopoiesis and the non-hematopoietic tissues of recipient origin. In ABO-incompatible HSCT the complete donor chimeric status implies a co-existence of donor ABO blood group expressed on RBC and platelets,^{140,201} and recipient-type ABH antigens ubiquitously distributed in tissues, body fluids (secretors)^{92,124,202} and possibly expressed at low levels on donor-derived RBC,^{203,265} as demonstrated in the present work.

Investigating the source of recipient-derived erythropoiesis

A possible source of detectable recipient-derived RBC after allogeneic HSCT, as determined by RBC-phenotyping, would be a progenitor cell common to the original malignant clone at diagnosis. Alternatively, the recipient RBC may emerge from residual non-malignant host stem cells.²¹⁶⁻²¹⁸ We used previously established (paper II) surface markers for neutrophil and erythroid lineage affiliation to investigate the possible presence of the original cytogenetic 20q deletion, initially observed at the time of diagnosis, in subsets of re-appearing host cells post HSCT.

Autologous erythropoiesis carries the del(20q)

A donor-derived lymphopoiesis and a recipient-derived myelopoiesis were revealed with the vast majority of erythroid and granulocytic cells being del(20q)-positive, suggesting an origin common to the reappearing autologous RBC and the original malignant cell clone. The lineage distribution of the del(20q) points to a common progenitor with preserved granulocyte/monocyte and megakaryocyte/erythroid potential. The finding of an apparently unaffected mature lymphopoiesis does, however, not rule out the involvement of a more immature lymphoid-myeloid progenitor/HSC.⁵⁷ Minor populations of erythroid and granulocytic cells were found to be negative for the del(20q). Possibly, these cells represent co-existing normal host cells which, as hypothesized by others,^{214,266} may have facilitated the re-emergence of malignant cells by inhibiting immune-competent donor effector cells. Alternatively, they may be neoplastic cells harboring cytogenetically cryptic mutations occurring prior to del(20q)^{267,268} or donor-derived, as indicated by a proportion of myeloid cells of donor origin in the STR-PCR chimerism analysis.

Del(20q)-positive erythropoiesis can provide a normal RBC count

The stable condition of the patient with normal peripheral blood cell counts, suggests that the del(20q) may be a relatively benign genetic change in myeloid progenitor

cells, still being capable of normal differentiation *in vivo*. This is further supported by the fact that del(20q) as a sole anomaly in MDS is considered a good-risk abnormality.⁵⁹ Perhaps, further genetic or epigenetic events are required for transformation of 'preleukemic' del(20q)-positive progenitors into leukemic cells. The post-remission chemotherapy administered to the patient prior to HSCT may have increased the risk of such events. Possibly, the administration of DLI upon relapse has eradicated or provided control of more malignant cells, explaining the patient's stable clinical condition, while 'preleukemic' del(20q)-positive progenitors have escaped the GvL effect.

The presence of autologous erythropoiesis may predict PRCA following DLI

PRCA is a known complication to major ABO-incompatible HSCT, representing a host versus graft reaction exhibited by persistent recipient B-lymphocytes producing ABO antibodies against donor-derived erythroid precursors.^{193,198} Others have reported that a high percentage of autologous RBC at the time of DLI may predict bone marrow aplasia and pancytopenia.²¹⁸ In the present investigation, the development of PRCA and transfusion-requiring anemia following DLI was interpreted as part of a GvHD response directed against autologous erythropoiesis, and was subsequently overcome by steroid treatment. With the sole involvement of the erythroid lineage, leaving other lineages unaffected, it is tempting to speculate on the role of polymorphic blood group gene products as minor histocompatibility antigens (mHag) targeting the GvHD response to erythroid cells. Others have demonstrated the capability of ABO transferases to elicit *in vitro* T-cell responses, based on a difference of 3 SNP in exon 3-5 between the *O*^{1^v allele and the *O*¹, *A*¹ and *B*¹ alleles, and suggested that the presence of *O* allele disparities in phenotypically ABO-compatible recipient-donor pairs is a potential risk factor for GvHD.²⁰⁸ In the present investigation, the recipient and the donor were both phenotypically blood group A, although a genotypic difference in *O* alleles cannot be ruled out.}

Investigating the source of A/B antigens acquired by donor O RBC

Acquisition *in vivo* of A/B antigens by group O RBC following transfusion or HSCT has previously been observed, using standard serological techniques.^{203-205,269} The availability of a highly sensitive flow cytometry assay developed in our laboratory for the semi-quantification of low levels of A/B antigens,^{97,270} prompted us to assess the extent of A/B antigen acquisition by RBC, and to investigate mechanisms alternative to the previously proposed adsorption from plasma of A/B-antigen-bearing glycolipids.¹¹¹⁻¹¹³ Donor group O RBC were found to express variable levels of acquired antigen, ranging from very small amounts in non-secretor individuals to subgroup A_x-like profiles, in group A₁ secretors. Our findings support the major role

of A/B antigen adsorption from secretor plasma, but indicate that secretor status is not an absolute prerequisite for the *in vivo* conversion of group O RBC.

Acquisition of A/B antigens by group O RBC in secretors

The marked difference in detectable levels of adsorbed antigen between secretor individuals of different ABO blood groups reflects a variable amount of soluble antigen available in plasma, governed by the complex interaction between the *ABO* gene, the secretor gene (*FUT2*) and the Lewis gene (*FUT3*), and is influenced by allelic variants at the three loci. A clear difference in A antigen levels on donor-derived group O RBC was found in two secretor patients genetically defined as A^1O^2 and A^2O^{1v} , respectively, corresponding to a higher level of soluble A antigen in A_1 than A_2 individuals of the Le (a-b+) phenotype,^{111,117,118} as determined by the quantitative difference in A_1 and A_2 glycosyltransferase activity at the site of A type 1 antigen synthesis.^{119,120} In contrast, only scarce amounts of B antigen were found to be adsorbed from secretor plasma by donor group O RBC, although the anti-B clone 9621A8 was specifically selected for its high sensitivity and ability to detect native ABO subgroups⁹⁷ and remnant A/B antigens after exoglycosidase treatment.²⁷⁰ Notably, this may point to a preference of the anti-B clone 9621A8 for type 2 based B antigen, although the degree of antibody specificity for type 1 versus type 2 oligosaccharides was not investigated in this study.

Acquisition of A/B antigens by group O RBC in non-secretors

In non-secretor plasma, A/B antigens are found, albeit in small quantities, on type 2 carbohydrate chains linked to glycoproteins and, predominantly to glycolipids, and are presumed to arise from endothelial or hematopoietic cells.^{111,117,126,127} Lactosylceramide, the major glycolipid precursor on RBC⁹⁰ is also expressed in endothelial cells. In plasma, glycolipids are associated with lipoproteins and may, thus, be exchanged between cells and lipoproteins, or vice versa, via shedding and/or receptor-mediated endocytosis.²⁷¹ In the recipient of a minor ABO-incompatible HSCT, the endothelium is, thus, a plausible source of A/B type 2 antigen available for transfer to transfused group O RBC. In a transfused group A or B, non-secretor patient the hematopoietic tissue may contribute to the total amount of lipid-linked type 2 A or B antigen available for uptake. A possible mechanism also for direct membrane exchange between adjacent RBC of type 2 A and B specific glycolipids was suggested by the *in vitro* experiments.

Possible conversion of H antigen by enzymatic action of A/B enzymes

The A/B glycosyltransferases are present in plasma irrespectively of secretor status, with only a minor part originating from hematopoietic tissue,^{119,206} and are generally considered incapable of enzymatic activity on the RBC surface, due to insufficient extracellular concentrations of donor-sugar nucleotides.⁸⁹ Also, inhibiting antibodies

directed against endogenous A/B glycosyltransferases may completely abolish enzymatic activity in plasma in the recipient of a minor ABO-incompatible HSCT.²⁰⁹ Conversion of H substance on the surface of donor-derived group O RBC by enzymatic action of A/B enzymes was hypothesized in this work, and was successfully demonstrated *in vitro*, by adding the appropriate donor sugar substrate. Also an active conversion of H substrate to A antigen without the addition of donor sugar was suggested, although further studies are needed to confirm this finding.

Transfusion policy in ABO-incompatible HSCT

Current guidelines for transfusion support in ABO-incompatible hematopoietic HSCT prescribe blood components which are compatible with both the recipient and the donor ABO types, until a complete change to donor blood group can be established. From this time point, the recipient is generally recommended to receive blood components of donor ABO type.^{189,200} In the setting of a minor ABO-incompatible HSCT, the switch to donor type transfusion support will inevitably expose the recipient to antibodies directed against blood group A/B epitopes, expressed on vascular endothelium, carried by soluble structures in plasma and, possibly, present on donor-derived RBC at ABO-sub-group like levels, as demonstrated in this work. Repeat transfusion of donor ABO-type plasma and platelet components have been demonstrated to cause immune-complex formation, endothelial cell damage and multiple organ dysfunction.^{212,213} In this setting, circulating donor-derived RBC have an important role in CR1-receptor mediated clearance of immune-complexes²⁷² but are, as shown here, also potential targets for direct ABO antibody binding and, possibly, hemolysis. In a clinical context, this work provides additional support for the proposed^{212,273} selection of plasma components compatible with both donor and recipient ABO blood group, also beyond the time of complete HSC engraftment.

Monitoring the complete (donor) ABO chimera

Blood group incompatible HSCT presents a challenge to cell processing, blood group serology and transfusion. In the ABO-incompatible setting, the risk of early and delayed complications must be addressed by means of graft processing and adequate blood component support. In the post-transplant period, standard ABO typing is performed to establish the conversion to donor blood group and components must be selected for transfusion support accordingly.

In the surveillance of ABO-incompatible HSCT recipients, detection of recipient ABO-type RBC as a mixed field agglutination reaction in standard blood typing may provide an alert of impending relapse or rejection. However, low levels of acquired A/B antigen by donor-derived group O RBC following minor ABO-incompatible

HSCT, as demonstrated in this work, may well present with a similar agglutination pattern. We developed a highly sensitive flow cytometry assay for the semi-quantification of low levels of A/B antigens, that demonstrated distinguishable patterns for several ABO subgroups and chimeras. Accordingly, this assay enables the distinction between HSC-donor-derived RBC expressing low levels of acquired A/B antigen as presented in this work and reappearing RBC of recipient origin which may herald relapse or HSC graft rejection.^{216,217}

In recent years, analysis of polymorphic DNA fragments by STR-PCR, has replaced RBC phenotyping as the standard method for monitoring chimerism. Nevertheless, detection of recipient RBC in routine ABO typing may be the first indication of relapse after ABO-incompatible HSCT, which implies transfusion of blood components compatible with both the recipient and the donor ABO types.¹⁹⁹

HSCT graft engineering

Optimizing the haploidentical graft

The best possible graft in haploidentical transplantation is a rigorously T-cell depleted PBPC product containing high numbers of viable CD34⁺ cells and other immunocompetent cells. Providing suitable grafts for children and adults, respectively, presents unique challenges. In adult patients, the target of $\geq 5 \times 10^6$ CD34⁺/kg recipient body weight in the final graft calls for collection and MACS processing of high CD34⁺ cell numbers, which must be optimally recovered in the process of immunomagnetic T-cell depletion. In children, the major challenge is not to exceed the clinical limit of $\leq 1 \times 10^5$ CD3⁺ cells/kg recipient body weight in the graft, and therefore extensive T-cell depletion is prioritized. Consequently, the available “tools” for graft engineering must be optimized to a reproducibly high performance, and adequately selected for graft engineering according to the specific requirements for every one patient.

Representing a development in MACS technology direct T-cell depletion, the D3.1 program was developed for high throughput, i.e. processing of high cell numbers in a short amount of time, while retaining the depletion efficiency of the standard D2.1 program. In our hands (paper III) the D3.1 was indeed superior in terms of processing time and CD34⁺ cell recovery. Also, a substantial number of immune-competent cells were retained in the graft, with the potential to enhance immune reconstitution.¹⁷⁷⁻¹⁷⁹ The T-cell depletion performance was however, much less efficient (log 2.3) compared to what we (log 3.6) and others^{175,176,179} have observed using the D2.1 program.

Modification of the original protocol to prevent nonspecific binding of non-T-cells by the anti-CD3 microbeads significantly improved D3.1 program T-cell depletion (log 3.4). Nonspecific binding results in an underestimation of the actual number of anti-CD3-labeled cells to be processed on the separation column, and may also lead to an insufficient number of microbeads available for specific T-cells binding. With the D2.1 program, cells are processed at a slower rate thus allowing a wider safety margin regarding a possible column overload, and also giving insufficiently labeled T-cells enough time to be retained. Nonetheless, adding the IgG blocking step to the D2.1 procedure resulted in a highly effective method for T-cell depletion (log 4.5), in the range of what is normally seen in CD34⁺ positive selection. The optimized D2.1 procedure is particularly useful when T-cell numbers in the final graft may be limiting, as in case of large donor-recipient weight differences (adult donor, pediatric patient), or when depleting a second or third PBPC product aiming to increase CD34⁺ cell numbers in the final graft.

Our current strategy for haploidentical graft engineering is to primarily apply a direct depletion technique. The choice between the D2.1 and D3.1 programs is based upon the estimated numbers of CD34⁺/kg and CD3⁺/kg in the final graft, which are dependent on the cell counts of the apheresis product, the weight of the recipient and the expected performance of the available methods. A blocking reagent is used in all direct depletion procedures and PBPC products are processed within a maximum of 24 hours from collection.

Platelets mean problem

The outcome of MACS graft engineering is dependent also on the quality of the starting product. As observed (paper III), a poor cell viability following prolonged PBPC product storage (> 24 hours from collection) will translate into an insufficient direct T-cell depletion.

Platelet contamination of PBPC products has been reported to have a negative impact on CD34⁺ positive selection in terms of target cell recovery²³³ and purity.²³⁵ Coating of CD34⁺ cells by apheresis product platelets has been demonstrated and was suggested to prevent the binding of progenitor cells by the anti-CD34 antibody.²⁷⁴ In our study, PBPC products that were sub optimally T-cell depleted were, indeed, comparatively rich in platelets before MACS separation, and a negative impact of platelets on depletion performance has been confirmed in subsequent direct T-cell depletion procedures.²⁷⁵ Low speed manual²³³ or automated²³⁵ centrifugation for PBPC product platelet removal prior to further processing typically results in 80-95% platelet depletion. However, sequential centrifugation steps are often required with additional processing time and mechanical stress on cells.

The unavoidable co-collection of platelets in centrifugation-based PBPC apheresis^{226,230,231} does also presents a risk to the donor of clinically significant thrombocytopenia. This is especially relevant for autologous donors, with already reduced pre-apheresis platelet counts due to prior administration of mobilizing chemotherapy.²⁷⁶ A gentle method for removal of intact platelets from the PBPC product for infusion to the donor would, thus, have the potential to improve both donor safety and PBPC product quality.

Acoustophoresis can be utilized for platelet removal

As demonstrated (paper IV), the acoustophoresis technique can be utilized to effectively deplete PBPC samples of platelets, whilst preserving the target leukocyte fraction, cell viability and progenitor cell colony-forming ability. With the future goal to deplete clinical scale PBPC products of intact platelets for re-transfusion to the donor, the possible impact of acoustophoresis on platelet functionality was investigated.

The alpha-granule protein P-selectin (CD62P) is exposed on the surface membrane of activated platelets, and is thus a widely used marker for assessment of platelet activation in the preparation and storage of blood bank platelet concentrates.²⁷⁷⁻²⁸⁰ We applied P-selectin for the evaluation of acoustophoresis-induced platelet stress, using a modified flow cytometry assay designed to minimize artefactual *in vitro* activation of platelets.²⁸¹⁻²⁸⁴ Acoustophoresis-induced platelet activation was comparable to previous reports on activation levels in standard platelet apheresis, using different cell separators,^{277-280,285-288} but did not reach the activation levels generally observed in platelet concentrates prepared from single donor apheresis or pooled buffy-coats stored at a maximum of 5-7 days before transfusion.^{277,278,285,288}

A negative correlation between CD62P expression of stored platelets and post-transfusion platelet survival has been demonstrated,^{277,286} suggesting a rapid clearance of *in vitro* activated platelets from circulation.²⁸⁹ Others, however, have not found such a correlation.²⁸⁷ Although the mechanisms governing clearance of activated platelets are not clear, adhesion of CD62P expressing platelets to circulating leukocytes²⁸⁹ are known to play an important part in the pathogenesis of inflammation, involving neutrophil degranulation and phagocytosis of activated platelets.²⁹⁰ Given the comparatively low-level of platelet activation induced by acoustophoresis and a preserved response to TRAP stimulation as demonstrated, it appears unlikely that acoustophoresis separated platelets would be less efficient in hemostatic function or confer a higher risk of adverse transfusion reactions than current standard blood bank platelets, that are routinely and safely used in clinical transfusion.

In PBPC apheresis, significant platelet activation may be induced as shown by detection of CD62P positive platelets in donor circulation.²⁹¹ Moreover, PBPC products containing platelet-platelet or platelet-leukocyte aggregates, which are sometimes visible during apheresis or in further processing of platelet rich PBPC products, may present a risk to the recipient. Currently used centrifugation for platelet removal from PBPC products may, despite low g-forces, induce interactions between platelets and adjacent leukocytes. In acoustophoresis, the size dependent cell fractionation provides an instant physical separation of platelets and leukocytes, thus preventing further aggregate formation, as demonstrated here.

Acoustophoresis in future graft engineering

In a future clinical setting, acoustophoresis has the potential to provide effective platelet depletion which, despite an increase in activation of the few platelets remaining in the target fraction, will result in a markedly reduced total number of activated platelets and also minimize platelet-leukocyte aggregate formation in PBPC products. As previously demonstrated in applications demanding high throughput, several acoustophoresis separation channels can be operated in parallel to increase the system flow rate to 0.5 ml/min.²⁵⁰ Such an approach would open up the possibility for 'in-line' platelet depletion of collected PBPC directly following separation in the collection chamber of the apheresis instrument, which would require a flow rate of 1-2 ml/min. The instant physical separation of collected leukocytes and platelets would prevent platelet activation in further handling of the PBPC product and, also, enable a simultaneous re-infusion of separated platelets to the donor, thus preventing apheresis-induced thrombocytopenia.

Acoustophoresis has recently emerged as a gentle method for continuous flow cell separation, with potential applications in the field of HSCT graft engineering. Acoustic transfer of intact target cells to a clean suspension medium^{256,292} with concomitant platelet depletion (paper III) is certainly an attractive alternative to the manual centrifugation steps currently applied for cell wash, with accumulating mechanical stress on target cells and risk of cell loss. Today, advanced processing such as progenitor cell selection or T-cell depletion requires a complete switch to second platform, i.e. MACS, which is restricted to one-parameter sorting. With the potential application of affinity-acoustophoresis,^{256,257} i.e. the use of targeted microbeads, for discriminative separation of multiple cell populations (T-cells, B-cells, NK-cells etc.), acoustophoresis has the potential to offer a single platform technique for multi-parameter cell separation in graft engineering.

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