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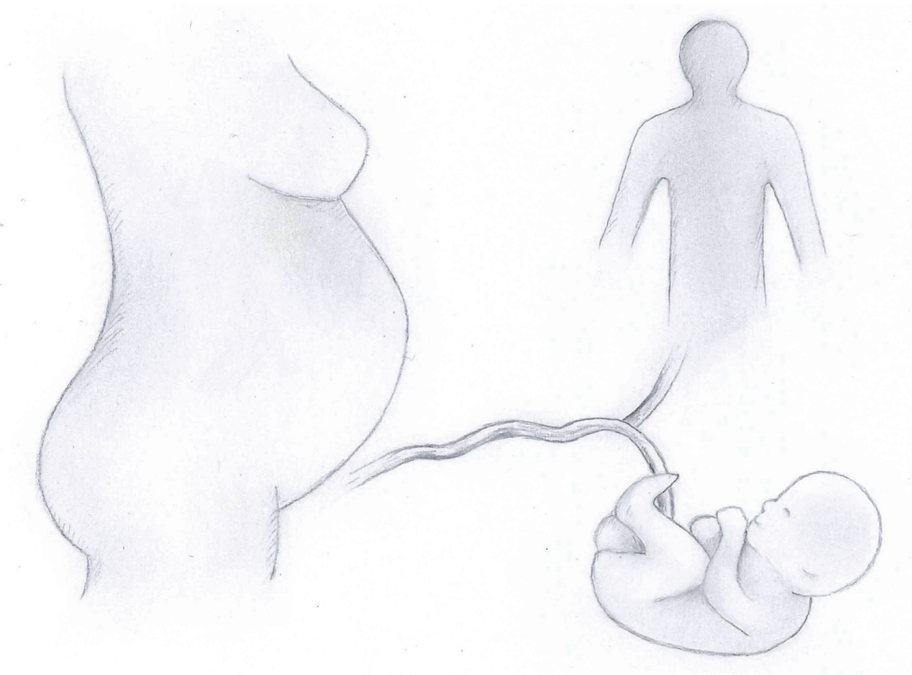

Jens Gertow

Karolinska Institutet

From DEPARTMENT OF LABORATORY MEDICINE
Karolinska Institutet, Stockholm, Sweden

NEW STRATEGIES FOR ALLOGENEIC HEMATOPOIETIC STEM CELL TRANSPLANTATION WITH UMBILICAL CORD

Jens Gertow

Karolinska Institutet

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ABSTRACT

Umbilical cord blood is enriched in hematopoietic stem cells. For this reason, cord blood units may be utilized for allogeneic hematopoietic stem cell transplantations when no adult human leukocyte antigen (HLA)-matched donor is found. Cord blood units are rapidly available from international cord blood banks and the naivety of cord blood cells allows the transplantation of HLA-mismatched units without an increase in graft-versus-host disease. But cord blood is also beset with some drawbacks compared to other stem cell sources, the most apparent being a slow immune reconstitution after transplantation leading to increased infection related mortality. The overall aim of this thesis work has been to develop new strategies and tools for handling patients transplanted with umbilical cord blood.

Donor lymphocyte infusions (DLI), i.e. an additional boost of donor lymphocytes, can be used to treat threatening rejections or malignant relapses in the adult donor setting. However, due to the limited cell dose, this treatment option is currently not available for cord blood transplanted patients. For this reason, we aimed to expand cord blood-derived T cells for possible use as DLI after transplantation. Starting with an aliquot from the original cord blood graft, we successfully expanded T cells in eight days to adequate numbers for DLI preparation. By studying the cells with multicolor flow cytometry for surface and intracellular markers, functional assays and spectratyping techniques we concluded that the T cells had polyclonal T cell receptor repertoire, were of central and effector memory phenotype and responded in a similar manner towards mitogenic and allogeneic stimulation compared to peripheral blood T cells.

The cytokine IL-7 has previously been shown to protect T cells from apoptosis induced by, e.g. cytokine withdrawal. This feature should be especially important for cord blood T cells due to their sensitivity to activation induced cell death as well as their high expression levels of the IL-7 receptor. Hence, we aimed to optimize our expansion protocol by adding IL-7 to a range of IL-2 concentrations. When IL-7 was added to low-dose IL-2, the resulting T cells presented with a higher degree of polyfunctionality and superior proliferation potential compared with cells expanded without IL-7. The T cells also had a higher CD4/CD8 ratio and a higher frequency of effector memory cells, which may have positive implications for their use as DLI.

The overall one-year 55% survival after cord blood transplantations at our center highlights the need for predictive risk markers for earlier interventions. We hypothesized that the T cell expansions could be utilized as indirect indicators of graft quality and, thus, as a tool for risk prediction. We correlated phenotypical and functional data from expanded cord blood T cells with clinical features after transplantation. The results indicated that higher frequencies of CD69+ T cells in the expansions were predictive of prolonged patient survival. Since many of the deaths

were due to infections, this marker may thus be used as an indicator for e.g. the administration of prophylactic antiviral drugs.

To overcome the problem of low cell dose, the strategy of double cord blood transplantations (DCBT) in which two cord blood units are transplanted simultaneously, has been effectively employed. This provides the patient with an increased total nucleated cell dose during the initial critical weeks after transplantation but, in the vast majority of cases, one of the units eventually prevails. However, three out of seven evaluable patients undergoing DCBT at our center presented with a mixed donor chimerism more than two years after transplantation. Since these patients are extremely rare we characterized the phenotype and functionality of their immune systems to gain insight into the significance of mixed donor chimerism. Results indicate that patients with long-term mixed donor chimerism after double cord blood transplantation have a less functional immune system compared to control patients with one donor immune system. This could be because one of the two immune systems had a more naive T cell profile with poor cytokine production. Moreover, we speculate that the mixed donor chimerism in part may be explained by a graft-versus-graft tolerance induced by our use of high-dose anti-thymocyte globulin and an inter-unit match of HLA-C.

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CONTENTS

LIST OF ABBREVIATIONS

aGVHD	Acute Graft-Versus-Host Disease
AIRE	Autoimmune Regulator
ALL	Acute Lymphocytic Leukemia
AML	Acute Myeloid Leukemia
APC	Antigen-Presenting Cell
ATG	Anti-Thymocyte Globulin
BCR	B Cell Receptor
BM	Bone Marrow
Bu	Busulphan
CAR	Chimeric Antigen Receptor
CAST	Center for Allogeneic Transplantation
CB	(Umbilical) Cord Blood
CBT	Cord Blood Transplantation
CD	Cluster of Differentiation
cGVHD	Chronic Graft-Versus-Host Disease
CLL	Chronic Lymphocytic Leukemia
CML	Chronic Myeloid Leukemia
CMV	Cytomegalovirus
^{51}Cr	Chromium-51
CsA	Cyclosporine A
CTLA-4	Cytotoxic T-Lymphocyte-Associated proten-4
Cy	Cyclophosphamide
DC	Dendritic Cell
DCBT	Double Cord Blood Transplantation
DLI	Donor Leucocyte/Lymphocyte Infusion
DNA	Deoxyribonucleic Acid
EBV	Epstein-Barr Virus
HLA	Human Leukocyte Antigen
HSCT	Hematopoietic Stem Cell Transplantation
HVG	Host-Versus-Graft
GMP	Good Manufacturing Practice
GVG	Graft-Versus-Graft
GVH	Graft-Versus-Host
GVHD	Graft-Versus-Host Disease
GVL	Graft-Versus-Leukemia
KGF	Keratinocyte Growth Factor
mAb	Monoclonal antibody
MAC	Myeloablative Conditioning
mHAg	Minor Histocompatibility Antigen
MHC	Major Histocompatibility Complex
MUD	Matched Unrelated Donor
NK cell	Natural Killer cell
NKT cell	Natural Killer T cell
IL	Interleukin
Ig	Immunoglobulin

IFN	Interferon
KIR	Killer Immunoglobulin-like Receptor
LCL	Lymphoblastoid Cell Line
LFS	Leukemia-Free Survival
PAg	Phosphoantigen
PB	Peripheral Blood
PCR	Polymerase Chain Reaction
PD-1	Programmed Cell Death 1
PHA	Phytohemagglutinin A
PMA	Phorbol 12-Myristate 13-Acetate
RIC	Reduced Intensity Conditioning
S1P	Sphingosin-1-Phosphate
SCID	Severe Combined Immunodeficiency
SDF-1	Stromal-Derived Factor 1
TBI	Total Body Irradiation
TCR	T Cell Receptor
Tcm	Central memory T cell
Tem	Effector memory T cell
Tfh	Follicular helper T cell
TGF-β	Transforming Growth Factor- β
Th	T helper cell
Th1	T helper cell type 1
Th2	T helper cell type 2
Th17	T helper cell producing IL-17
Tn	Naive T cell
TNC	Total Nucleated Cell dose
TNF	Tumor-Necrosis Factor
Treg	Regulatory T cell
TRM	Transplant-Related Mortality
Tscm	Memory Stem T cell
Ttd	Terminally differentiated T cell

1 INTRODUCTION

1.1.1 The beginning

It began with a big bang. The atomic bombs dropped over Hiroshima and Nagasaki at the end of World War II were not only horrific weapons of immediate mass destruction. The intense ionizing radiation that followed also severely affected survivors for years to come. Damage to the bone marrow of some of these survivors halted the division of blood-forming stem cells, resulting in diverse blood disorders and leukemia. With these consequences of radiation in mind, and with the Cold War increasing the fear of nuclear warfare in the 1950's, researchers began to investigate ways to restore bone marrow function after radiation injury. Hence, the concept of hematopoietic stem cell transplantation (HSCT) was born.

A common characteristic of radiation-sensitive cells is that they divide quickly, exposing the DNA to radiation-induced free radicals during every cell cycle. Researchers quickly realized that the undifferentiated, rapidly dividing phenotype characteristic of stem cells was shared by another cell type: malignant cells. Thus, patients with solid tumors were among the first to be transplanted with blood-forming stem cells in humans (1). By saving patient bone marrow before treatment, clinicians were able to increase the intensity of the radiation therapy to tumor-responsive but also bone marrow-damaging levels. Subsequently, the bone marrow of the patient was rescued by re-infusing the saved cells. This method of using the patient's own cells would later be known as *autologous* HSCT.

Previous animal studies had demonstrated that shielding the spleen with lead foil could save mice from otherwise lethal irradiation, and that non-shielded irradiated mice could be rescued by intravenous infusion of bone marrow cells from other mice of the same strain (2-4). However, in contrast to inbred mice, humans are genetically diverse, which would turn out to be of outmost importance when Mathé *et al* tried to treat five patients in need of a new bone marrow after accidental exposure to high dose radiation (5). Since the bone marrow of these patients was already destroyed, much like those in patients after the nuclear explosions, Mathé built on the pioneering work by Edward Donnall Thomas (6) by attempting *allogeneic* HSCT, *i.e.* the transplantation of cells from another individual. However, no patient survived this procedure or subsequent efforts in, for example, a patient group suffering from leukemia (7). Instead, patients succumbed to graft rejection, infections or secondary symptoms including skin rashes, weight loss and diarrhea. These latter symptoms were later attributed to the phenomenon of graft-versus-host disease (GVHD), in which cells of the donor immune system recognize healthy tissue of the recipient as foreign and, therefore, attack it.

These initial results were disappointing and parts of the scientific community abandoned the concept of allogeneic HSCT, believing that suitable donors would never be found. However, already during the Second World War, Medawar and co-

workers had studied skin graft survival and rejection of burn victims and came to the conclusion that successful transplantations depended on compatibility of donor and recipient tissues (8)(Fig. 1). The major breakthrough for the allogeneic field came with the identification of the major histocompatibility complex (MHC). In mouse studies, Gorer and Snell discovered an association between tissue rejection and genetic differences, and located one of these differences to a gene locus they entitled the histocompatibility locus 2 (9-12). Since a grouping, i.e. a complex, of similar genes was later discovered at that same site, the name major histocompatibility complex (MHC) was created.

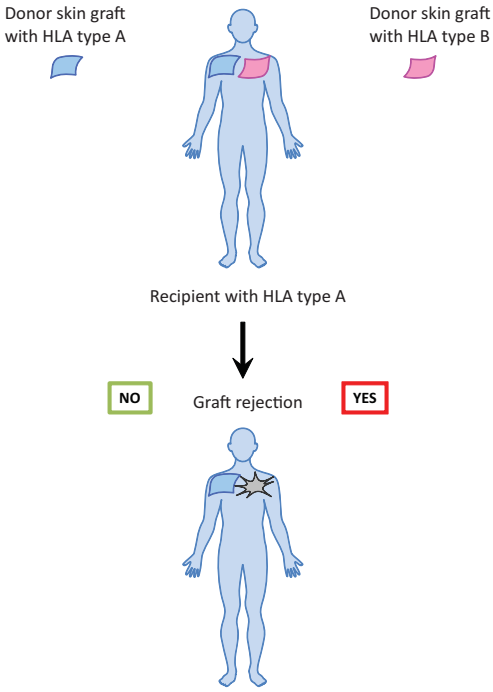

Fig. 1 The concept of donor and recipient histocompatibility. When a recipient is transplanted with tissue of a matched HLA-type, in this example a skin-graft of HLA type A, the graft will be accepted by the immune system of the recipient. However, if the tissue graft comes from a donor with other HLA molecules on the cell surfaces, in this example a skin-graft of HLA type B, the immune system of the recipient may recognize the graft as being foreign and therefore attack and reject it.

When human MHC molecules were finally discovered, they were named human leukocyte antigens (HLA) as they were first described in lymphocytes by van Rood, Dausset and Payne (13-15). Ten years later, in 1968, Storb and colleagues studied dogs, outbred in contrast to mice, and reported that dog leukocyte antigen compatibility between donors and recipients improved the outcome of allogeneic HSCT (16). This finding, together with improved drug regimens for controlling GVHD (17), stimulated the field towards selecting HLA-compatible family donors for allogeneic HSCT, vastly improving the results (18, 19)).

One could stop here and wonder why allogeneic HSCT was sought after; why not just stick to autologous HSCT using the patient's own stem cells and decrease the risk of rejection and GVHD to a minimum? First of all, in the case of radiation injury it is not possible to procure patient cells, necessitating stem cells from another source. Additionally, autologous transplantations were, and still remain, not suitable for all disease diagnoses. For example, in patients with inborn errors, such as severe combined immunodeficiency (SCID), the genetic disorder is present in all cells and hence cannot be cured by just "restarting" the system. In the case of leukemia, autologous stem cells are difficult to obtain without contaminating malignant cells in the graft. The only semi-successful transplant for leukemic patients in the early days occurred in those patients given transplant from an identical twin, i.e. a *syngeneic* transplantation (20). However, these patients died of leukemic relapse, and it eventually became apparent that an allogeneic graft could confer some sort of reaction towards leukemic cells to a much higher degree than syngeneic or autologous

grafts. This effect was later termed the graft-versus-leukemia (GVL) or graft-versus-tumor effect (7, 21, 22).

1.1.2 The dawn of umbilical cord blood as stem cell source

Not only did Edward Donnall Thomas pioneer the field of allogeneic HSCT, he was also deeply involved in the continued work on patients with e.g. acute leukemia (23, 24) and was ultimately awarded the Nobel Prize in Physiology and Medicine in 1990 for this work. By steady progress in solving problems regarding supportive care, conditioning regimens and immunosuppressive treatments, allogeneic HSCT became available as treatment for a wide variety of hematopoietic diseases (25-28). However, approximately only one third of patients in need of a transplant have an HLA-matched donor among their family members. Although efforts had been made to utilize HLA-matched unrelated donors, finding such donors presented as a huge problem (29, 30). For this reason, the first registry collecting information of volunteers for stem cell donation was created in England, known as the Anthony Nolan foundation. Several national and international registries followed, among them the Tobias Registry in Sweden. With an increasing availability of potential unrelated HLA-typed donors through registries and the advent of T cell depletion techniques to further reduce the risk of GVHD (31), the frequency of HLA-matched unrelated donor transplantations increased. Eventually it was shown that the patient outcomes with stem cells from unrelated donors were comparable to those with related donors (32-34).

While depleting the unrelated donor graft of lymphocytes such as the T cells reduces GVHD, it also increased the risks of malignant relapse and opportunistic infections after transplantation (35). In search of additional options, researchers began to look for alternative sources of hematopoietic stem cells, i.e. umbilical cord blood (CB). The history of CB transplantations has been thoroughly reviewed by two of the pioneers in the field, Eliane Gluckman and John E. Wagner (36). Briefly, the proof-of-concept for using CB for transplantations was made by Boyse et al in mouse models, demonstrating that lethally irradiated mice could be rescued by transfusion of small volumes of blood from neonatal donors. Human CB, being of fetal origin, was hence recognized as a possible source of hematopoietic stem cells. Some potential benefits over adult stem cells were seen, such as absence of viral contamination, immediate availability of pre-frozen CB units, and a naivety of the cells possibly reducing the risk of GVHD (37). The very first CB transplantation (CBT) was performed in 1988 by professors Gluckman and Broxmeyer when a six-year old boy with Fanconi anemia was reconstituted with CB from his sister (38).

Methods for easy collection and storage of CB units were established by Broxmeyer et al (39, 40) and the banking of unrelated donor CB was initiated in 1992 (41, 42). Due to these CB banks, the first unrelated CBT was carried out in 1993 with a CB unit mismatched with the recipient at two HLA-antigens (43). Despite the mismatches, the patient did not develop GVHD, supporting the theory of reduced risk of GVHD.

Moreover, since the adult bone marrow registries were skewed in the favor of Western European and North American volunteers, the apparent HLA-permissiveness of CB increased the hopes of finding donors for patients of ethnic minorities.

Because of the limited cell dose in a CB unit, the initial transplantations were performed in children. The first adult treated with CB was a leukemic patient in 1995 (44). Although successful, it became obvious that only smaller patients could benefit from the lower cell dose obtained from a CB unit. To overcome this problem, double cord blood transplantation (DCBT), in which two unrelated CB units are co-infused, emerged as a promising alternative (45-48). However, both CBT and DCBT were, and still are, plagued by a slower neutrophil engraftment and T cell recovery after transplantation compared to adult donors (49-52). This extended engraftment time leads to e.g. increased risk of opportunistic infections, one of the very factors initially sought to remedy with this alternative stem cell source.

1.1.3 The present, or, the aim of my research on cord blood

Since the start in 1975, with Professor Olle Ringdén as driving force, the nurses and medical doctors at the Center for Allogeneic Transplantation (CAST) in Huddinge work hard day and night to make allogeneic hematopoietic stem cell transplantation possible, regardless of the stem cell sources (53, 54). The overall aim of my research has been to provide them and the transplantation community with additional tools to improve the possibility of a successful transplant, specifically when using cord blood stem cells. Therefore, the thesis work presented here centers on my research to improve immune reconstitution and predicting complications after CBT and DCBT. However, before digging into my manuscripts, the reader might benefit from some more details concerning the immune system and its relation to transplantation.

1.2 DEVELOPMENT AND COMPONENTS OF THE IMMUNE SYSTEM

Hematopoietic stem cell transplantation is based on the knowledge that self-renewing hematopoietic progenitor cells have the ability to differentiate into all blood cells. At birth, these blood-forming stem cells populate the bone marrow and give rise to the two cell lineages: lymphoid and myeloid. These lineages further differentiate into mature immune cells and subsequently migrate to peripheral lymphoid organs, blood and tissues. In this context, a transplanted patient resembles a new-born; the donor stem cells need to repopulate the bone marrow of their new host, differentiate and give rise to the completely new immune system of the transplanted patient. Indeed, much like after birth, patients may receive their first vaccination shots six months after a successful transplantation (55). Furthermore, closely resembling the protective content of breast milk, patients may be infused with pooled human antibodies to fight off early infections post-transplantation (56).

A complete view of the immune system is beyond the scope of this thesis; for that I recommend a textbook like Janeway's Immunobiology (57) or Cellular and Molecular

Immunology (58). I will, however, try to give the reader a brief and relatively T cell focused introduction of the immune system, enough to understand the results of my research.

Thus, the immune system is in charge of protecting its host from pathogens. That is, to drive back viruses, bacteria and parasites that try to harm us. The common myeloid progenitor mentioned above gives rise to granulocytes, macrophages, some dendritic cells (DC), mast cells, erythrocytes (red blood cells) and platelets, whereas the lymphoid progenitors differentiate into the lymphocytes B cells, T cells and Natural Killer (NK) cells (Fig. 2). T cells are so called because they are thymus-dependent, that is, their precursors leave the bone marrow early in development and migrate to the thymus, located in the upper part of the chest in the human body, where they subsequently differentiate. Once fully mature, they migrate to secondary lymphoid organs.

Another way of dividing the immune system is on the basis of function, usually named the *innate* and the *adaptive* arms. The innate arm comprises not only cells, like the granulocytes and NK cells, but also physical barriers, such as the skin and mucus layers, and blood circulating inflammatory mediators, such as the complement system. If the physical barriers are broken, the pathogen is greeted by the other innate components equipped with receptors that recognize evolutionary conserved pathogenic patterns, such as single-stranded viral DNA and bacterial surface molecules. Usually, recognition of a pathogenic pattern means that the intruder will be captured and killed.

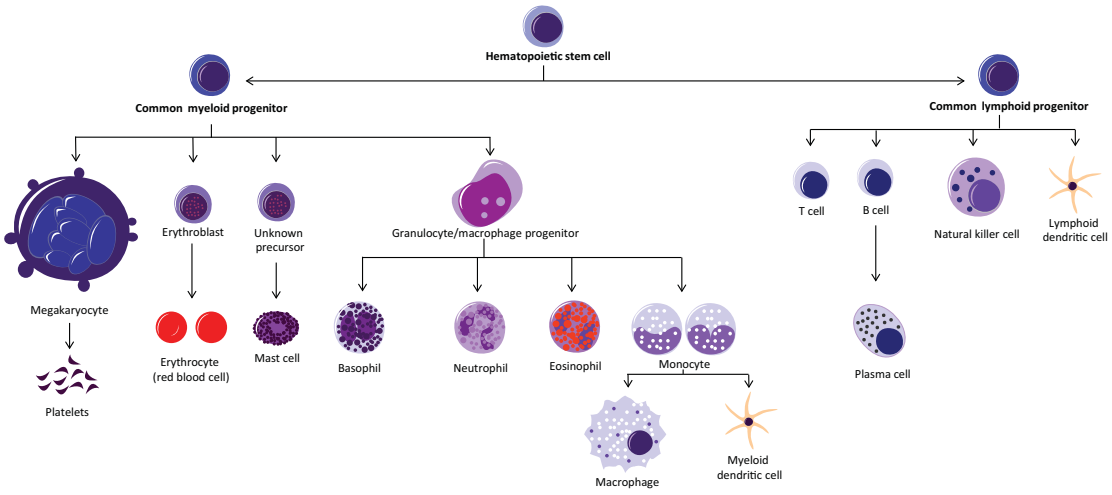

Fig.2 All cells of the blood arise from hematopoietic stem cells in the bone marrow. The stem cells give rise to two types of progenitor cells: the common myeloid progenitor and the common lymphoid progenitor. From these two lineages, all other cells of the immune system arise.

An adaptive response is only necessary if the first line of defense gets overwhelmed. Because it takes time to adapt and mount a proper response, parts of the recognized pathogen are transported from the site of infection to the closest secondary lymph node already at the time of capture. These pathogen elements are called antigens, short for "*anti*body *gen*erators", and are presented to the adaptive B- and T cells in the lymph nodes. Each of these cells possess a unique antigen receptor, formed by

gene rearrangements during maturation, and each of these B- or T cell receptors (BCR and TCR) is specific for only one particular antigen. Since we have hundreds of billions of different lymphocytes circulating our bodies, this makes up a vast diversity of antigen receptors and hence potential targets to respond to.

B-cells recognize their cognate antigen in its native form and may subsequently differentiate into antibody-producing plasma B cells. However, most of them do require activated T cell help to become activated. In contrast, most T cells are a little bit pickier, and need to have their antigen properly digested and presented before going to work. This service is provided by professional antigen presenting cells (APC), of which the DC is the most effective. The APCs present short peptides (protein fragments of 8-24 amino acids in length) of the pathogen bound to MHC molecules on their cell surface which may be recognized by the TCR of the T cell. Hence, the TCR is not specific for the antigen itself, but for the combination of peptide and specific MHC molecule it is bound within.

A T cell samples many different DC in search of its cognate antigen and, once found, the T cell becomes activated and starts to divide and produce cytokines. Cytokines are soluble proteins that act as messengers, relaying information between different players of the immune system. They can be produced by various hematological and non-hematological cells and induce functions like differentiation, proliferation and activation upon binding receptors on target cells. Cytokines can act on the same cell that produced them (autocrine action), on neighboring cells (paracrine action) or on cells in a different part of the body (endocrine action), as long as the target cell expresses the correct receptor. Examples of cytokines are interleukins (e.g. IL-2, IL-7, IL-17), interferons (e.g. IFN-γ, IFN-α), and chemokines which induces cell migration, given they express the correct receptor.

Of note, despite encountering *antigen*, T cells do not produce antibodies as a response like plasma B cells, but instead acquire other effector functions partly dependent on the surrounding inflammatory milieu. Some of these effector functions will be described next.

1.2.1 Development of T cells

T lymphocyte progenitors migrate from the bone marrow to the thymus in response to a chemokine gradient. In the thymus, the TCRs are developed and rearranged and expressed on the cell surface of each T cell. The TCR comes in two flavors: the most studied "conventional" T cells have TCRs made up of α- and β-chains and respond to antigen in the way described above. In contrast, the T cells with TCRs composed of γ- and δ-chains can recognize conserved pathogenic and "altered self" patterns, making them resemble innate immune cells. The γδ T cells have been proposed to be an evolutionary bridge between the innate and adaptive systems, although increasing evidence shows that they are not just relics, but are indeed essential components of

the immune system (59). The term "T cell" in this thesis will, however, refer to the conventional αβ T cells unless stated otherwise.

Newly generated T cells undergo "schooling" in the thymus, where their TCR needs to pass two tests before the cell is allowed to enter the rest of the body. These tests are known as the positive and the negative selection. According to the avidity hypothesis, the first T cell test is to bind weakly to a self-HLA molecule; a T cell that cannot bind autologous HLA will not receive survival signals and, therefore, "dies by neglect". If this first positive selection is passed, the final task is to *not* bind self-HLA conjugated to self-tissue antigen too strongly. Self-tissue antigens are expressed by thymic medullary epithelial cells by the action of a transcription factor in these cells called "autoimmune regulator" (AIRE) (60). If a T cell reacts too strongly with self-HLA:self-antigen it is potentially dangerous and, therefore, gets deleted. This negative selection mechanism that prevents self-reactive (autoimmune) T cells from circulating the body, is called central tolerance (61). A similar selection process applies for B cells in the bone marrow. This is thus the process that allows the adaptive immune system to distinguish antigens as foreign or self: any antigen not selected against during the negative selection process can potentially be recognized as foreign (62).

Depending on the class of the HLA molecule (class I or class II) they first bind to during the positive selection process, the T cells progress into either Cluster of Differentiation (CD)4+ or CD8+ expressing cells (γδ T cells leave the thymus negative for both). HLA class I molecules are expressed by all nucleated cells of the body and present intracellular peptides to CD8+ cytotoxic T cells that kill the target cell if binding occurs (63). A common cause of recognition is a target cell infected by virus presenting viral peptides on its HLA class I molecule. In contrast, HLA class II is only expressed by professional APCs such as DCs, B cells and macrophages. The HLA class II molecules present peptides derived from exogenous proteins, e.g. from bacteria or viruses that were captured outside the cell, to CD4+ T cells, also called helper T cells.

1.2.2 Activation and differentiation of T cells

The T cells that pass the dual selection process, leave the thymus as naive CD4+ or CD8+ T cells (Tn) and start scanning the scene, especially the lymph nodes, in search of their TCRs matching HLA:peptide-combination. However, the interaction between a TCR and its cognate HLA:peptide complex is only one of the two signals needed for successful activation of a naive T cell. Mature and activated professional APCs also need to provide a co-stimulatory signal to lower the activation threshold of the T cells. This is mediated through the APC molecules CD80 and CD86 binding to CD28 on the surface of the T cell (64)(Fig. 3). A pre-requisite for expression of these co-stimulatory molecules is that the antigen was taken up in the presence of "danger signals", i.e. evolutionary conserved molecular patterns of pathogens or cell damage that previously provoked the innate immune system. A T cell encountering antigen without co-stimulation will not become activated, but instead enters a state of anergy

rendering it functionally unresponsive. A similar requirement exists for B cell activation and this restriction is part of a process called peripheral tolerance which helps to ensure that autoreactive T cells that escaped negative selection in the thymus remain harmless.

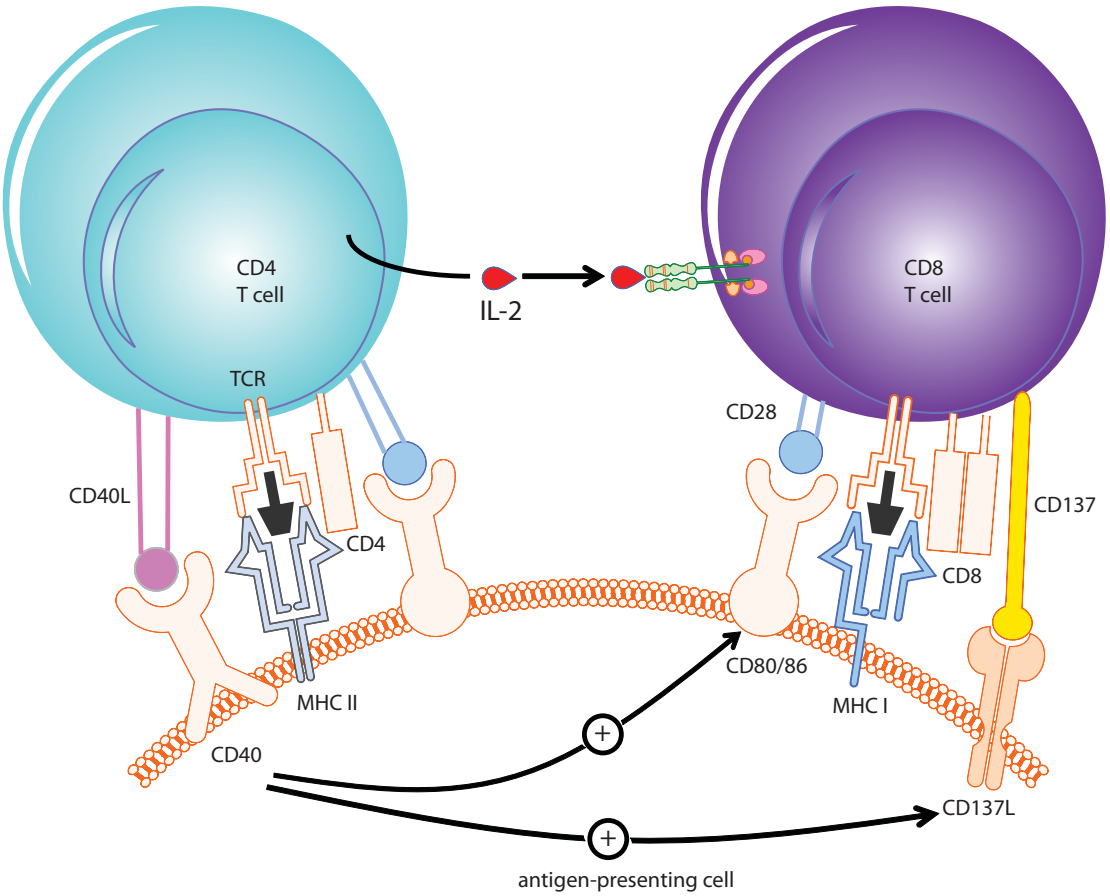

Fig.3 Most CD8 T cell responses require CD4 T cell help. Naïve CD8+ cytotoxic T cells require more stimulation to become activated than do naïve CD4+ helper T cells. In many cases, the naïve CD8+ T cells cannot become activated if not an effector CD4+ T cell interacts with the same antigen-presenting cell (APC). The activated APC presents potential pathogenic peptides to CD4+ T cells through MHC class II, and to CD8+ T cells through MHC class I. If the APC shows the correct antigen, the T cells bind to the MHC molecules with their TCRs and co-receptors CD8 or CD4. This binding is commonly called signal 1. Signal 2 is provided by the co-stimulatory receptor CD28 binding to CD80/86 on the APC. The surface expression of CD80/86 may be increased by CD4+ T cells to aid the stimulation of CD8+ T cells. The CD4+ helper T cell does this through the interaction of CD40 ligand (CD40L) and CD40. This interaction also upregulates CD137 ligand (CD137L) on the APC, which provides additional co-stimulatory signals to the CD8+ T cells by binding to CD137. Finally, the CD4+ T cell produces IL-2 and thus help drive CD8+ T cell proliferation.

APC: antigen-presenting cell, TCR: T cell receptor, IL-2: interleukin-2, CD40L: CD40 ligand

The type of effector T cells that arises is influenced by the cytokines produced by the activated APC and/or the surrounding. These additional cytokines are sometimes termed signal 3 (signal 1 being TCR engagement and signal 2 the co-stimulatory CD28-binding), and the inflammatory milieu, such as the type of pathogen, determines which cytokines the APC will produce.

Effector CD4+ T helper cells (Th) have been divided into subclasses partly based on their cytokine secreting profile (65). If the activated DC produces IL-12, the CD4+ T cell will differentiate into Th1 cells. Th1 cells produce IFNγ which, among many other functions, stimulates macrophages to more effectively engulf pathogens. DC acting upon a naïve CD4+ T cell with IL-4 turns it into a Th2 cell. Th2 cells produce IL-4, -5 and -13 which activate mast cells, eosinophils (cells of the innate arm) and

immunoglobulin E (IgE)-producing plasma B cells helping to control parasites. T follicular helper cells (Tfh) have been described as the T cell subset that helps proper activation of B cells in the lymph nodes (66). The Tfh cells can release both Th1 and Th2 typical cytokines helping to shape the class of antibody eventually secreted. A fourth major CD4+ T cell subset is the Th17 cells, characterized by their production of IL-17. This subset has anti-bacterial and anti-fungal properties through stimulation of neutrophils of the innate immune response (67). They also have immune suppressive functions (68, 69), just like the last CD4+ subset I would like to mention: the T regulatory cells (Tregs). Tregs are clearly part of the peripheral tolerance process, ensuring that healthy immune responses do not spiral out of control and also help to prevent autoimmune reactions. They are identified by the expression of transcription factor FoxP3 and the IL-2 receptor α-chain (CD25) and suppress immune responses through production of IL-10 and transforming growth factor-β (TGF-β)(65).

The CD8+ cytotoxic T cells directly kill their target cells by the targeted release of the pore forming protein perforin and subsequent granzymes inducing programmed cell death, also called apoptosis. Target cells are most often recognized by the surface expression of intracellular viral or malignantly- transformed self-proteins presented within HLA class I. The lethal and potent content of cytotoxic T cells makes them especially devastating if autoreactive. Perhaps because of this, naive CD8+ T cells require even more co-stimulation to become activated than do naive CD4+ T cells. If the APC cannot activate the naive CD8+ T cell on its own, an already activated CD4+ T cell can come to the rescue. The antigen-specific CD4+ helper T cell binds to the same APC, increasing its co-stimulatory activity partly by the interaction of CD40 and CD40-ligand (CD40L)(70). This interaction induces the APC to increase the expression of CD28 and another co-stimulatory molecule, CD137L, on its surface (Fig 3). The CD4+ T cell may also produce IL-2 and IL-21 driving the proliferation of the CD8+ T cell (71).

1.2.3 T cell memory formation

Upon activation, one of the first surface molecules to appear is CD69 which retains the T cell in the lymph node, allowing it to clonally expand and potentially become restimulated. Shortly thereafter, the co-stimulatory CD28 is down-regulated (72-74). As noted, IL-2 is produced by CD4+ T cells but also by activated CD8+ T cells, and CD25 (the α-chain of the IL-2 receptor) is accordingly upregulated on these cells allowing IL-2 to act in both an autocrine and paracrine fashion. The activated cells clonally expand, meaning that a cell bearing one specific type of antigen receptor (TCR) divides into numerous identical effector daughter cells that subsequently leave the lymph node and migrate to the site of infection. Once the pathogen has been killed and cleared, the expanded pool of clonal T cells contracts by apoptosis. Importantly though, some of the activated antigen-specific T cells will persist as memory cells which can be rapidly and efficiently re-activated if the same pathogen tries its luck again. This feature is the most important hallmark of the adaptive immune system and is described in more detail below.

The linear differentiation model postulates that when a naive T cell (Tn) encounters its cognate antigen, it differentiates into effector cells as described above. Once the invader in cleared, during the T cell pool contraction some effectors differentiate into memory cells by not undergoing apoptosis (75, 76). An asymmetrical model has also been proposed, where naive cells differentiate into distinct daughter cells, which are either short-lived effectors (that may only terminally differentiate) or long-lived memory cells (that may both regenerate and terminally differentiate) (77). Regardless of which model is correct, four T cell subsets with diverse differentiation status have been defined: the previously mentioned naive T cells (Tn), the central memory T cells (Tcm), the effector memory T cells (Tem) and the terminally differentiated T cells (Ttd) (78). These subsets are usually identified by the expression pattern of surface markers CD45RO, CD45RA, the adhesion molecule CD62L and/or the chemokine receptor CCR7. In the papers included in this thesis work, we have defined Tn as CD45RO-CCR7+, Tcm as CD45RO+CCR7+, Tem as CD45RO+CCR7- and Ttd as CD45RO-CCR7- (Fig. 4). As seen, Tn and Tcm express the chemokine receptor CCR7, allowing them to migrate to lymph nodes. In contrast, Tem and Ttd cell lack CCR7 expression, indicating they are committed to perform effector functions at the site of infection (79). Thus, Tcm cells have limited effector functions but proliferate and acquire effector cell characteristics after secondary stimulation, whereas Tem cell are already armed and involved in the immediate protection versus the specific pathogen.

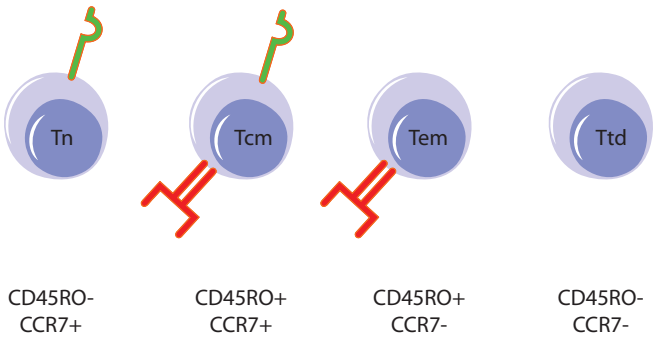

Fig.4 T cell memory subsets. The T cell subpopulations are defined by the differential expression of two surface molecules: The chemokine receptor CCR7 and CD45RO, an isoform of the receptor-linked protein tyrosine phosphatase CD45. Naïve T cells (Tn) express only CCR7, central memory T cells (Tcm) express both molecules, effector memory T cells (Tem) express only CD45RO, and terminally differentiated T cells (Ttd) express neither.

1.3 TRANSPLANTATION IMMUNOLOGY

Transplantation puts the immune system in a seemingly peculiar situation. After having evolved in parallel with pathogens for millions of years, this defense mechanism is, in the example of human organ transplantation, suddenly faced with foreign but indeed human tissue. However, the adaptive immune defense does not care about the source of the foreign agent. Rather, it just discriminates "self" from "non-self". Thus, a host-versus-graft (HVG) reaction, or rejection, of an organ or tissue is an ever-present risk after transplantation. This discrimination process is also one reason for the recognition of malignant cells which, due to their transformation, may acquire "altered-self" or "non-self" features known as tumor-associated antigens (80, 81).

In addition to the risk of a HVG reaction, transplantation of an immune system also carries the risk of a graft-versus-host (GVH) reaction in which donor immune cells

attack healthy tissue of the recipient. Due to these potentially lethal consequences, HSCT comes with much higher requirements of histocompatibility compared with organ transplantations. Ideally, organs would be well-matched as well, but since there usually is a shortage of donors, physicians accept mismatched organs and instead treat the patient with immunosuppressive drugs for the rest of their lives. In contrast, many potential donors are registered for HSCT but only a few, if any, are compatible enough for the specific patient.

1.3.1 Donor and recipient compatibility

As stated in section 1.2, the products of the MHC genes, called HLA in humans, are proteins that can present antigens to T cells. The MHC region is located on chromosome 6 and there are several genes in this region that encode diverse HLA antigens. The HLA is hence said to be polygenic, and all of these HLA genes are usually expressed simultaneously. HLA-A, -B and -C are classical MHC class I molecules that present antigen to CD8+ T cells, while HLA-DP, -DQ, and -DR are classical MHC class II molecules that present antigen to CD4+ T cells. Each cell has two sets of chromosomes (one of maternal and one of paternal origin) and HLA genes are co-dominantly expressed. Thus, every nucleated cell may express up to 12 different classical HLA molecules. Since the different HLA molecules bind different peptides, the polygenic feature of HLA allows an increased range of pathogens to be recognized by a given individual. Importantly, the MHC regions are not only polygenic, but also polymorphic, meaning that there are several isoforms of the same gene in the human population. As of April 2013 more than 9000 different HLA alleles have been identified and named, with the majority of them being discovered in the volunteers for the bone marrow (BM) registries (82).

Because of their importance in adaptive immune responses and their extensive diversity, the classical HLA molecules are the main factors determining histocompatibility. As a result, HLA-matching is an important part of HSCT. There are also non-classical HLA molecules (e.g. HLA-E, -F, -G, and the CD1 family members) which are more evolutionary conserved. They may present pre-defined peptide or lipid antigens and regulate NK and NKT cell responses. Because of their conserved (i.e. non-polymorphic) nature, their impact on HSCT outcome is considered negligible and are usually not included when tissue-typing donor and recipient before HSCT.

HLA genes are inherited as a whole on the chromosome, making up a so-called haplotype. Since each individual inherits two haplotypes, one from each parent, there is an approximate 25% chance that a sibling has the same HLA setup of 12 different classical HLA molecules, i.e. a 12/12 match (83). Improvements in polymerase chain reaction (PCR)-based techniques have allowed for genomic HLA-typing and identification of possible allele disparities. If no HLA-identical sibling is available, clinicians are left with four options: (i) an HLA-matched unrelated donor from the BM registries, (ii) a haploidentical donor, i.e. a donor with one shared haplotype, most

often a parent, (iii) an HLA-mismatched unrelated donor, or (iv) an HLA-matched or -mismatched cord blood unit, i.e. the focus of this thesis. Several diagnosis-, disease status-, and center-specific factors determine which alternative donor stem cell source is ultimately chosen.

Common to all stem cell sources is that the risk of GVH and HVG reactions (and, hence, transplant related mortality) increase with the number of HLA mismatches (83, 84). HLA molecules bind and present a massive amount of peptides from self-proteins made within the cell. Of importance for histocompatibility, not only HLA molecules are polymorphic. Other proteins in the human population also exist in different isoforms, resulting in different peptides being produced in different individuals. In a transplantation setting, these peptides can be recognized as foreign minor histocompatibility antigens (mHags), which may doom the mHag-expressing cell to destruction. The term "minor" indicates that these antigens are weaker inducers of immune reactions than the "major" histocompatibility complex-encoded HLA-proteins, but they have indeed been shown to impact transplantation outcome (85). Some polymorphic proteins that potentially could be mHAgs have been identified, but their clinical relevance is unclear (86). The only mHags currently possible to account for when choosing donor are for proteins encoded by the male Y-chromosome. Since Y-chromosome encoded genes are not expressed in females, female anti-male mHag-responses may occur, whereas male anti-female reactions do not. Using a female donor to a male recipient is associated with worse outcome and an increased risk for acute GVHD (87). This was also the only risk-factor for acute GVHD found in a large retrospective analysis by the Center for International Blood and Marrow Transplant Research (88). Donor sex is thus a non-HLA factor that may be included when searching for a suitable donor. Other non-HLA factors include donor age, previous pregnancies, cytomegalovirus (CMV) serostatus, and ABO blood type, although many of these have been shown to have only weak impact on transplantation outcome (89-91).

The HLA disparity in a haploidentical transplantation setting increases the risk of GVHD and graft failure. There are however some perks to using a family-member as a donor: the rapid availability of stem cells makes this choice attractive for patients whose disease progresses too fast for the time required to carry out an unrelated donor search. Moreover, the fact the graft is from a related individual increases the chance of matching other polymorphic proteins that otherwise might have become mHags and cause e.g. GVHD. However, because of the HLA-mismatch, haploidentical grafts were initially depleted of T cells to reduce the risk of GVHD unfortunately also increasing the risk of opportunistic infections and rejection (92, 93). More recently, protocols including post-transplant administration of the chemotherapeutic drug cyclophosphamide have been employed to wipe out early expanding allo-reactive T cell clones, which reduce GVHD while sparing other T cells (94).

If an unrelated donor search is initiated, the most important HLA genes to match are HLA-A, -B, -C, and the genes encoding for the β-chains of the class II molecules HLA-DR (-DRB1) and HLA-DQ (-DQB1) (83). Matching these five genes situated on two different chromosomes makes for a so-called 10/10 match, also known as the "gold standard", and transplantations with matched unrelated donors (MUD) have shown comparable relapse-free survival to that of matched related donor transplantations (34, 95). However, this "gold standard" is not always met when searching for an unrelated donor. Studies have tried to elucidate acceptable numbers and sorts of HLA mismatches and results include a tolerance of single mismatch in HLA-DQB1 (giving a so-called 8/8 match) (89, 96). There is however no consensus, and our single-center experience at Karolinska University Hospital shows that neither an HLA-C nor HLA-DQ mismatch negatively affected the outcome (97, 98). Conflicting results like these might reflect different effects of HLA-matching in different patient cohorts. Thus, although HLA-match is undeniably important, patient characteristics such as age and stage of the disease might also play major roles in contributing to outcome. Adverse effects of using an HLA mismatched donor may, for example, be less serious than waiting for a perfect match to then transplant a patient with a more advanced disease.

To hasten the process of identifying a suitable and available unrelated donor, Confer et al have proposed an electronic communication system to always have an updated view of volunteer engagement and motivation as well as a donor center ranking score system for the stem cell isolation performance of each collection site (99). These non-HLA donor related factors might in the future help rapid and successful searches. The speed of an adult donor search can, however, never be as readily available as a CB graft, since this graft source has already been collected, HLA-typed and stored. Thus, if a suitable CB unit is found, it can be shipped immediately. Another major advantage of CB as a stem cell source is that the HLA-matching criteria are less strict, increasing the chances of finding a donor. These benefits, as well as downsides, will be described in more detail under a separate section (1.4).

1.3.2 The allogeneic hematopoietic stem cell transplantation

After identification of a suitable donor, the practical work begins at the hospital ward. Prior to infusion of hematopoietic stem cells, the patient is subjected to conditioning therapy in which radiation and/or chemotherapeutic agents destroy recipient cells to (i) create space for the donor cells, (ii) suppress recipient immune responses against the graft, and (iii) to kill malignant cells if the patient suffers from malignant disease. Specific immunosuppressive drugs are also administered before infusion, to dampen recipient-mediated anti-graft responses, and after infusion, to reduce risk and severity of GVH reactions. Compared to the major surgeries associated with organ transplantation, the infusion of stem cells is a straight forward procedure, closely resembling a normal blood transfusion (Fig. 5). However, the complications that may follow are severe. After transplantation, patients are closely monitored by the ward staff. In most cases, the first days after transplantation are characterized by

pancytopenia, basically meaning that the patient completely lacks an immune system. Risk for infections is high during this period and patients are often kept isolated and administered prophylactic antibiotics. Within a few weeks, the new immune system starts to populate its new host and the patient is discharged from the transplantation ward. He/she still needs to be monitored for potential complications, but on an outpatient basis. After a couple of months to a few years, depending on many transplant-related factors, immunosuppression may eventually be tapered and a state of tolerance between the immune system and its new host is finally established. Thus, in a successful transplantation, there is no need for life-long immunosuppressive treatment.

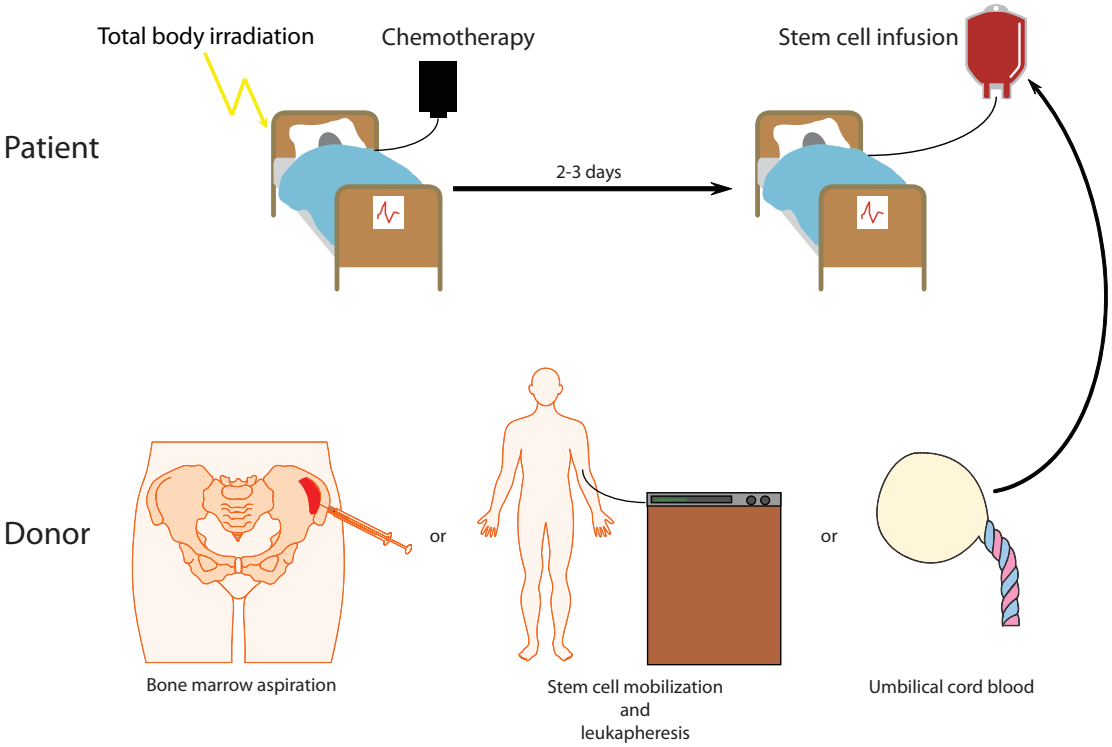

Fig.5 The allogeneic stem cell transplantation procedure. The patient is subjected to conditioning therapy involving chemotherapy and/or total body irradiation to destroy bone marrow cells and make space for the donor cells, to kill off as many malignant cells as possible (if underlying malignant disease), and to weaken the recipient immune system and reduce the risk of rejection. Donor stem cells are aspirated from bone marrow, or taken from peripheral blood if first mobilized by granulocyte-colony stimulating factor, or obtained from donor umbilical cord blood unit. The donor cells are subsequently infused to the patient through a central venous catheter.

1.3.2.1 Indications for allogeneic hematopoietic stem cell transplantation

The disease diagnoses eligible for allogeneic HSCT are changing every year and generally more diagnoses are added to the list than taken away. Transplantations were initially restricted to acute leukemia, severe aplastic anemia and SCID (18, 19). Later on, the more slowly progressing chronic leukemias were added to the list, although recently developed tyrosine kinase inhibitors have vastly improved the disease status of patients with e.g. chronic myeloid leukemia (CML) decreasing their need for transplantation (100). Nowadays, patients with diverse hematological disorders such as lymphomas, myelodysplastic syndromes, multiple myeloma, inborn immunodeficiency and metabolic disorders may all be treated by allogeneic HSCT. Autologous transplantations have been quite successfully performed for certain solid

tumors and autoimmune diseases (101, 102). Also, allogeneic transplantations which seem to benefit from an enhanced graft-versus-tumor effect compared to autologous transplants, show early promising results for patients with e.g. kidney cancer (renal cell carcinoma) (102). The European group for Blood and Marrow Transplantation (EBMT) and its American sister-organization, Center for International Blood and Marrow Transplant Research (CIBMTR), recurrently publish updated recommendations regarding practices and indications for HSCT (103).

1.3.2.2 Conditioning therapy before stem cell transfusion

The conditioning treatment is employed to eradicate recipient hematopoietic and malignant cells before transplantation. This is accomplished by aggressive cytotoxic chemotherapy, sometimes in combination with controlled radiation. Both techniques lead to DNA damage and cell death, especially for rapidly dividing malignant cells and stem cells. There are two major groups of regimens: myeloablative conditioning (MAC) and the later introduced reduced-intensity conditioning (RIC). Simply put, the MAC regimens are lethal and require infusion of hematopoietic stem cells to save the patient. With the less intense RIC regimens, the patient's own hematopoiesis may recover.

The aim of the myeloablative treatment is to kill off as many malignant cells as possible without causing lethal toxicity. Two common protocols include the chemotherapeutic agent cyclophosphamide (Cy) together with either total body irradiation (TBI) (104) or another cytotoxic drug called Busulfan (Bu) (28). These myeloablative protocols are often referred to as "Cy/TBI" and "Bu/Cy". As alluded to, myeloablative treatments are highly toxic and may cause e.g. veno-occlusive disease of the liver and damage to the central nervous system. Thus, monitoring of drug concentrations in the serum and individual dose adjustment is important to reduce toxicity (105).

At least since the late 70's, researchers have observed that allogeneic cells can mediate anti-cancer effects (21, 22). Being less intense, the RIC protocols rely on these donor cells to eradicate recipient hematopoietic cells and to control malignancy. This less toxic strategy has extended the use of allogeneic HSCT to older and sicker patients that otherwise could not tolerate conditioning therapy. There are many different and center-specific RIC-regimens but most of them include the chemotherapeutic agent Fludarabine in combination with immunosuppressive (mycophenolatemofetil or cyclosporin-A) or other chemotherapeutic (Cy, Bu, or melphalan) drugs (106-110).

1.3.2.3 Reconstitution of the new immune system

After transfusion of donor stem cells and, most importantly if not *ex vivo* depleted, mature donor T cells present in the graft, the new immune system start to take its place in the recipient. Generally, the different types of immune cells reconstitute in an orderly fashion (111, 112). The first donor cells to engraft are the monocytes, rapidly followed by other cells of the innate immune system: granulocytes, NK cells and megakaryocytes producing platelets. The appearance and persistence of neutrophil granulocytes, the most abundant type of white blood cell in our blood, are used as a marker of engraftment. This is a sign that the donor stem cells have found their way to the bone marrow and have started to generate the components of the immune system. Neutrophil numbers normalize approximately two to three weeks after transplantation with mobilized peripheral blood (PB) stem cells or BM grafts, but after four weeks with CB (111, 112).

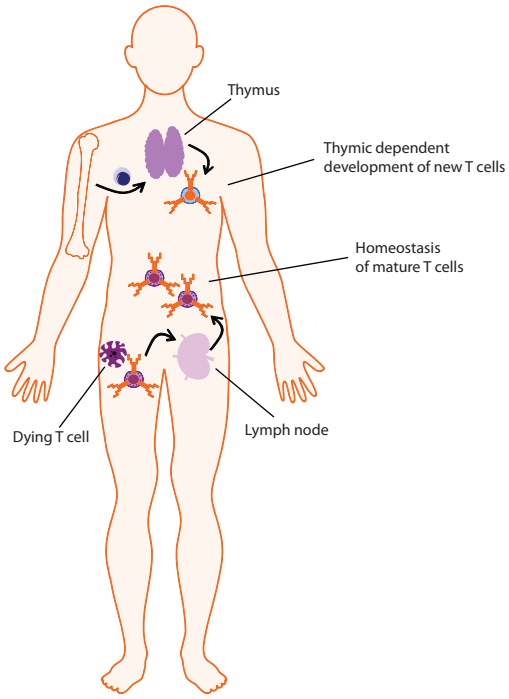

Fig.6 Two ways for T cells to increase in number: Thymic dependent *de novo* development and homeostatic expansion of mature T cells. Thymocytes emerging from the bone marrow migrate to the thymus where they undergo positive and negative selection. The few cells that pass the selection process are allowed to enter the periphery as naïve T cells. Mature T cells maintain their numbers by dividing and dying in roughly even pace. This process is called T cell homeostasis and is driven by cytokines such as IL-7. The mechanism is important in the immediate post-transplant situation where a few mature donor cells have access to high levels of cytokines, allowing them to fill the empty space left after conditioning therapy by a process called "homeostatic expansion".

The recovery of cells belonging to the adaptive arm is slower compared to the innate cells, partly reflecting the higher thresholds for activation and proliferation of these cells. As seen above, the source of stem cells influences the rate of immune reconstitution suggesting that non-stem cells following the graft are major players in shaping the initial repopulation of the host. This feature is most evident for T cells, where still one year post-transplantation many of them are descendants of the mature donor T cells that were originally co-transfused with the stem cells (113). The cell damage induced by the conditioning treatment results in high levels of e.g. IL-7 and IL-15 which, together with the empty space available, allows these T cells to proliferate through a mechanism called homeostatic expansion (Fig. 6). Depending on the graft source (T cell numbers in mobilized PB > BM > CB) and immunosuppressive regimens (*ex vivo* or *in vivo* T cell depletion or inhibition), this T cell population is more or less restricted in its TCR diversity and, hence, quite limited at recognizing pathogens. The T cell pool eventually diversifies from three (PB, BM) to six (CB) months post-transplantation and onwards by the emergence of newly developed T cells that have undergone "education" in the thymus (52). Of note, thymic function fades with age, and the production of naive T cells decreases in adults in comparison with children (114, 115). The thymus may also

be damaged by conditioning treatment and alloreactive cells and, hence, highly intense myoeloablative regimens and GVHD negatively affects the recovery of the patient's immunity (114, 116).

The CD8+ T cell numbers rise faster than the CD4+ T cells, reaching normal levels approximately one year post-transplantation compared with two years for the CD4+ cells. This imbalance partly accounts for the characteristic inversed CD4/CD8 ratio seen compared with healthy controls. CD8+ T cells binding cognate antigen without help from CD4+ T cells may develop normally but they usually have both reduced proliferative capacity and lack memory formation leading to their name, “helpless T cells” (117). This phenomenon may, for example, also be responsible for the poor CD8+ T cell responses seen when the CD4+ T cell numbers drop in HIV-infected patients (118). The CD4/CD8 ratio is shifted even more in patients suffering from GVHD (119) or cytomegalovirus (CMV) infection (120) suggesting a dominant role for homeostatic and antigen-driven expansion in the regeneration of CD8+ T cells, and less so for CD4+ T cells (121).

The restricted diversity and disproportionate characteristic of the T cell pool gives rise to inadequate T cell immunity after transplantation. Although cell numbers eventually normalize, patients remain at higher risks of infection during the first two years after transplantation compared with healthy and age-matched controls, and even beyond for patients with chronic GVHD (122).

1.3.2.4 Three post-transplant events involving T cells: graft-versus-host disease, graft-versus-leukemia effect and rejection

The conditioning therapy regimens used today kill the recipient immune system to varying degrees but seldom eradicates them completely. As a result, both recipient and donor immune systems transiently co-exist in the early phase post-transplantation and may react against each other and against recipient tissue. These bidirectional immune reactions, or alloreactions, are the cause of three major post-transplant events: graft-versus-host disease (GVHD), graft rejection and the graft-versus-leukemia (GVL) effect (21, 123-125) (Fig. 7).

T cells are thought to be the main effectors in all three events (126-128). Not surprisingly, risk of graft rejection increases when recipient T cell immunity remains strong and the donor T cell compartment is weak. This is the case after RIC regimens (129), after T cell depletion of the graft (128), and when haplo-identical (130) and CB grafts (131) are used.

Two forms of GVHD have been defined: acute and chronic (132). These forms differ in time of onset and clinical features, where acute GVHD (aGVHD) usually develops within 100 days and chronic GVHD (cGVHD) usually beyond 100 days post-transplant. Clinical manifestations are also distinct. While aGVHD is characterized by fast and severe tissue destruction, cGVHD more resembles slowly progressing rheumatic

autoimmune diseases (133-135). Similar to the adaptive immune response towards a pathogen, allogeneic reactivity first needs components of the innate arm to ignite the flame of T cells. This feature is apparent in the currently accepted model of aGVHD pathophysiology (136). According to this model, aGVHD begins with the tissue damage inflicted by the conditioning therapy which disrupts the physical epithelial barriers inducing interaction between innate immune receptors and microbial structures. This concept explains why aGVHD most often affects organs having microbes on their epithelial surface, such as the skin and the gastrointestinal tract. This interaction leads to substantial release of inflammatory cytokines and chemokines, attracting and

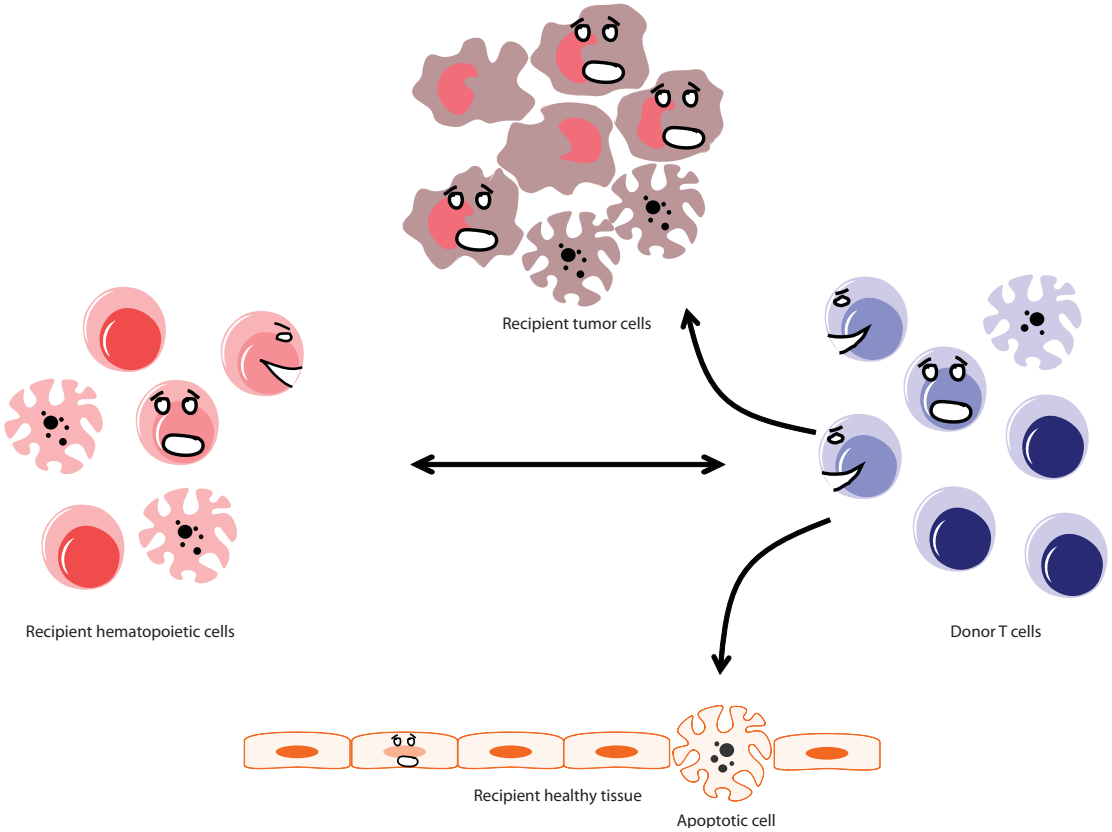

Fig.7 Three post-transplant events involving T cells. Depending on degree of suppression, available antigens, and the pro-inflammatory state, donor T cells may attack recipient cancer cells, remaining hematopoietic cells and healthy tissue. Remaining recipient immune cells may also recognize donor cells as foreign and react to them, in worst case scenario rejecting the graft.

activating other parts of the immune system, as has been described in section 1.2. Of importance, activated recipient and donor APCs may present recipient antigen to donor alloreactive T cells initiating their effector functions, among them the cytotoxic (cell-killing) function of CD8+ T cells (137).

The GVHD-process is swamped with positive feedback loops which amplify the reactions further and explain why aGVHD is so hard to treat. Current standard therapy for development of GVHD is high-dose corticosteroids, but it is not always successful (138). Of course, the best would be to prevent development of GVHD which is why patients receive prophylactic treatment, described in section 1.3.2.5.

The GVL effect and GVHD often go hand-in-hand. Already early on in the allogeneic field, investigators observed that leukemia patients suffering from GVHD had less risk

of relapse, suggesting that alloreactive cells within the graft could mediate anti-cancer effects (21, 123). Further support came when leukemia relapses were shown to respond to donor lymphocyte infusions (DLI) (123, 127, 139-141). For T cell-mediated allogeneic reactions to take place, antigens must be recognized by TCRs and T cell activation threshold need to be reached. The activation threshold could potentially be lowered by the proinflammatory milieu created by the conditioning regimen which, in turn, may be propelled forward by the GVHD process (21, 142). Thus, leukemia-reactive T cells might benefit by the mere presence of inflammatory cytokines produced by other alloreactive cells. However, the antigen(s) associated with the GVL effect are often unknown. Some tumor-specific antigens have been identified, be it mutated endogenous or viral-associated proteins (143). However, many recipient antigens may also be shared between malignant and healthy cells and, thus, the dangerous GVHD-inducing T cells could in theory be the very same cells that eliminate leukemic cells. Despite this potential overlap, evidence of tumor-specific and tumor-associated antigens (80, 81, 144) as well as tumor-infiltrating T cells in cancer patients (145, 146) continues to raise the hope of dissecting the GVL effect from GVHD in the future (147).

NK cells have also been suggested to take part in the GVL effect, especially in situations where T cell immunity is absent, e.g. after T cell-depleted haplo-identical transplantations (148). NK cells recognize "missing self" when inhibitory receptors on donor NK cells lack matching ligands on recipient hematopoietic cells. This feature can be exploited by deliberately choosing a donor with mismatched inhibitory NK cell ligands, and has shown some promising results for patients with ALL after haplo-HSCT (149).

1.3.2.5 Immune suppression as prophylaxis for rejection and graft-versus-host disease

Since memory T cells divide slowly in the absence of their cognate antigen, they are quite resilient to chemotherapy (150). Hence, there is a risk that recipient T cells remain after conditioning treatment. If these cells recognize donor cells as foreign, graft rejection may follow (151). To counteract this, recipient T cell reactivity can be inhibited before undergoing HSCT. Immunosuppressive drugs act on a more specific level than the broadly reactive chemotherapeutic drugs. For example, Cyclosporine A (CsA) is a calcineurin inhibitor that blocks transcription of IL-2 reducing T cell activation (152, 153). One of the most common immune suppressive treatment protocols is CsA in combination with the cytostatic drug Methotrexate (154, 155). Several other drugs with similar mechanisms of action may be employed, e.g. Tacrolimus (156) that blocks IL-2 secretion and Sirolimus (157) that blocks responsiveness to IL-2, all with the common feature of inhibiting T cell activation.

After transplantation, the donor immune system would ideally be given room to develop and populate its new host. However, as has been described in section 1.3.2.4,

there is a risk for T cell mediated GVH reactions. Therefore, the immunosuppressive treatment initiated before transplantation is continued after the transfusion of donor cells. Since T cells have been acknowledged as the main mediators of GVHD (126), a more direct way of inactivating donor T cells is to deplete them *ex vivo* or *in vivo* with neutralizing anti-T cell antibodies. These antibodies may be monoclonal (OKT-3, Campath) or polyclonal (anti-thymocyte globulin (ATG)) and have the potential to eliminate GVHD (158-161). However, without donor T cells, the risk of rejection and opportunistic infections increases and chance of GVL effect decreases and, thus, the treating physician needs to constantly fine tune the immune suppressive treatment.

1.3.2.6 Common infections after transplantation

Post-transplantation, virtually any pathogen can infect the severely immunocompromised patient. As the various components of the immune system gradually recover (as described in section 1.3.2.3) fewer pathogens will have the opportunity to bloom. Three different phases can be distinguished after transplantation based on the incidence of certain infections (162). During the first month, when mucus layers are still damaged by chemotherapy and neutrophils have yet to appear, bacteria normally residing in and on our body may enter the blood stream. These can often be controlled by prophylactic antibiotic treatment (163). Furthermore, diverse fungi genera like *Candida* and *Aspergillus* may see a chance to blossom during this period and prophylactic anti-fungal treatment is common for this reason (164, 165).

After engraftment, during the intermediate phase between 30 and 100 days after transplantation, the risk of bacteremia decreases. However, since T cells are the last cells to recover, viral infections remain common during this period (166, 167). The usual suspects are herpesviruses, especially CMV and Epstein-Barr virus (EBV). These viruses are extremely common in the human population but are normally controlled by an immune competent individual. When T cell control is lost at time of transplantation, the latent viruses are suddenly able to replicate unchecked. Anti-viral drugs such as diverse nucleoside analogues that incorporate into or interact with viral DNA or RNA blocking their replication are available for treatment (168). However, these drugs are associated with toxic side-effects, especially kidney damage, and they are not always effective (169). Alternative treatment options include adoptive transfer of virus-specific T cells obtained from stem cells or a third party donor (170, 171).

Although cell numbers are on the rise during the late phase, after 100 days, T cell immunity is not fully recovered. Thus, reactivation of herpesviruses like Herpes-Simplex and Varicella-Zoster is seen as well as infections of encapsulated bacteria (166). In addition, patients with chronic GVHD and patients transplanted with alternative donor sources, e.g. CB, have delayed immune reconstitution and may remain at threat of infections from all phases (132, 166, 172).

1.3.2.7 Ways to improve restoration of T cell immunity after transplantation

Since a lack of T cells post-transplantation contributes towards several post-transplant complications, considerable research is aimed at facilitating the donor T cell restoration after engraftment. This can be achieved either by infusing the patient with additional donor T cells, better known as DLI, or by boosting thymic function to stimulate the *de novo* production of T cells. Quite a few agents to boost thymic function have been used in mice studies including IL-7 and keratinocyte growth factor (KGF) (113). In humans, administration of IL-7 resulted in proliferation of already mature naive T cells but did not increase *de novo* production (173). Since this kind of thymic-independent expansion in theory is unable to discriminate between donor or recipient-derived T cells, there is a risk in the RIC setting that recipient-derived T cells expand increasing the risk of graft rejection. However, a recent phase I trial of recombinant human IL-7 (CYT107) for patients with T cell depleted grafts has provided promising observations of enhanced TCR diversity suggesting a thymopoietic effect (174). The stimulatory effect of KGF on human thymus remains to be tested, although animal studies suggest a role in protecting the thymus from conditioning- or GVHD-induced damage (175, 176).

DLI can be administered therapeutically in combination with tapering of immune suppression to treat a threatening relapse or to pre-emptively assist restoration of donor T cell immunity (139). When referring to DLI, one usually means a non-manipulated donor lymphocyte product. However, since collection methods vary, the cell content and proportions of various subsets are often undefined and may vary (177). In its heterogeneous form, therapeutic DLI can effectively treat patients with relapsing chronic myeloid leukemia (CML) (139, 178, 179) but has been less successful for acute leukemias and other malignancies (127, 140, 180-182). This difference in response incidence may be due to the inherent aggressiveness of acute leukemia compared with the more slowly progressing CML. Furthermore, phenotypical characteristics of CML cells may play a role, e.g. potentially better antigen presentation compared to other malignant cells (183). However, efficient DLI-responses have been described for diseases other than CML but often in combination with other therapeutic regimens (184). For example, patients relapsing with acute myeloid leukemia (AML) can respond to DLI if the tumor burden is first reduced by chemotherapeutic drugs (185). Moreover, in a pre-clinical study, DLI had enhanced effect on diverse B-cell lymphomas if given in combination with the monoclonal anti-CD20 antibody Rituximab (186).

Efforts have been made to manipulate the lymphocyte product to increase DLI effectiveness. For example, pre-activation of donor T cells with anti-CD3/CD28 beads before infusion has shown promising results in a phase I study for patients with leukemic relapses other than CML (187). Unfortunately, the phase II study was prematurely terminated due to insufficient funds (ClinicalTrials.org identifier: NCT00674427). The timing of DLI administration is also of importance and our group

has shown responses in patients with acute leukemia when DLI is administered in the early stages of relapse (188).

To control the severity of GVHD induced by donor lymphocytes, a dose-escalation regimen has been successfully employed although it does delay the GVL effect (178, 179). Furthermore, the DLI product may be purged of cells reactive to host antigens as defined by cellular expression of activation-induced antigens such as CD25, CD69 or CD137 (189-191). The identified cells can be separated from the non-reactive cells using various techniques and reducing the risk of GVHD. Selective depletion could also eradicate reactivity towards hematopoiesis-restricted mHags although potential negative effects on the GVL effect remain unclear (192). An interesting mouse study has provided insight into different cell killing mechanisms used by GVHD and GVL-mediating T cells for myeloid leukemia: the GVL effect was mainly perforin-mediated whereas GVHD was primarily carried out by the interaction of Fas:Fas-ligand (193). It would be interesting to selectively manipulate these mechanisms in favor of the GVL effect.

With advances in T cell engineering techniques, the DLI product can also be custom-made to reduce risk of GVHD while attacking specific malignant or viral targets. There are multiple types of these specific adoptive immunotherapies. For instance, we and others have selected virus-specific T cells from the donor and infused them directly into the patient to treat opportunistic viral infections without causing GVHD (171, 194). Other strategies include priming naive T cells with viral or tumor antigens *in vitro* before infusion (195, 196), and active transfer of e.g. tumor-specific TCR genes to donor T cells (197).

For acute and chronic lymphocytic leukemia (ALL and CLL), both diseases with poor response to conventional DLI (198, 199), a hope for the future is genetically manipulated T cells bearing chimeric-antigen receptors (CARs) (200-202). A CAR is basically an extracellular, antibody-derived recognition moiety fused together with intracellular T cell activation domains. A few T cells can be transfected by gammaretroviral or lentiviral vectors encoding the CAR gene and subsequently stimulated to proliferate to adequate numbers using anti-CD3 monoclonal antibodies (mAbs) or anti-CD3/CD28 beads. Most CARs tested to date have the antibody part directed against CD19, i.e. a surface molecule of most B-cell malignancies, and intracellular signaling domains of the TCR ζ-chain in combination with domains from co-stimulatory molecules such as CD28 and CD137 (203, 204). As of yet, promising results have been reported for patients that not have undergone allogeneic HSCT (205, 206). We eagerly await the results of phase 1 and phase 2 studies of CAR-expressing T cells as prophylaxis for patients with high risk of relapse after transplantation (ClinicalTrials.gov identifiers NCT01475058 and NCT00840853)

1.3.2.8 Development of chimerism, or when two immune-systems share host

In some cases after transplantation, especially after RIC regimens, the recipient immune system remains to co-exist with the donor system turning the patient into what is commonly called a "mixed chimera" (207) (the chimera in Greek mythology was a fire-breathing creature made up of parts from several different animals) (Fig. 8). Chimerism development can be monitored by PCR- based techniques looking at genomic differences between the recipient and donor cells (208).

Depending on what cell subset(s) are mixed, treating physicians can get clues as to what direction the post-transplant events are taking. For example, if the percentage of recipient stem cells and T cells are on the rise in consecutive measurements, the likelihood of graft rejection is high (209, 210). Similarly, relapse of an underlying malignant disease can be detected by chimerism analysis as an increasing amount of cells belonging to the malignant cell linage (211-213). This is most evident in the MAC setting during which recipient hematopoiesis is replaced fast and entirely by donor cells. In the RIC setting, the picture is much more uncertain and persistent mixed chimerism is not always a sign of relapse (214). Both relapse and rejection can be reverted by administering a boost of donor lymphocytes (DLI) (179, 180, 188, 215).

Fig.8 Development of chimerism. The Chimera was in Greek mythology a supernatural creature made up of parts from several different animals. This myth serves as an analogy to the chimerism development after hematopoietic stem cell transplantation, when two or more immune systems co-exist within the patient. Illustration by Melissa M Norström.

Remaining recipient hematopoietic cells are not always bad though. Immunologically competent recipient cells could potentially help to fight opportunistic infections commonly seen after transplantation. Moreover, following myeloablative conditioning, mixed chimerism is associated with a decreased risk of acute GVHD (216). Patients with non-malignant diseases such as severe aplastic anemia or sickle cell anemia may have symptoms remedied without full donor chimerism (Walters, Patience, Leisenring 2001, Saito, Chiba, Ogawa 2007). Since these non-malignant patients cannot benefit from a GVL effect and since DLI increases the risk of GVHD, some mixed chimeras have been left unaddressed for several years with sustained mixed chimerism (217-219). The mixed characteristics of these patients indicate that allogeneic reactions between immune systems does not always lead to rejection or GVHD and that some sort of tolerance may develop.

Reasons behind chimerism development are still vague but lessons from the CBT field suggest that the immune systems do reject each another through the action of CD8+ cytotoxic T cells (220). In the referred study, CD8+ T cells of the "winning" unit of

double cord blood transplantation (DCBT, in which two cord blood units are co-transplanted to increase cell numbers) produced IFNγ in response to the non-engrafting unit but not vice versa. Similar CD8+ T cell-mediated reactions may well be responsible for the GVH and HVG reactions occurring after transplantation. Interestingly, our group has observed long-term mixed chimerism between the two CB units after DCBT for over two years after transplantation (221). The study of these mixed chimeric patients is the basis for Paper II in this thesis.

1.4 UTILIZING UMBILICAL CORD BLOOD AS STEM CELL SOURCE

There is an approximate 25% chance for patients in need of a stem cell transplantation to find an HLA-matched donor among their siblings. For patients lacking such a donor, there are three alternative stem cell sources potentially available: unrelated adult donor from the bone marrow registries, related but only partially HLA-matched ("haploidentical") donor, or umbilical CB obtained from cord blood banks (222). Since the four papers included in my thesis concern CB transplantations (CBT), I will in these last introductory sections focus on CB as stem cell source.

1.4.1 The pros and cons of cord blood transplantations

With the thousands of successful CBT performed to date (47, 223), it is almost inconceivable that just 20 years ago the placenta and the umbilical cord were considered biological waste. Since the realization that neonatal blood was enriched with stem cells, the field virtually exploded (37). Four advantages of CB can be identified as explanations for its success (222): (i) Compared to the invasive procedure for collecting adult stem cells, CB collection comes with no risk to the donor. (ii) Due to the maternal-fetal barrier of the placenta, there is a low risk that the CB is infected by pathogens. (iii) As soon as a suitable CB unit is found, it is rapidly available from the cord blood bank. Depending on the disease status, the time to transplant might be critical and the fact CB units are pre-harvested stands in contrast to adult donors where a stem cell collection is first required. (iv) HLA mismatch requirements are reduced without an increase in aGVHD (131, 224) enabling most patients to find a 4-6 of 6 HLA-allele matched donor (225).

Disadvantages can be summed up in two points: (i) The number of cells in a CB unit is limited. This makes it challenging to obtain the minimum cell dose for adult patients and heavier children. In addition, since all blood is used for the transplantation it is impossible to infuse a boost of stem cells or lymphocytes, should that be needed. (ii) There is a delay in the time to engraftment which may lead to increased transplant-related mortality (TRM) (51, 226) in the majority of cases due to infections (172, 225, 227).

Despite the delayed engraftment and reduced alloreactivity (as seen by the reduced risk of aGVHD), leukemia-free survival (LFS) is comparable to that seen with unrelated

adult donors. This feature has been observed in both children and adult patients with acute leukemia transplanted with a single CB unit (4-6 of 6 HLA-matched) in two large registry-based studies (51, 226). In fact, children with well-matched CB had superior LFS when compared to 7-8 of 8 HLA-matched unrelated adult blood (226). Similarly, adult leukemia patients undergoing double cord blood transplantation have shown higher TRM but lower relapse rates compared with matched unrelated donors (15% vs. 43%), resulting in a net-effect of equal LFS between the donor sources (228). These findings of acceptable LFS have not been corroborated in our single-center experience, where a relapse-rate of 29% at one-year post-transplantation has been recorded for CB transplanted patients (229). We can imagine multiple reasons for this discrepancy, among them a possible overly dampened immune system by the action of both ATG and third party mesenchymal stem cells previously used at our center (230).

From an international perspective however, higher TRM but equal or superior LFS points towards a GVL effect in CBT patients distinct from the immune reactivity that causes GVHD and protects from infections. The mechanism of this feature is unclear, but clues can be found when looking at the biology of cord blood (section 1.4.2).

1.4.2 The biology of umbilical cord blood

The developing immune system of a neonatal is, naturally, young. In the context of transplantation, this naivety has some positive implications. For example, the small size of a CB unit is counteracted by an increased proliferative potential of CB-derived stem cells. Compared with adult BM, CB stem cells generate higher number of daughter cells (231, 232) which might be explained by the “early” phenotype (CD34+CD38-) (233) and longer telomeres observed in CB stem cells (234, 235). Telomeres are the end sections of chromosomes which are shortened for every round of replication. If the telomere becomes too short, the risk for DNA damage increases and the cell stops dividing to avoid mutations. The telomere length can thus be seen as a marker of cell age. Some highly dividing cells and cells that possess the ability to self-renew, such as CD34+ stem cells, express the enzyme telomerase that extend telomeres and preventing them from becoming too short. Hence, young CB stem cells with longer telomeres have an increased proliferative potential allowing transplantation with approximately 10 times fewer cells compared with adult bone marrow (236).

In adults, the constant turnover of mature T cells in the periphery in combination with the decreased thymic output of fresh T cells into the circulation results in a T cell population with reduced telomere lengths (237). Over time, these aged T cells experience diminished TCR signal transduction and other deleterious alterations (238) collectively termed immunosenescence (239). Interestingly, CB-derived T cells express telomerase (240) and the implications of this expression in the transplantation setting will be speculated upon next.

A pregnancy can be considered a highly successful albeit transient transplantation. Not to say that all is quiet on the placental front; for the duration of the pregnancy, the fetal allograft actively defends itself from attack by maternal T cells by producing diverse T cell inhibiting factors. These placental derived factors do not only inhibit maternal T cells, but developing fetal T cells as well. Thus, fetal T cells develop in the presence of increased levels of progesterone, IL-4 and prostaglandin E2 (241) and reduced tryptophan levels (242). Therefore, CB-derived T cells produce for example only low levels of IFNγ (243). Indeed, antigen-inexperienced and inhibited CB-derived T cells probably contribute to the poor T cell immunity during the first months after CBT resulting in increased death rates due to opportunistic infections (172). However, time is key here, as the slow initial reconstitution is followed by considerable improvements of immunity: at day 100 post-transplantation, infection-related deaths are similar between recipients of CB and of 7-8 of 8 HLA-matched unrelated adult donors (244). Nine months after CBT, T cell recovery is comparable (245) or even superior to that of unrelated adult donor transplantations at the same time point (246). These improvements could well be attributed to the telomerase activity of CB-derived T cells mentioned above, possibly preserving telomere lengths despite a high cellular turnover during homeostatic expansion. Moreover, an efficient thymic-dependent pathway for the *de novo* production of CB T cells has been suggested by the observation that CB recipients have a higher TCR diversity two years post-transplantation compared with recipients of adult BM (247). An increased thymic output and TCR diversity might be just what is needed to maintain a GVL effect without causing GVHD. Additionally, CB-derived NK cells have comparable or better cell killing function than NK cells from adult BM grafts (248) and these could contribute to the comparable relapse rates seen.

1.4.3 Strategies to accelerate cord blood stem cell engraftment

Several studies have correlated high total nucleated cell dose (TNC) and CD34+ stem cell dose in CB grafts with a high rate of neutrophil engraftment, while a slow rate of neutrophil engraftment correlates with graft failure and TRM (249-251). Because of these connections, methods to enhance the engraftment of CB transplanted patients have been developed. One of the most accepted and standardized strategies is the somewhat non-intuitive use of two different CB units simultaneously, a so called double cord blood transplantation (DCBT) (45). The idea is not to provide the patient with dual immune systems, but is to increase the TNC during the initial critical time after transplantation. In a matter of weeks, one of the CB units generally prevails and alone makes up the new immune system (252-254). Reasons behind unit predominance remain unclear and there is no correlation between unit dominance and TNC, CD34+ or CD3+ graft dose, degree of HLA mismatch, viability, or order or route of infusion (46, 255). However, the capacity of the engrafting unit to reject the non-engrafting unit has been shown (220). Possible collateral damage to recipient cells during this initial GVG interaction may account for the increased incidence of

aGVHD as well as the lower relapse rates observed compared with single unit CBT (252, 256). Reduced relapse rates after DCBT have also been reported as compared to sibling and unrelated BM donors, which, in combination with increased TRM, result in a similar progression-free survival (228).

DCBT have also been used as a platform to expand CB-derived stem cells. With one unit as a backup, diverse manipulations have been carried out on the other unit (257). However, extrinsic and/or intrinsic signals that favor optimal self-renewal instead of differentiation are not yet completely defined. Cytokine-based expansion systems have been tried in clinical trials with little change in engraftment kinetics (258, 259). More recently, Delaney et al. took advantage of the Notch signaling pathway, which has been shown to enhance self-renewal of CD34+ cells (260). Using an engineered form of Notch ligand, an adequate number of CD34+ stem cells could be expanded and initial clinical result seem promising as to shortening the time to engraftment (257).

Stem cell responsiveness to chemoattractants can be utilized to increase homing of infused cells to the BM, enhancing engraftment. For example, the stromal-derived factor 1 (SDF-1) is secreted by stromal cells in the BM and stem cells migrate towards the SDF-1 gradient through its interaction with the chemokine receptor CXCR4 (257). This signaling axis can be positively modulated by various molecules involved in inflammation, e.g. the third complement component (C3), and CB grafts may thus be primed before infusion to enhance the BM homing characteristics (261). However, to the best of my knowledge, a clinical trial employing this strategy has yet to be performed.

To accelerate engraftment, Frassoni and co-workers injected CB cells intraosseous (i.e. directly into the BM) and reported excellent outcome (262). However, we did a prospective randomized study following myeloablative conditioning and infusion of bone marrow grafts and found no difference in engraftment or other outcome parameters if the stem cells were infused intraosseously or with standard intravenous technique (263). The distribution in the body of hematopoietic stem cells marked with I^{111} after intraosseous or intravenous infusion was also similar.

Most retrospective multicenter analyses of outcome after CB transplantation suggest that cell dose is more important for engraftment and survival than HLA compatibility between donor and recipient (225). However, a recent study suggested that HLA-identical CB grafts had a better outcome than those who were only partially matched (264).

1.4.4 Strategies to enhance cord blood T cell immunity

Patients transplanted with CB have a higher death rate due to infections compared to transplantations from adult donors (131, 226, 265). In part, this is due to a low CD34+ count in the CB graft, but also a lower CD3+ T cell dose has been associated with

higher probability of infection-related death at 6 months post-CBT (172). This indicates that mature T cells in the graft provide protective anti-viral immunity for several months before the recovery of the thymic pathway. However, as was described in section 1.4.2, CB-derived T cells are largely naive and have developed in the presence of inhibiting placental factors. As a form of irony, CB is the only stem cell source where DLI is not possible. Since all blood collected from the CB unit needs to be used for transplantation to ensure adequate cell dose, there is no opportunity for DLI preparation at the time of graft procurement. Ethical and logistic reasons also exclude contacting the donor for extra cells.

To enable DLI for CB transplanted patients, researchers began expanding CB-derived T cells *ex vivo* for possible subsequent infusion (266-268). Expansion was feasible with anti-CD3 mAbs and cytokines (267, 268) but superior expansion rates were found when adapting the T cell activation and expansion protocols originally developed for adult peripheral blood, described in section 1.3.2.7 (187, 269, 270). In these protocols, T cells are activated by the action of paramagnetic beads conjugated to anti-CD3 and anti-CD28 antibodies and then allowed to proliferate in the presence of IL-2 (266, 271). The paramagnetic beads operate as artificial APCs that interact with TCRs while providing co-stimulatory signals via CD28, essential for naive cells to become activated. In addition, CD28 signaling has been shown to maintain telomere lengths of T cells (272, 273) and induce telomerase activity even in adult derived T cells (187). Thus, the anti-CD3/CD28 beads might not only activate naive T cells but also improve the persistence of adoptively transferred T cells (272, 273).

Although details and routes of T cell differentiation remain undefined (see section 1.2.3), it is clear that different T cell subsets have specialized functions and that not all would be beneficial in adoptive T cell therapy (274, 275). For example, naive cells are unable to perform effector functions such as killing tumor cells. T cells with effector memory phenotype (Tem) are superior to central memory T cells (Tcm) at acquiring cytolytic- and cytokine-producing effector functions *in vitro* (79, 276). However, in mice and non-human primates, the Tcm cells have shown to exhibit superior therapeutic effects, probably due to a more long-lived phenotype *in vivo* (275, 277, 278). According to these findings, an optimal expansion protocol would generate high frequencies of Tcm cells. Therefore, recently reported protocols aim at shortening the expansion time as well as adding homeostatic cytokines such as IL-7 and IL-15 to the protocol (279, 280).

2 AIMS

The general aim of this thesis work was to develop new strategies and tools to handle patients transplanted with umbilical cord blood. The studies aimed to address the following observations and limitations of everyday clinical work:

- After umbilical cord blood transplantation, a donor lymphocyte infusion to treat e.g. threatening rejection or relapse is not possible due to low cell numbers of the original graft.
    - We specifically aimed to develop a protocol to bypass this current limitation. This was achieved by expanding T cells from cord blood grafts used for transplantation with anti-CD3/CD28 magnetic beads and interleukin (IL)-2 for possible use as donor lymphocyte infusion **(Paper I)**.
- Three out of seven evaluable patients that received a double cord blood transplant at our center presented with a mixed donor chimerism more than two years post-transplantation. As such long-term mixed chimerism is extremely rare, with only a few cases reported globally, we investigated the underlying transplant characteristics in an effort to elucidate this relatively high incidence in our center and/or patients.
    - We specifically aimed to characterize the phenotype and functionality of the immune system(s) of patients with mixed donor chimerism **(Paper II)**.
- Diagnostic criteria for e.g. graft-versus-host disease are based on already developed clinical features after onset and, therefore, therapies might be too late to have an effect. Ideally, predictive markers for different complications could identify high-risk patients allowing for well-timed and appropriate treatments. The overall one year survival of only 55% after cord blood transplantation at our center underscores the need for early markers of such transplant-related complications. We hypothesized that expansion of cord blood derived T cells could function as a surrogate marker for graft quality and, hence, a tool to predict cord blood transplantation outcome.
    - We specifically aimed to correlate phenotypical and functional data from expanded cord blood T cells with clinical features after cord blood transplantation (**Paper III**).
- The cord blood T cell expansions are intended for clinical use and are, therefore, limited to culture reagents of “Good Manufacture Practice” (GMP)-grade. As this became more common practice, more GMP-grade cytokines have become commercially available, among them the cytokine IL-7. This cytokine has been shown to be important for homeostatic proliferation of T cells leading us to hypothesize that the addition of IL-7 to our expansion protocol would improve the expansion rate.
    - We specifically aimed to phenotypically and functionally study the effect of IL-7 on expanded cord blood T cells in conjunction with increasing doses of IL-2 (**Paper IV**).

3 RESULTS AND DISCUSSION

The advent of using unrelated umbilical cord blood (CB) as hematopoietic stem cell source has dramatically increased the chances of finding a donor for patients in need of a transplant but lacking an HLA-matched sibling. The rapid availability, apparent lower risk of graft-versus-host disease (GVHD) and higher permissiveness for HLA-mismatches (281) gave CB a positive reputation, increasing its popularity in transplantation centers from the early nineties and onwards (36, 223). However, like all known stem cell sources, CB comes with some drawbacks, the most evident being the limited cell dose and naive cellular status. With high probability, these features cause the delayed immune reconstitution observed in CB transplants, resulting in high risks of graft rejection and infectious complications (172, 282-285). This limitation, together with improved transplantation protocols for e.g. haploidentical donors (94, 286) has resulted in a hesitation of using CB at our center.

The aim of this thesis work was to provide novel tools to treat and predict complications in patients after CB transplantation. Despite the mentioned disadvantages, CB remains a promising stem cell source with extraordinary proliferative potential (287), high telomerase activity (240), vast T cell receptor diversity (287) and its full potential has probably not yet been realized. Indeed, the debate of preferred alternative stem cell donor is ongoing (50, 222, 288).

Specifically, **Papers I and IV** in this thesis address the challenges imposed by delayed immune reconstitution after CB transplantation. As donor lymphocyte infusions (DLI) has been unavailable for CB transplanted patients, the primary aim of these two papers was to develop a protocol to make DLI available also for these patients. DLI, in the form of expanded T cells, may now be employed to counteract threatening rejections or relapses.

By successfully expanding polyclonal and functional T cells from CB, we also created a platform enabling sophisticated adoptive T cell therapies of selected or constructed antigen-specificities in the future. Furthermore, in **Paper III** we focused on predicting transplant complications for timely and suitable treatments, e.g. administration of DLI, by correlating the phenotype of expanded T cells to clinical outcome post-transplantation. Finally, in **Paper II** we characterized the immune systems of local mixed double donor chimeric patients to obtain insight as to why both units persist, and what the implications may be. Thus, the results presented here in **Papers I-IV** might offer physicians more factors and treatment options to consider when choosing an alternative stem cell donor.

In this section I will present the main results from the four scientific papers included in this thesis and discuss their implications in the field of allogeneic stem cell transplantation. The papers are numbered chronologically but will be mentioned in order of suitability for the discussion. For more detailed results, experimental setup and complete figures, please refer to the respective papers.

3.1 MAKING DONOR LYMPHOCYTE INFUSIONS AVAILABLE FOR CORD BLOOD TRANSPLANTED PATIENTS

In the adult stem cell transplantation setting, an unmanipulated DLI may be adequate to address an emerging rejection or relapse (139, 178). Donor lymphocytes have unknown, but possibly multiple advantageous antigen specificities that can potentially target recipient hematopoietic cells as well as malignant and virus infected cells. Hence, all disease diagnoses have the potential to respond to DLI, although, for reasons still unidentified, the graft-versus-leukemia (GVL) effect of DLI has up to now been effective mostly in patients suffering from chronic myeloid leukemia (CML) (289, 290). In light of the slow immune reconstitution as well as increased risks of infection and rejections (172, 244, 282), DLI could provide a considerable advantage after CB transplantation. Unfortunately, as the CB graft is limited, it is not possible to save cells from the graft at the time of transplantation, and ethical and logistic reasons makes it impossible to contact the original donor. Therefore, DLI has previously not been a feasible treatment modality for patients receiving an unrelated CB unit.

With the introduction of clinically safe cell expansion protocols, the community began to experiment with expanding leukocytes as well as hematopoietic stem cells from the CB graft (257, 291). Clearly, the most potent effector cell in the lymphocyte population is the T cell, driving both anti-viral and anti-leukemia responses in addition to the detrimental GVHD (123, 126). Expansion of CB-derived T cells presented as a rational approach to enable DLI for CB transplanted patients.

In **Paper I**, T cells were expanded from a 5% aliquot taken from 13 CB grafts used for transplantation at CAST. By cross-linking cell surface CD3 and CD28 with antibody-coated magnetic beads, luring T cells into activation, we could both activate and magnetically separate the T cells for subsequent expansion in medium supplemented with 600 international units (IU)/mL IL-2 for 8-10 days (Fig. 9). Compared to previously reported expansion protocols, we could show a superior proliferation rate (median 150-fold expansion vs. 100-fold) (266, 271). Medium was added continuously to maintain a concentration of 0.3 million cells/mL in our cultures, lower than the 1 million cells/mL reported by Parmar et al (266). This difference could, in part, explain the improved proliferation rate observed in our expansions.

One aspect missing in previous literature is deep descriptive data of the expanded cells. That is, there has been an unsatisfactory understanding of what cell subsets are expanded and their functionality (266, 271). Therefore, we set out to characterize the expanded CB-derived T cells regarding their phenotype, clonality and responsiveness to allo- and mitogenic stimuli. To this end, we used multicolor flow cytometry for surface and intracellular staining, standard ^{51}Cr-release assays, mixed lymphocyte reactions and spectratyping techniques.

Encouraging observations of polyclonal, cytokine-producing and proliferating effector memory cells led us to conclude that expanding CB T cells is a feasible way of

producing DLI for CB transplanted patients. Specifically, our expansion protocol resulted in T cells of predominantly CD45RO+ central (CCR7+) and effector (CCR7-) memory phenotype. One concern we had regarding this activated phenotype was a risk of exhaustion (239, 277). To study this, we monitored the proliferative potential of the cells after expansion in response to allogeneic mismatched and haploidentical target cells and found that it was comparable to that of non-expanded CB derived and peripheral blood (PB) T cells.

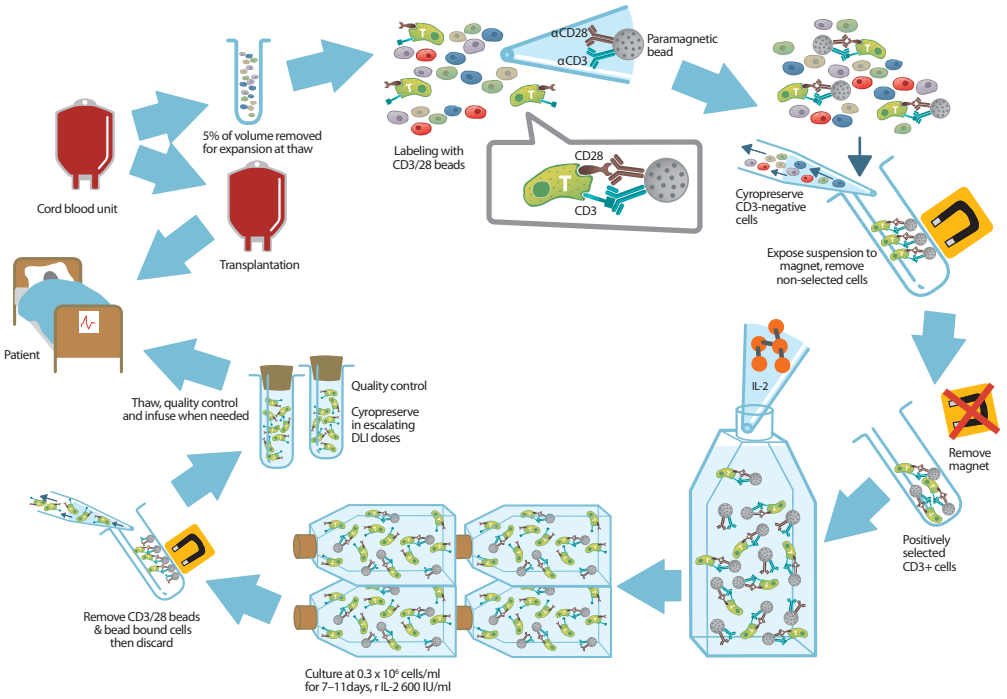

Fig.9 A schematic picture of the procedure of expanding cord blood-derived T cells. Adapted from Okas, M, doctoral thesis 2010

Despite the relatively high dose of IL-2 added, we did not observe an increase in the frequency of FoxP3+CD25+CD4+ regulatory T cells (Tregs) compared to non-expanded CB or PB T cells. This is in contrast to previous studies (292, 293) and is also somewhat surprising since Tregs have a high expression of IL-2 receptor and, hence, should be highly responsive to IL-2 stimulation. However, for DLI, lower number of Tregs is undoubtedly an advantage as their presence could suppress the very immune reaction we are trying to induce by the DLI. In other words, with many Tregs in the DLI product we would push the gas pedal and the brake simultaneously. Importantly, the "gas pedal" was indeed functional as expanded cells could produce cytokines in response to non-specific stimulus and perform cytotoxic actions following allogeneic and malignant stimuli (Fig. 10). The responsiveness to multiple stimuli was also aptly reflected in the polyclonal distribution of T cell receptors among the expanded T cells (Fig. 11). Thus, in our hands, CB-derived T cells could be successfully expanded to functional and polyclonal conventional T cells in sufficient numbers for DLI in just over one week.

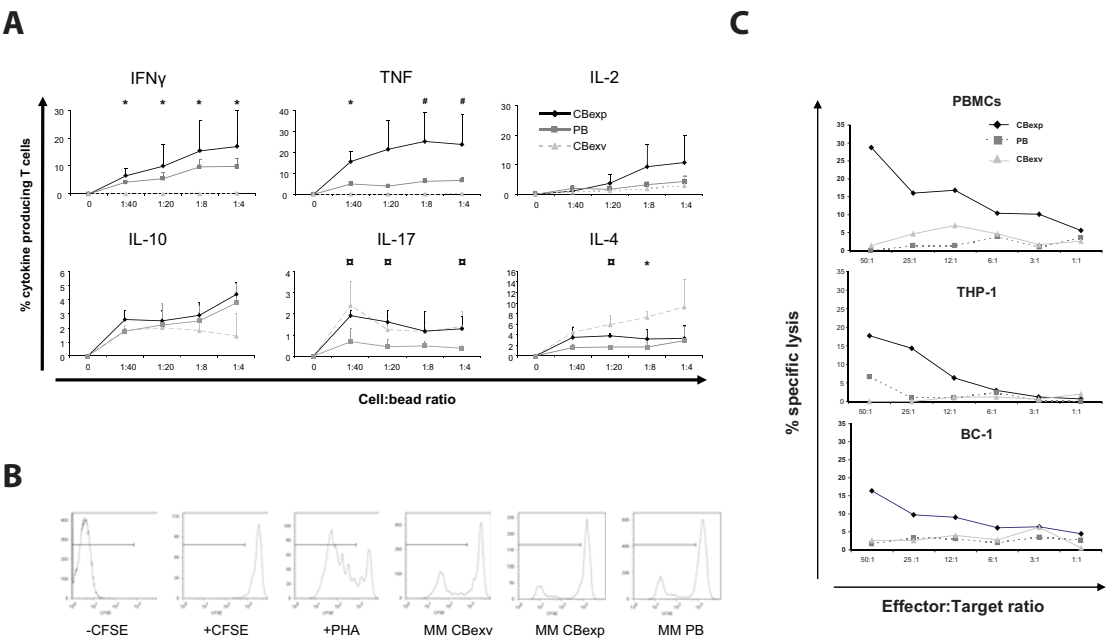

Fig.10 Expanded cord blood-derived T cells respond to non-specific stimuli, lyse malignant cells, and proliferate when exposed to allogeneic targets in a comparable manner to non-expanded T cells from cord blood or adult peripheral blood. A. T cells were stimulated with anti-CD3 beads at different ratios for six hours, stained for intracellular cytokines and acquired by flow cytometry. Graphs display mean % ± SD of T cells positive for indicated cytokines. *Significant difference ($p<0.05$) between CBexp and CBexv, ¤Significant difference between Cbexp and PB, #Significant difference between both Cbexp and CBexv, and CBexp and PB. **B.** Negatively selected T cells from PB, CBexv and CBexp were stained with CFSE and exposed to irradiated mismatched (MM) PBMCs, or PHA as a positive control. MM was defined as <3 matches regarding HLA A, B and DRB1. On day six, cultures were harvested, stained with antibodies, and acquired by flow cytometry. Decreased fluorescent intensity of CFSE indicates T cells proliferation. **C.** Chromium-labeled target cells were incubated with T cells purified from CBexp, CBexv and PB. Specific lysis of allogeneic PBMCs, THP-1 (leukemic cells) or BC-1 (lymphoma cell line) target cells was measured as release of chromium from dying cells at indicated effector:target ratios. Figure adapted from Paper I.

CBexp: expanded cord blood-derived T cells, CBexv: *ex vivo* non-expanded cord blood T cells, PB: adult peripheral blood, MM: HLA mismatched, CFSE: carboxyfluorescein succinimidyl ester, PHA: phytohemagglutinin A

However, although highly functional *in vitro*, we were concerned about the fate of the expanded cells after infusion *in vivo*. CB-derived T cells expanded by our protocol have been infused on three occasions on vital indication with only limited effects (unpublished observations). The cells were infused in low doses to decrease risk of toxicity which could explain the absence of a positive effect. However, it is unknown whether these cells might have experienced cytokine deprivation after infusion when moved from an environment of high IL-2 concentration to a human body with lower levels as has been suggested before (271, 279). If true, cells may quickly have become apoptotic after infusion due to a lack of survival signals. To address this, and to study other possible effects, we attempted to lower the dose of IL-2 by adding GMP-grade IL-7 to the expansion protocol (**Paper IV**).

The cytokine IL-7 has been shown to protect T cells from apoptosis induced by cytokine withdrawal *in vitro* (294). This characteristic of IL-7 is a result of both upregulation of anti-apoptotic and inhibition of pro-apoptotic signals as well as an increase of telomerase activity slowing down telomere shortening (295). The anti-apoptotic feature of IL-7 could be especially important for CB-derived T cells due to their higher expression levels of the IL-7 receptor α-chain (296) and their particular sensitivity to activation-induced cell death compared to naive adult T cells (297).

Despite the anti-apoptotic effect of IL-7 described above, there was no decrease in the frequency of Annexin V positive, i.e. apoptotic, cells when expanding CB-derived T cells in IL-2 and IL-7 compared to IL-2 alone (**Paper IV**). However, several other differences emerged between the different culture conditions. We collected and froze down cord blood units from the maternal ward at Karolinska sjukhuset, Huddinge. After thawing, we activated and selected T cells from the units in the exact same way as in **Paper I**, but instead of culturing the cells in 600 IU/ml IL-2 only, we used 12 different conditions of increasing IL-2 concentrations ranging from 0 to 600 IU/mL, with or without addition of IL-7 at 20 ng/mL.

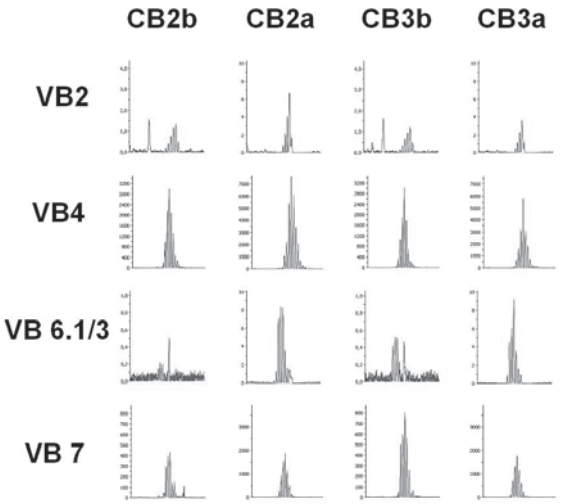

Fig.11 Expanded cord blood-derived T cells display a polyclonal pattern of T cell receptors with a Gaussian distribution of CDR3 fragment lengths. The variable domain of the T cell receptor β-chains (VB) has a hypervariable region named complementarity determining region 3 (CDR3) that is partly responsible for recognizing antigen. The CDR3 lengths of different VB were investigated by PCR-based spectratyping before and after expansion. Representative plots from two cord blood units before (CB2b, CB3b) and after (CB2a, CB3a) expansion are organized in rows for four different VB. In each plot, CDR3 fragment size distribution in base-pairs is shown on the x-axis and relative fluorescence on the y-axis. Figure adapted from Paper I.

At all concentrations of IL-2, the addition of IL-7 further increased the proliferative rate of the T cells, as has been reported (279). This increased proliferation could have been the result of less cell death, although undetectable by Annexin-V staining. Importantly, however, the increased expansion rate will allow us to shorten the expansion phase by at least a day, making the DLI product more readily available for the patient. Of all culture conditions tested in **Paper IV,** condition 6 displayed the highest proliferative rate despite having a low IL-2 dose (100 IU/mL) (Fig. 12). Due to this, we considered this combination the most promising for clinical use.

This cytokine combination (i.e. 20 ng/mL IL-7 and 100 IU/mL IL-2, condition 6) increased the frequency of CD45RO+CCR7- effector memory cells, especially evident in the CD4 compartment. This finding stands in bright contrast to the study by Davis et al using similar culture conditions, where a majority of cells maintained naïve CD45RA+CD62L+ phenotype (279). Reasons behind this discrepancy are unclear. CD45RA generally has an inverted relationship to CD45RO and CD62L is, to a large extent, co-expressed with CCR7 (78). Thus, the definition of a naive T cell used by Davis et al in theory concur with our own (CD45RO-CCR7+) and is most probably not the explanation of the difference seen. Further studies will elucidate whether the target cell concentration (0.3 million/mL vs. 1 million/mL), the IL-7 concentration (20 ng/mL IL-7 vs. 10 ng/mL IL-7), the time of expansion (7 days vs. 12-14 days) or selection method (simultaneous positive selection and activation vs. negative T cell selection before activation is the cause for the observed differences.

Despite their differences, the expansion protocols proposed by us and by Davis et al might both bring something to the table. With a high proportion of naive cells, an infused DLI product should be able to home to secondary lymphoid tissues, be primed by cognate antigens, and result in an *in vivo* expansion and differentiation into effector cells. Indeed, Davis et al demonstrate *in vitro* that their cells may be primed by mitomycin-C-treated leukemic cell lines resulting in cytotoxic capacity of the T cells towards leukemic cell lines (279). However, to see any effect, the expanded cells needed to be restimulated with the cell lines for three weeks. In contrast, cells of memory phenotype expanded by us, killed both leukemia and lymphoma cell lines readily and directly without the need for priming (**Paper I**) (Fig. 10c). Since polyfunctional effector memory cells are exactly what is missing in a CB graft (298) with, as a result, little transfer of protective memory specific for either malignant cells or viruses, we propose a role for these expanded cells to quickly bridge that gap. Interestingly, the addition of IL-7 to our expansion protocol resulted in a higher frequency of polyfunctional cells, i.e. cells producing three or four of the cytokines IFNγ, tumor-necrosis factor (TNF), IL-2, and degranulation marker CD107a after mitogenic stimulation, compared to cells expanded with IL-2 alone (**Paper IV**) (Fig. 13). Polyfunctional T cells have been shown to be more effective than T cells producing only one cytokine in mediating responses towards malignancies as well as viruses (299-301), an encouraging sign for the future clinical use of these expanded cells. Future studies will reveal whether a "golden mean" may be paved, where, for example, negatively selecting T cells before activation (as Davis et al) but using 20 ng/mL IL-7 (as we did) could lead to a polyfunctional, but still less differentiated, T cell product with enhanced *in vivo* proliferative potential.

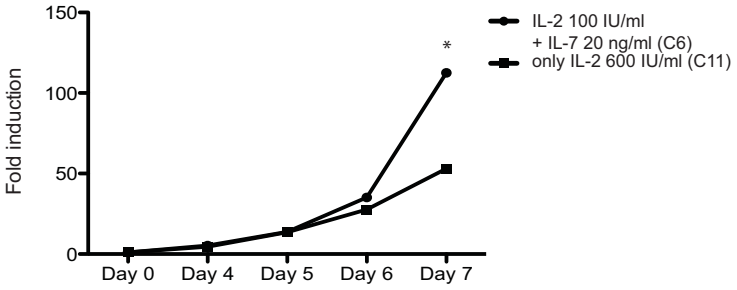

Fig.12 A combination of IL-7 and low-dose IL-2 results in higher proliferate rate compared to high-dose IL-2 alone. Cord blood derived T-cells were positively selected, stimulated with anti-CD3/CD28-coated paramagnetic beads and cultured for seven days in different concentrations of IL-2 with or without IL-7. The figure shows the kinetics of the mean fold induction of the 10 expansions in C6 versus the 10 expansions in C11. Figure adapted from Paper IV.

C6: condition 6 (20ng/mL IL-7 and 100 IU/mL IL-2), C11: condition 11 (600 IU/mL IL-2)

As mentioned in **Paper I**, no increase of Tregs was observed after expansion compared to *ex vivo* non-expanded CB despite a high dose of IL-2. Addition of IL-7 to the expansion protocol (**Paper IV**) slightly decreased the proportion of Tregs, probably due to a combination of a lower IL-2 dose and lower IL-7 receptor expression resulting in a decreased Treg response to IL-7 compared to conventional T cells (302, 303). This finding supports the use of IL-7 in our expansion protocol further.

In vivo, IL-7 is primarily produced by non-hematopoietic stromal cells and is usually accumulated during lymphopenia, e.g. after a transplantation (304). This accumulation could provide the continuous cytokine stimulation needed for an *in vivo* persistence of

infused T cells. Unfortunately, we did not study the expression of the α-chain of the IL-7 receptor (IL7Rα, CD127) in the expanded population (**Paper I and IV**), making it hard to judge their responsiveness to further IL-7 stimulation *in vivo*. Davis et al showed a downregulation of CD127 by their protocol, but also an ability to *in vitro* upregulate CD127 after thaw and culture for 24 hours without supplemented cytokines (279). Their finding points towards a possibility for re-expression also *in vivo*, but since our protocol differs on a few mentioned parameters this feature remains to be studied in our expansion setting.

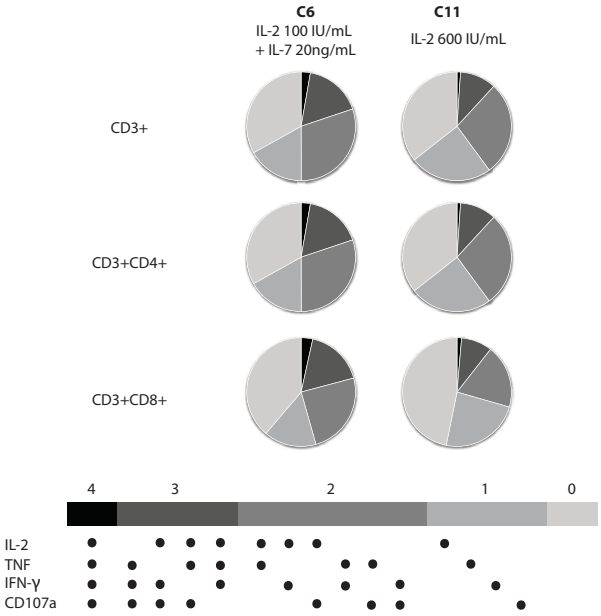

Fig.13 Addition of IL-7 to expansion protocol yield higher frequencies of polyfunctional T cells. Expanded cord blood-derived T cells were stimulated with Ionomycin and PMA, incubated in 37°C overnight and subsequently stained for degranulation marker CD107a and cytokines TNF, IFNγ and IL-2 and analyzed by flow cytometry. The mean frequency of five experiments is presented for condition 6 (C6) and condition 11 (C11) for CD3+, CD3+CD4+ and CD3+CD8+ cells positive for 0,1,2,3 or 4 of the analyzed factors. The bottom graph details the different combinations of factors included in the analysis. Figure adapted from Paper IV.

The transplantation community has, by the two studies reported in **Paper I and IV,** gained an additional tool to potentially treat threatening relapses, rejections and, although not tested, possibly viral reactivations in the CB transplantation setting. The expanded cells from clinically used CB grafts were in **Paper I** frozen in a dose-escalating scheme, allowing a safe but effective T cell dose to be found. This methodology relies on the hypothesis that a yet unknown effector:target cell ratio exists that increases the GVL effect without causing GVHD and it has been shown to function when infusing adult donors with CD3+ cells with decent tolerability (11% aGVHD II-IV, 8% extensive cGVHD)(305).

Further support for the applicability of cell expanded and activated by our protocol comes from the June lab, the pioneers of anti-CD3/CD28 stimulation procedure (270, 306). They have shown in the adult donor setting that pre-activated donor T cells induced complete remission in 8 out of 18 relapsing patients with diagnoses usually poorly responsive to conventional unmanipulated DLIs (ALL, AML, CLL, non-Hodgkin lymphoma) (187). In this study, Porter et al introduces an interesting hypothesis of disease-induced suboptimal activation and, thus, anergy of T cells *in vivo* that may be overcome by activating donor cells *in vitro* before infusion. Besides the promising remissions seen, this phase I study also showed a reasonable safety profile of just 7 cases of aGVHD (≈40%) after DLI, with only two of these showing grades over II. Although shown in an adult PB transplantation setting, with an expansion protocol devoid of supplementary cytokines, higher bead:cell ratio and an unreported resulting memory phenotype, this method of pre-activating T cells could have great impact for

CB transplantation. Not only is there a limitation in the number of CB lymphocytes, they have also been shown to be inefficient cytokine producers (307, 308), have reduced cytolytic activity (309) probably owing to immunomodulatory placental factors (241, 242) and have an absence of cognate antigens *in utero* (172). These deficiencies are evidently alleviated by the activation and expansion protocols described in **Paper I and IV**.

The reduced functionality of CB-derived lymphocytes together with the low cell dose explain the delayed immune reconstitution. Leukocyte count is recovered approximately 1 year after CB transplantation but the CD4/CD8 ratio does not return to normal until around 2 years post-transplant (172). Notably, in **Paper IV** the addition of IL-7 significantly increased the CD4/CD8 ratio in the expanded product, possibly due to a higher IL-2 dependence of the CD8+ T cell compared to the CD4+ T cells. These observations also advocate the pre-emptive use expanded T cells to facilitate immune reconstitution as has been proposed for DLI in PB transplantation (310-312). Hence, we await the results of a clinical phase I study involving infusion of expanded CB cells 14 days after transplantation (ClinicalTrials.gov identifier: NCT00281879). With an activation and expansion phase of only 7 days, these expanded T cells could be infused pre-emptively one week after transplantation to e.g. eliminate minimal residual disease, control viral reactivation, and reduce the CD4+ and CD8+ T cells imbalance earlier.

3.2 PREDICTING TRANSPLANT RELATED FEATURES AND COMPLICATIONS AFTER CORD BLOOD TRANSPLANTATION

Normally after a transplantation, treating clinicians rely on diagnostic markers such as skin rashes, viral titers and recipient/donor chimerism for the delicate balance of immune-suppressive versus immune-enhancing treatments. However, such diagnostic markers are only detectable after the onset of clinical symptoms and, since timing of treatment is important, i.e. the earlier the treatment the higher the chance of success, predictive biomarkers would be most beneficial. The overall one-year survival of 55% after CB transplantation at our center highlights the need for early risk markers (229). Early insights could be important to address transplantation-related hazards as well as seemingly neutral features such as which CB unit "wins" after a double cord blood transplantation (DCBT).

In **Paper III**, we aimed to correlate phenotypic and functional characteristics of T cell expansions to diverse clinical features after CB transplantation. That is, we hypothesized that the expansion could be utilized as an indirect indicator of graft quality and, thus, as a tool for risk prediction. For this study, we used T cells from 33 CB grafts expanded with the same protocol introduced in **Paper I** (i.e. anti-CD3/CD28 beads, 600 IU/mL IL-2, no IL-7, and 8 days of expansion). A cryotube containing T cells from each expansion not needed for DLI was thawed and subsequently stained for analysis by multicolor flow cytometry. The cells were also stimulated non-specifically

with phorbol 12-myristate 13-acetate (PMA) and ionomycin and their cytokine production studied by Luminex assays and intracellular staining before flow cytometric acquisition. These phenotypical and functional data were then correlated to clinical features of the transplanted patients collected from the records at CAST.

The most striking finding from **Paper III** was that patients having T cell expansions with a frequency of CD69+ cells above the median were more likely to survive long-term post-transplant than those with a CD69+ cell frequency below (Fig. 14). The same association was observed for CD94+ T cells and Tregs, although not as clear and significant. CD69 was expressed to a high level relatively to CD94 (median 32% vs. 1.5%) and is also an easily accessible surface molecule (as opposed to the intracellular FoxP3 in Tregs), making it the most interesting biomarker for further studies. Reasons for the association are unclear, and it is only possible to speculate on why a high proportion of CD69+ T cells in this artificial model system would indicate a survival benefit.

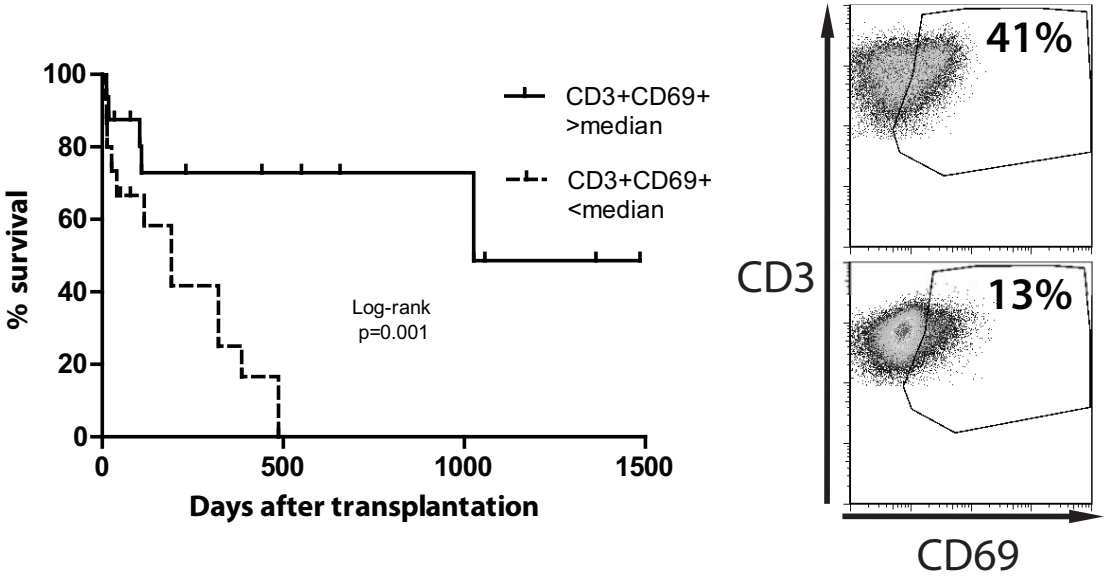

Fig.14 Cord blood T cell expansions with a frequency of CD69+ cells above the median are more likely to survive long-term after transplantation than those with a CD69+ cell frequency below. T cells from cord blood grafts were stimulated with magnetic beads coupled to CD3/CD28 monoclonal antibodies and cultured in medium with 600 IU/mL recombinant IL-2 for 8 days. Expansions were divided into two groups on the basis of expressing above- or below median levels of activation surface marker CD69, evident by flow cytometric analysis. The two groups were subsequently compared to the overall survival of the corresponding patients using a Kaplan-Meier analysis. Representative FACS-plots of the expanded T cells from the groups with above-median or below-median levels are shown to the right of the Kaplan–Meier curve. Figure adapted from Paper III.

The glycoprotein CD69 is upregulated early after T cell activation and appears to internalize the sphingosin-1-phosphate (S1P) receptor $S1P_1$, retaining T cells in lymphoid organs for possible restimulation *in vivo* (313, 314). Assuming that the expansion reflects immune reconstitution *in vivo*, the activated CD69+ population could prolong survival by increasing anti-viral or anti-tumor reactions rather than reducing the risk of GVHD. If anything, CD69+ T cells could increase the risk of GVHD. Indeed immunomagnetic allodepletion of CD69+ cells from graft or DLI has been tried to control GVHD for adult stem cell transplantation (191, 315). Although the CD69 negative population in the referred study was devoid of alloresponses and had preserved anti-viral and -tumor responses, a good proportion of allo-, viral-, and tumor-reactive T cells were CD69+ and thus this depletion method appears too crude

(191). With our data in mind, CD69+ depletion could even be detrimental in CB transplantation. Although not correlated to lower relapse rates or reduced viral reactivations, a combined effect provided by these activated CD69+ T cells might have been responsible for the observed survival advantage.

Regardless of mechanism, the frequency of CD69+ T cells in the expansions could be prognostic. Thus, if a future CB T cell expansion shows a CD69+ frequency below 32%, special attention could be given to the patient. Similar to previous literature (172, 225), infection was a relatively common cause of death in the cohort (5 of 15 deaths), hence, additional infectious prophylaxis and/or DLI could be considered. Notably, we observed the same positive correlation of prolonged survival and frequency of CD69+ T cells in a more recent study of non-expanded CB grafts, confirming our conclusion of CD69 as a functional indicator for the state of the graft (316). While studying non-expanded grafts is more direct it suffers from small numbers of accessible cells, allowing only limited analyses. Thus, expanding the cells may be a way to increase the number of potential analyses while preserving the observational intrinsic quality of the graft.

Lowering the dose of IL-2 and adding IL-7 to our expansion protocol rendered lower frequencies of CD69+ T cells (C6 vs. C11 in **Paper IV**). Further studies will determine whether/how this will associate to patient survival. On a side note, it is interesting that addition of IL-15 to expansions of CB-derived T cells upregulates CD69 (317). Hence, if the assumption that expansions are reflections of *in vivo* immune reconstitution hold true, the use of IL-15 to tweak the expansion protocol (see section 4.2) could potentially increase patient survival if infused.

Lymphocyte recovery at day 60 after CB transplantation has been associated to prolonged survival (318). This finding endorses the use of a pre-emptive DLI product proposed in section 3.1 if a patient presents with a low lymphocyte count at day 60. If the T cell expansion is indicative of graft quality and predicts clinical outcome, it would be expected that the *in vitro* T cell proliferative potential would be positively correlated to e.g. lymphocyte recovery and subsequent prolonged survival. Surprisingly, despite fold inductions ranging from 0 to 1650 (n=33), the *in vitro* proliferation rate did not correlate to any studied clinical parameter. One possible explanation is that the *in vitro* conditions are indeed artificial and cannot be considered exact reflections of *in vivo* conditions, but only as indicators of graft quality evident by markers such as CD69.

Other interesting markers identified in **Paper III** were CD45RO and CCR7, surface molecules commonly used to characterize the degree of T cell differentiation (78). After TCR engagement, the naive CD45RO-CCR7+ T cells usually upregulate CD45RO and, according to the linear differentiation model (319), either continue to express CCR7 (central memory phenotype) or downregulate it (effector memory phenotype) (78). The vast majority of expanded T cells in **Paper III** expressed CD45RO, probably since they had met an artificial antigen presenting cell (anti-CD3/CD28 beads) to

crosslink their TCR complexes. Nonetheless, a small proportion of the cells did not express CD45RO, and the larger this proportion the lower the risk for skin and liver GVHD was in our patient group. Thus, a more naïve status of the expanded cells was predictive of protection from GVHD. To complicate matters, CD45RO is downregulated as the memory cells terminally differentiate (again, according to the linear differentiation model) and, as a result, both naive and terminally differentiated T cells may be within this observed CD45RO negative population. This could be the case, since both naive and terminally differentiated T cells are poor responders and, hence, might be poor inducers of GVHD.

To nuance the differentiation status further, a high frequency of central memory T cells CD45RO+CCR7+ in the expansion was associated with decreased incidence of relapse in the patient group suffering from malignant disease (n=23). T cells of central memory phenotype can utilize CCR7 to recirculate to secondary lymph nodes for restimulation and have been shown to exhibit superior proliferative potential (275, 277, 278). Therefore, it is not hard to imagine this cell population capable of mounting an anti-leukemic response while also belonging to the GVHD-inducing CD45 positive population described above. Indeed, GVHD and GVL most often go hand-in-hand (21).

As said, it is assumed that 8 days of *in vitro* expansion somewhat reflects the *in vivo* situation. Our findings here fit that assumption, where activated (CD69+, CD94+), yet not very differentiated (CCR7+) T cells could provide a survival advantage with less relapse but an increased risk of GVHD. Further support comes from our more recent study on non-expanded CB grafts where a higher frequency of naïve (CD45RO-CCR7+) CD8+ T cells is associated to both prolonged survival and an increased incidence of aGVHD (316). I speculate here that it might be this naive non-expanded subset that becomes the CCR7 and CD69 expressing cells observed after expansion in **Paper III**.

We also studied the functionality of the expanded cells and correlated it to clinical features. All expansions contained high frequencies of IFNγ and TNF-producing T cells after mitogenic stimulation as evident by intracellular staining and flow cytometric analysis. However, the diverse production of these cytokines could not be correlated to any clinical parameter. Significant differences were found in production of the Th2 cytokine IL-4 (more IL-4 positive CD4+CD8+ T cells in expansions corresponding to patients alive than dead) and the Th17 cytokine IL-17 (less IL-17 positive CD4+ cells in expansions corresponding to patients suffering from bacteremia) but frequencies were below 1% in all groups making these cytokines hard to use as predictive markers.

High risk patients, especially for GVHD, have previously been identified by recipient or donor genotypes (320, 321). We have also identified other risk factors that have a negative effect on survival after CB transplantation, such as mesenchymal stem cell treatment possibly due to a blockade of thymic function (52, 229). With **Paper III**, the CB transplantation community has gained a non-invasive tool for potential identification of patients with a poor survival prognosis.

3.3 CHARACTERIZING THE IMMUNE SYSTEM(S) OF PATIENTS WITH MIXED DOUBLE DONOR CHIMERISM

The predictive markers revealed in **Paper III** could also be utilized to identify the most suitable unit if more than one HLA-matched CB unit is available. As an example, a patient with non-malignant disease has no benefit of GVL, and could be given the graft with the least amount of CD45RO+ T cells after *in vitro* expansion (predictive of less skin and liver GVHD). Similarly, when a double cord blood transplantation (DCBT) is planned due to low cell dose, not uncommon for adult patients, the two units could be selected on their recognized complementary characteristics.

Since only one of the two units usually engrafts after a DCBT to form the new immune system of the patient, understanding the factors that determine the predominant unit after DCBT could have implications for unit-selection. For example, a smaller HLA-matched unit predetermined to engraft could be co-infused with a larger HLA-mismatched unit that could carry out significant GVL effect before disappearing (thoughts from (322)). Moreover, if it was possible to make reliable predictions on the dominant unit, time and money could be saved by, e.g. expanding T cells for DLI from that unit only. Because of this, several studies have tried to elucidate predictive factors for unit predominance with conflicting results concerning CD34+ dose (254, 256, 323), CD3+ dose (252, 254, 256, 323), early dominance of T cells (324, 325) and HLA-match (252, 323).

We previously observed a relatively high frequency of mixed double donor chimerism at our center (221). Between June 2004 and May 2008, a total of 12 patients received DCBT at CAST and both donor immune systems co-existed in three out of seven evaluable patients in all cell lineages at 90 days after DCBT. Two of these patients still had a mixed double donor chimerism 28 and 45 months after DCBT, respectively. These patients are extremely rare and, to my knowledge, there are eight published studies describing the phenomenon where both donors are present but no recipient cells at day 60 or more post-transplantation (221, 252, 326-331). For only five of the patients described in these eight papers, including the two patients at our center, the double donor chimerism was apparent for more than one year after transplantation. Thus, out of at least a thousand DCBTs performed to date (47) only a handful long-term double donor chimeras have been reported.

Factors predicting unit predominance should be the same as the factors determining donor unit co-existence, only inversed. However, the two patients studied here did not differ from the five other DCBT transplanted patients for proposed predictive factors such as HLA-matching, cell dose or viability of given cells (221). Notably, neither of the two patients developed GVHD suggesting tolerance between the two units and the recipient. This finding spurred our interest in the immunological consequences of having two stable donor immune systems, something that has not been reported before. Theoretically, two immune systems could maximize the number of target antigens that can be seen by donor-derived effector cells. Hence, if

mixed double donor chimerism would be beneficial for transplantation outcome and could be predicted, CB units with high chance of tolerability for each other could be chosen. However, results from **Paper II** do not support this notion.

In **Paper II** we used flow cytometry and chimerism analysis to characterize the phenotype and functionality of the mixed double donor immune system. Using specific HLA antibodies and PCR-based chimerism analyses of cell subpopulations, we were able to study the two immune systems in the patients separately (Fig. 15). In addition, we compared the complete immune system of the mixed double donor chimeric patients to the immune system of patients with one prevailing donor immune system. Due to low patient numbers, it was not possible to perform statistical analyses. Thus, the results presented below should be interpreted with care and used as hints for further studies.

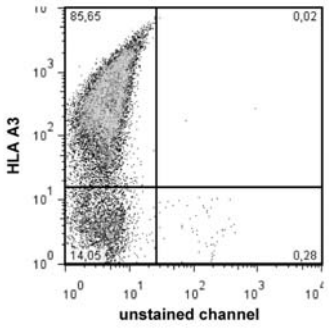

Fig.15 The two co-existing immune systems can be visually separated by the aid of fluorescently labeled anti-HLA antibodies. For the patient with two cord blood units mismatched for HLA A, flow cytometric typing assay with anti-HLA A3 antibodies were used to separate the two units. Figure adapted from Paper II.

In the only assessable patient, the two units had comparable TCR repertoires, as evident by studying 15 different Vβ chains, possibly invalidating our hypothesis of maximizing the number of recognized target antigens. Moreover, the two immune systems were neither phenotypically nor functionally equal. Although fluctuating somewhat over time, both patients presented with one dominant unit consistently occupying a larger part of the total immune system (Fig. 16). In both patients, this dominant donor unit had more T cells and fewer NK cells compared to the minor unit, although the differences were not striking. Intriguingly, a larger proportion of the T cells in the minor unit were naive (68% vs. 58% CD45RO-CCR7+ T cells). This naivety probably translated into the lower cytokine production (IFNγ, TNF, IL-17) seen for the minor unit after mitogenic stimulation. In addition, more NK cells of the dominant unit upregulated the degranulation marker CD107a when stimulated with K562 cells (that lack MHC class I molecules) compared to NK cells of the minor unit (Fig. 17). In other words, the functionality of the minor unit was lower than for the dominant unit.

In consequence of the naive minor unit, the two co-existing donor units had collectively a more naive phenotype and a less responsive functionality compared to control patients with a single CB unit (n=4). Both patients had a remarkably high percentage of naive T cells around 50% two years post-transplant compared to the 10% median observed in the single CB unit controls. Moreover, overall cytokine production by the two units was lower in response to mitogenic stimulation compared to the single CB unit controls. These findings might point towards a tolerability which may be associated to the absence of GVHD and mixed double donor chimerism. This

tolerability was probably not mediated by Tregs as no increase of these suppressive cells was observed in the mixed chimerism patients compared to controls.

Depleting donor T and NK cells *in vivo* using high-dose anti-thymocyte globulin (ATG) (8mg/kg) has been shown to be effective in reducing aGVHD (332) but has also been associated with delayed development of donor chimerism after HSCT (333). All of the patients included in this study received high-dose ATG which we previously proposed to play a role in development of mixed chimerism (221). Gutman et al provided compelling evidence for a CD8+ T cell-mediated graft-versus-graft interaction as the cause of unit predominance by showing that cells from the engrafting unit produced IFNγ in response to lymphoblastoid cell lines (LCL) from the non-engrafting unit but not vice versa (220). This data suggests that the engrafting unit rejected the non-engrafting unit, although the responsible antigens remain unknown. Interestingly, in the same study, three patients with persistent mixed chimerism showed no response in either direction, suggesting that mutual tolerance is associated with mixed double donor chimerism. The IFNγ producing cells in the study by Gutman et al were of CD45RO+/-CCR7- memory phenotype and were detected only transiently after transplantation. Thus, the high-dose ATG treatment used at our center could increase the chances of choking their alloresponsiveness, possibly enough for tolerance to develop. This hypothesis is consistent with a murine study where T cell depletion and subsequent infusion of HLA mismatched donor and recipient bone marrow resulted in tolerance and mixed chimerism (334).

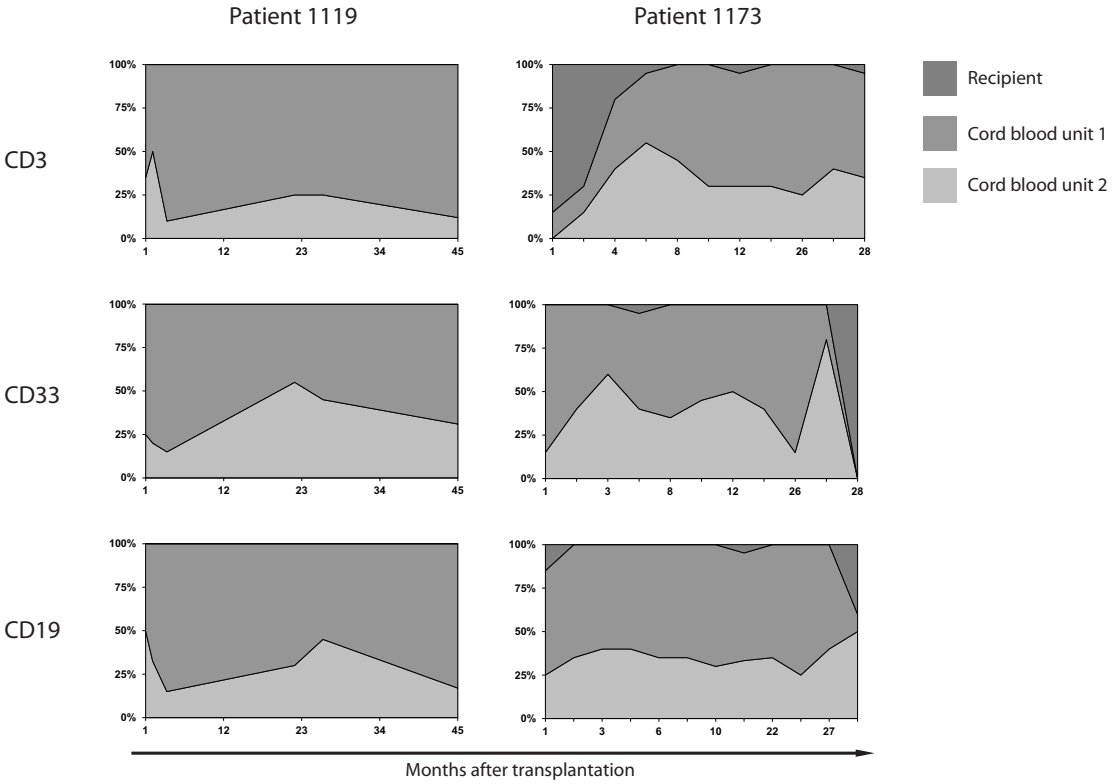

Fig.16 Both patients with mixed double donor chimerism consistently had one dominant unit for all cell subsets. The figure shows contributions of the three different immune systems (recipient and the two cord blood units) to the total immune system of the patient, measured by PCR-based analysis of short tandem repeats unique for each of the three systems. Figure adapted from Paper II.

Although depleted by ATG (335), NK cells reconstitute much faster than T cells (49, 336) and might play a role in determining dominant unit engraftment given that killer immunoglobulin-like receptor (KIR)/KIR ligand differences between units favor a one-sided alloreactivity (337). Notably, when studying the response against functional targets like K562 cells, higher frequencies of NK cells of the mixed double donor patient described in **Paper II** responded with upregulating CD107a compared with single CB unit controls, possibly indicating the importance of NK cell function in these patients.

NK cell function is to a large extent regulated by inhibitory and activating KIRs recognizing certain HLA molecules (KIR ligands) and the majority of KIRs respond to just two known epitope groups of HLA-C (338). In **Paper II**, we HLA-typed the patients by PCR-sequence specific primers for both HLA class I and II antigens and found a complete HLA-C match between the units in both of our mixed double donor patients. Thus, this match of KIR ligands might have supported NK cell tolerance and, accordingly, we did not observe an HLA-C match in three of the four patients with a single prevailing unit after DCBT. One possible reason why the fourth control patient presented with single unit predominance despite an HLA-C match of donor units is an extremely high inter-unit difference in CD34+ numbers (22 to 1) in favor of the prevailing unit (221).

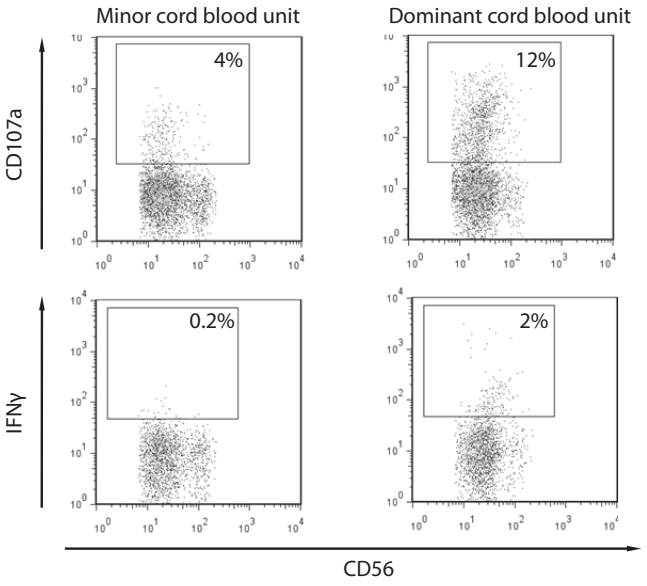

Fig.17 NK cells of the dominant unit are more responsive than NK cells of the minor unit. PBMCs were stimulated for with K562 cells lacking MHC class I molecules and hence provoking NK cell "missing self"-mediated reactivity. Upregulation of degranulation marker CD107a and IFNγ production was studied on CD3-CD56+ NK cells using flow cytometry. Figure adapted from Paper II.

DCBT has been associated with lower risk of relapse compared to single CBT possibly due to the initial clash between the two CB units if, for example, the non-engrafting unit would share mHags with the leukemic cells (256). Compared to matched adult unrelated donors, DCBT patients are at increased risk of infections but no actual increased risk of relapse suggesting that although T cell reconstitution is delayed, the GVL effect is maintained possibly and partly due to the early reconstitution of NK cells (50, 339). One of the two patients studied in **Paper II** suffered from leukemic relapse 28 months post-transplantation (seen as an increase of CD33+ cells of recipient origin in Fig. 16) perhaps due to a lack of NK cell alloreactivity.

Based on the findings in **Paper II**, I speculate here that development of mixed double donor chimerism was due to the increased tolerance induced by high-dose ATG and

HLA-C matching between units, and that this tolerance may have played a role in the relapse seen in one of the patient. As this study was made from a very small number of patients, further work is needed to support these ideas.

4 CONCLUSIONS AND FUTURE PERSPECTIVES

4.1 SPECIFIC CONLCUSIONS

- Umbilical cord blood derived T cells can be expanded in eight days to sufficient numbers using anti-CD3/CD28 magnetic beads and interleukin-2 for use as donor lymphocyte infusion after transplantation. Using this protocol, expanded cord blood derived T cells…
    - are of central and effector memory phenotype.
    - are cytokine-producing in response to mitogenic stimulation.
    - proliferate in response to allogeneic stimulation comparable to non-expanded T cells from both peripheral and umbilical cord blood.
    - have a polyclonal T cell receptor repertoire.
    - have a superior cytolytic capacity in response to allogeneic peripheral blood mononuclear cells, leukemia and lymphoma cell lines compared with non-expanded T cells from both peripheral and umbilical cord blood.
- Patients with long-term mixed donor chimerism after double cord blood transplantation have a less functional immune system compared to control patients with a single donor immune system.
    - Decreased functionality of the mixed donor chimerism might be because one of the immune systems has a naïve T cell profile and a reduced cytokine production in response to mitogenic stimulation.
    - Human leukocyte antigen-C match of donor units and use of high dose anti-thymocyte globulin may contribute to the development of mixed donor chimerism.
- Expansions of cord blood derived T cells might not only be used as donor lymphocyte infusions but also as *in vitro* indicators providing predictive information on the transplantation outcome, e.g…
    - higher frequencies of CD69+ T cells in the expansion are associated with prolonged patient survival.
    - higher percentages of CCR7+ T cells in the expansion are associated with less relapse.
- Cord blood derived T cells cultured with IL-7 in addition to IL-2…
    - are more polyfunctional and have a superior proliferation potential, allowing the IL-2 dose to be lowered without impacting the expansion rate.
    - results in a higher CD4/CD8 ratio and a higher frequency of effector memory T cells.

4.2 FURTHER TWEAKING OF THE EXPANDED PRODUCT AND OTHER POSSIBILITIES

The unknown epitope specificity of the cells in a DLI has both pros and cons. On the beneficial side, remaining recipient hematopoietic, as well as cancer cells and virus-infected cells, may be targeted by the different clones in an expanded T cell product. But, if recipient dendritic cells present self-antigens in the presence of danger signals, which often is the case after conditioning treatment and tissue damage, alloreactivity towards other recipient tissues may also ensue resulting in aGVHD. Indeed, the risk of GVHD is an ever present risk when administering unselected DLI (340).

Since CD8+ T cells are suggested to be the primary mediators of GVHD while the remaining CD4+ T cells could provide the desired GVL effect, an unrefined way to decrease the risk of GVHD consists of depleting CD8+ T cells from the DLI product (341-343). However, another risk of expanded T cells is that they are exhausted and unable to perform efficiently in the patient. Although proven to be functional *in vitro*, we cannot be certain that cells expanded using our protocol will work *in vivo.* Methods to enhance the effect of infused T cells include antibody-mediated blockade of co-inhibitory signaling pathways such as the programmed cell death 1 (PD1)/PD-L1 (344) or the cytotoxic T-lymphocyte-associated proten-4 (CTLA-4)/B7 axis (345). Unfortunately, as we did not study the expression of either PD1 or CTLA-4 on these cells, we cannot tell whether such a treatment would be effective in this setting.

The modulatory methods described above are all quite crude and the proposed blockade of co-inhibitory molecules might lead to a general lowering of activation threshold further increasing the risk of awakening alloreactive T cells. Ideally, we would like to separate the anti-viral and anti–tumor effects from the attack of healthy recipient tissue. For this purpose, the expansion protocols in **Paper I and IV** might be utilized as a platform for selective adoptive immunotherapy. In the following section I will present potential ways to direct the immune response of CB-derived expanded T cell products towards antigenic targets of choice.

The principle of bispecific antibodies is as easy as it is clever. By conjugating an anti-CD3 antibody to another antibody, e.g. anti-CD20, CD3+ T cells are targeted to specific antigens, e.g. CD20-expressing malignant B cells (346, 347). These bispecific antibodies likely work in similar way as the magnetic beads used for activation where interaction between the CD3-expressing cell and the CD20-expressing target cell crosslinks the TCR complexes on the T cell allowing it to perform cytotoxic action. This method has proven efficacious also for CB- derived T cells which, after 14 days of IL-2 expansion with OKT3 (anti-CD3) and armed with anti-CD3 x anti-CD20 bispecific antibodies, could mediate specific cytotoxicity versus CD20+ targets *in vitro* (348). It is unclear how T cells in the referred paper can perform cytolytic action without apparent co-stimulation. Perhaps, co-stimulation is provided by target B cells raising the question of functionality versus target cells that do not express CD80/CD86. Speculatively, the effector memory cells produced by our expansion protocol, which provides both signal

1 (anti-CD3) and signal 2 (anti-CD28) as well as IL-2 and IL-7, could prove to be just what is needed for the bispecific antibodies to have adequate effect *in vivo*.

Since antibodies have a relatively short half-life *in vivo*, repeated infusion of bispecific antibodies would probably be needed for clinical effect (347). A more persistent way of inducing HLA-independent but specific reactivity is to genetically modify T cells to express chimeric antigen receptors (CARs) before expansion (202). A CAR is basically a Fab-part of a pre-determined antibody coupled to the intracellular machinery of a TCR, allowing it to bind antigen and transduce T cell activation signals. These receptors can be engineered in DNA plasmids and inserted in target T cells by diverse viral or non-viral vectors and will after expression eventually overrun the function of the endogenous TCR. CARs versus CD19 and CD20 have successfully been expressed in CB-derived T cells and expanded cells are able to lyse target tumor cells specifically and repetitively (349, 350). Second generation CARs possessing co-stimulatory 41BB (CD137) and CD28 domains have also been tested in CB-derived T cells showing improved performance (351, 352). Furthermore, to improve persistence of infused cells, anti-CD19 CARs have been transfected into already pre-generated virus-specific T cells from CB (353). These CB-derived CAR-bearing T cells showed comparable anti-viral and anti-tumor function to similarly transfected PB-derived T cells *in vitro* and could potentially be useful in the clinic. Like in the case of bispecific antibodies, most investigators have concentrated on B-linage malignancies, e.g. B-ALL and lymphomas, diseases that do not respond well to conventional DLI. However, the concept may lend itself to all kinds of antigen targets in the future. Our lab will pursue the method of transducing CARs into T cells with gammaretroviruses as described by the Rosenberg lab (202, 354). In the first stages we will focus on the adult stem cell setting and, if successful, we will also turn to CB-derived T cells and use the expansion protocol proposed in **Paper IV** to increase cell numbers after plasmid transduction.

Instead of genetically modifying T cells, investigators could of course make use of the pre-existing TCR repertoire in a CB graft. Although priming of fetal T cells does occur *in utero* (355-358) most T cell clones are naive and need extensive priming by peptide-pulsed autologous APCs *in vitro* before tumor or viral antigen specificity is sufficient in both cell number and function for effective adoptive immunotherapy (298, 359). Compared to our method of T cell expansion, these methods need a larger portion of the CB graft (20% vs. 5%) to generate DCs or LCLs and several weeks of re-stimulations. In contrast, both our and the expansion protocol by Davis et al maintain the TCR polyclonality while activating the T cells, allowing subsequent and fast priming against leukemic and lymphoma cells without the need for autologous APCs (Davis, (279) and **Paper I**). However, compared to laborious techniques proposed by Merindol et al and Hanley et al (298, 359), this fast kind of priming probably results in an antigen- specific T cell population of much lower purity. Hence, to reduce the risk of unwanted alloreactivity, antigen-specific cells could be positively selected after priming. This could be achieved either by recombinant MHC molecules conjugated to peptide of choice (tetramers/pentamers) or by HLA-independent procedures like the

IFNγ- and CD137-capture techniques, the later having been successfully used in CB transplantation (171, 360-362). It remains to be seen whether cells expanded using our protocol may be stimulated to also respond to viral antigens or whether viral antigen priming is required before expansion as has been reported by Hanley et al (298). In any case, pending results from a clinical phase I study regarding CB derived anti-Adenovirus/Cytomegalovirus T cells primed with autologous DCs (ClinicalTrials.gov identifier: NCT01017705) will shed further light on the applicability of virus-specific adoptive immunotherapy after CB transplantation.

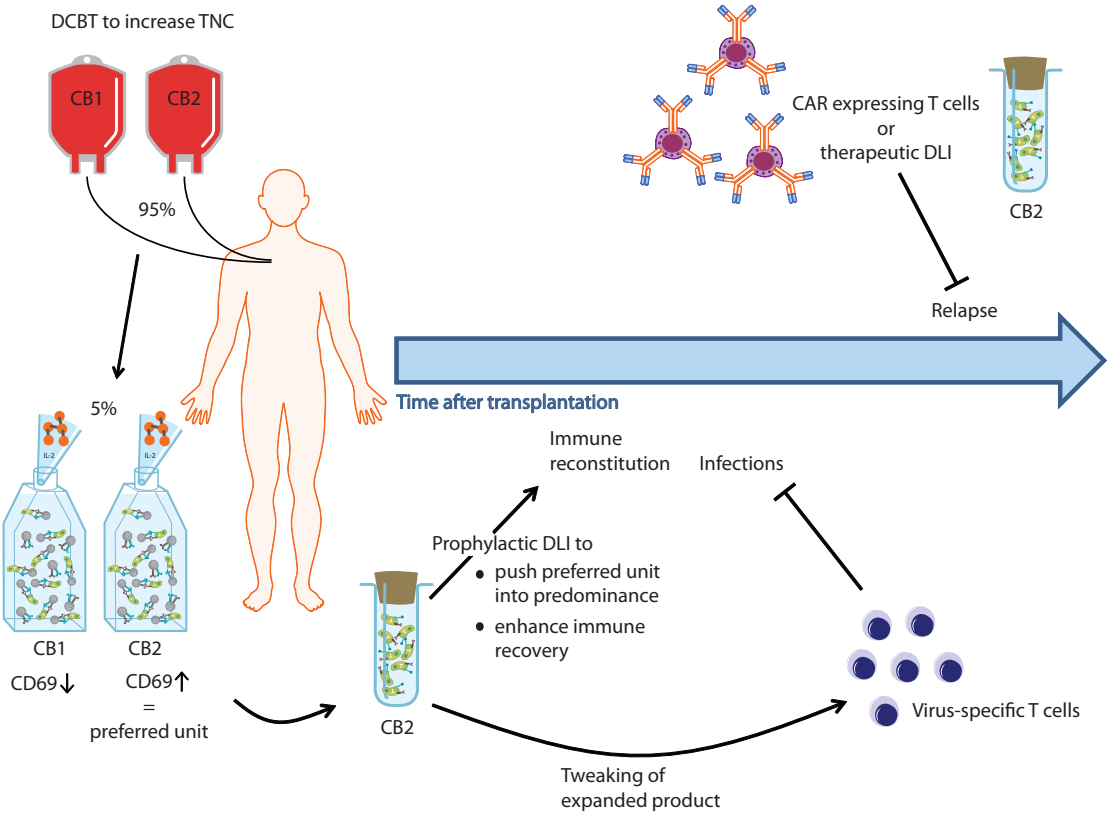

Fig.18 A theoretical future for the results presented in this thesis. A T cell expansion is done from both cord blood units of a DCBT. The unit with the most favorable predictive value, in this case a high frequency of CD69+ T cells, is infused as prophylactic DLI. The expanded product may be primed and/or selected to generate virus-specific T cells to fight off opportunistic infections. The expanded product might also be used as therapeutic DLI to address a threatening relapse. Alternatively, the naïve non-expanded T cells from the original graft can be transduced with CAR-gene containing vectors and subsequently expanded to generate CAR expressing T cells. Figure inspired by Hanley et al 2010.

DCBT: Double cord blood transplantation, TNC: total nucleated cell dose, CB: cord blood unit, DLI: donor lymphocyte infusion, CAR: chimeric antigen receptor

Lastly, we might try to further improve our expansion protocol. For example, the AB-serum batch used as supplement in the expansion medium in our studies has a number of poorly defined components with batch variations potentially complicating culture standardization. In the future, fully-defined serum-free medium might become a necessity for T cell expansions (363). Furthermore, the release of GMP-grade IL-15 is of interest as it has been shown to protect antigen-specific CD8+ T cell survival (364). Similarly, IL-12 in GMP-grade may come in handy for future experimental protocols, as it has been shown to be important for optimal IFNγ production by CD8+ T cells (365).

IL-15 is also appealing as, in combination with phosphoantigens (PAgs), it has been shown to enrich γδ T cells from CB (366). This subset of T cells have innate-like

features, recognizing "altered self"-patterns induced by e.g. bacterial, protozoal or viral infections as well as malignant transformations in an MHC-independent manner (59). The γδ T cells have also been shown to enhance immune reconstitution after HSCT by an unclear mechanism (367) and have, as of yet, not been associated with GHVD-induction (368). Interestingly, Cairo *et al* did not expand their cells with anti-CD3/CD28 beads but only with IL-15 and PAgs (366). γδ T cells can definitely be expanded by the present protocols, their frequency ranging from 0-8% in the expansions performed to date (**Paper I, III and IV**). Hence, it is possible to imagine a different approach in the future where CB-derived γδ T cells are primed with PAg, expanded with anti-CD3/CD28 beads and cytokines, and subsequently infused for enhanced immune reconstitution after transplantation. Importantly, γδ T cells are divided into two major subsets: the Vδ2-positive cells which dominate in peripheral blood, and Vδ2-negative cells that are more frequent in the mucosa (59). The Vδ2-negative but not the Vδ2-positive cells have been implicated in anti-CMV immune responses (369) and future studies will reveal whether the CB-derived γδ T cells can provide adequate immunity.

Yet another use for IL-15 is the generation of the recently described memory stem T cells (Tscm) (280). These cells, defined as CD62L+CCR7+CD45RA+CD45R0+IL7Ra+CD95+, seem to have the ability to both self-renew and differentiate into effector cells and may show superior *in vivo* persistence after adoptive transfer. Tscm can be enriched from naive adult T cells by stimulation with anti-CD3/CD28 beads and culture with IL-7 and IL-15, and might be a prospect for our lab in the future, especially considering that the immaturity of CB cells make them an ideal starting cell source.

The last alternative expansion protocol I would like to mention is that for Tregs. CB-derived Tregs have been shown to be superior immune suppressors compared to PB Tregs (293, 370). If infused DLI prove to induce GVHD, a back-up reservoir of expanded Tregs could alleviate problems. For this purpose, one could imagine two expansions in parallel, one for conventional T cells and one for Tregs, where a CD4+CD25+ isolation is performed before expansion (without IL-7) and subsequent rapamycin treatment is used to kill off activated conventional T cells before freezing (expansion procedure from Godfrey, Spoden et al (293). Whether this back-up proves necessary is of yet unknown.

Naturally, the easiest, fastest and most cost-effective way of using the expanded CB-derived T cells is directly without further tweaking of the protocols or the expanded cells. For this cause, a phase I clinical study is highly encouraged.

The results from **Paper II** indicated that long-term mixed chimerism was associated with a less functional immune system. Mixed recipient/donor chimerism in patients with malignant disease is routinely used as a marker for risk of relapse, and is usually quenched by e.g. DLI (371, 372). Thus, I recommend that an expanded DLI product (**Papers I and IV**) should be used pre-emptively to push the preferred unit into a

"winning" position. Which unit is to be preferred may partly be decided by the appearance of predictive markers such as CD69 in the T cell expansions reported in **Paper III** (see Fig. 18).

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