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UMBILICAL CORD BLOOD TRANSPLANTATION: CLINICAL OUTCOME, CHIMERISM DEVELOPMENT, AND IN VITRO EXPANSION OF T-CELLS FOR CLINICAL USE

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Umbilical cord blood transplantation: clinical outcome, chimerism development, and in vitro expansion of T-cells for clinical use

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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ABSTRACT

Umbilical cord blood (UCB) is an alternative graft source in allogeneic hematopoietic stem cell transplantation (HSCT). The unique advantages with UCB as a stem cell source are found in the permissibility for human leukocyte antigen (HLA)-mismatch, rapid availability, and comparatively risk-free graft collection. Important drawbacks to UCBT are delayed engraftment, poor immune reconstitution and the lack of donor cells for post-transplant therapy. In this thesis we aimed to: 1) evaluate clinical outcome and chimerism development after UCBT, and 2) develop treatment alternatives to donor lymphocyte infusions (DLI) for UCBT recipients.

In **scientific paper I**, we performed a retrospective single-center analysis of chimerism development and clinical outcome in 50 single and double UCBTs (DCBTs) in children and adults with hematological malignancies and non-malignant diseases. Complete donor chimerism was found to be associated with total body irradiation. A negative correlation was seen between 5-year survival and age, acute graft-versus-host disease (GVHD) grades III-IV, and mesenchymal stem cell (MSC) treatment. In **scientific paper II,** we focused on chimerism development in seven patients undergoing DCBT. Two patients developed long-term mixed donor–donor chimerism (MDC) in all cell lineages, at 25 and 35 months after DCBT. The work in **scientific paper III** was performed to study the immunological phenotype and function of these patients with MDC. We found that there were differences between the engrafted units: one unit had more naïve, less functional T-cells and more NK-cells. When the patients with MDC were compared with patients with single UCB unit chimerism, they had a more naïve T-cell profile. The results also indicated that inter-unit matching of the HLA-C genes and high-dose anti-thymocyte globulin (ATG) might influence MDC development after DCBT. We have expanded T-cells from clinical cord blood grafts with anti-CD3+/CD28+ beads and interleukin (IL)-2 for possible use as alternative DLI. In **scientific paper IV** we studied the effect of adding IL-7, and found that it conferred a greater proliferation rate, higher CD4+/CD8+ ratio, and less central memory T-cells. We also noticed a higher percentage of polyfunctional T-cells with IL-7. The cultured UCB DLI has been used clinically in four patients with mixed chimerism (MC), minimal residual disease (MRD) and graft failure without certain adverse effects. The results were presented in **scientific paper V**. In the patient treated due to MRD, the malignant cell clone was undetectable at treatment and for 3 months after infusion. In one patient with MC, the percentage of recipient cells decreased in temporal association to the treatment. These events indicate possible treatment benefits, but without certain causal association to UCB DLI treatment.

In summary, we found that MSC treatment was negatively correlated to survival in our UCBT recipients. High-dose ATG and HLA-C match might predispose for MDC. Cultured UCB DLI products are feasible to produce, and have been used in patients with no certain adverse effects, but with possible benefits.

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I. **Berglund S**, Le Blanc K, Remberger M, Gertow J, Uzunel M, Svenberg P, Winiarski J, Ljungman P, Ringdén O, Uhlin M, Mattsson J. Factors With an Impact on Chimerism Development and Long-Term Survival After Umbilical Cord Blood Transplantation. *Transplantation.* 2012 Nov 27;94(10):1066-74.

II. **Berglund S**, Okas M, Gertow J, Uhlin M, Mattsson J. Stable mixed donor-donor chimerism after double cord blood transplantation. *Int J Hematol.* 2009 Nov;90(4):526-31.

III. Gertow J, **Berglund S**, Okas M, Uzunel M, Berg L, Kärre K, Mattsson J, Uhlin M. Characterization of long-term mixed donor-donor chimerism after double cord blood transplantation *Clin Exp Immunol.* 2010 Oct;162(1):146-55.

IV. **Berglund S**, Gertow J, Magalhaes I, Mattsson J, Uhlin M. Cord blood T cells cultured with IL-7 in addition to IL-2 exhibit a higher degree of polyfunctionality and superior proliferation potential. *J Immunother*. 2013 Oct;36(8):432-41.

V. **Berglund S,** Gertow J, Uhlin M, Mattsson J. Expanded umbilical cord blood T-cells used as donor lymphocyte infusions after cord blood transplantation. *Cytotherapy*. 2014 Nov;16(11):1528-36.

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II. Gertow J, **Berglund S,** Okas M, Kärre K, Remberger M, Mattsson J, Uhlin M. Expansion of T-cells from the cord blood graft as a predictive tool for complications and outcome of cord blood transplantation. *Clin Imm.* 2012 May;143(2):134-44.

III. Uhlin M, Sairafi D, **Berglund S**, Thunberg S, Gertow J, Ringden O, Uzunel M, Remberger M, Mattsson J. Mesenchymal stem cells inhibit thymic reconstitution after allogeneic cord blood transplantation. *Stem Cells Dev.* 2012 Jun 10;21(9):1409-17**.**

IV. Uhlin M, Gertow J, Uzunel M, Okas M**, Berglund S,** Watz E, Brune M, Ljungman P, Maeurer M, Mattsson J. Rapid salvage treatment with virus-specific T cells for therapy-resistant disease. *Clin Infect Dis*. 2012 Oct;55(8):1064-73.

V. **Berglund S,** Uhlin M, Mattsson J. Chimerism and use of mesenchymal stem cells in umbilical cord blood transplantation. *Chimerism*. 2013 Jan-Mar;4(1):34-5.

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LIST OF ABBREVIATIONS

ACT	Adoptive cell therapy
AIRE	Autoimmune regulator
ALL	Acute lymphoblastic leukemia
AML	Acute myeloid leukemia
APC	Antigen-presenting cell
ATG	Anti-thymocyte globulin
CAR	Chimeric antigen receptor
CAST	Center for Allogeneic Stem cell Transplantation
CCR	Chemokine receptor
CCR7	Chemokine receptor 7
CD	Cluster of differentiation
CGD	Chronic granulomatous disease
CLL	Chronic lymphatic leukemia
CML	Chronic myeloid leukemia
CMV	Cytomegalovirus
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
DC	Full donor chimerism
DCBT	Double umbilical cord blood transplantation
DLI	Donor lymphocyte infusions
DNA	Deoxyribonucleic acid
EBV	Epstein-Barr virus
Fox	Forkhead box protein
FoxP3	Forkhead box protein P3
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GMP	Good manufacturing practice
GVHD	Graft-versus-host Disease
GVT	Graft-versus-tumor effect
HLA	Human leukocyte antigen

HLH	Hemophagocytic lymphohistiocytosis
HR	Hazard ratio
HSC	Hematopoietic stem cells
HSCT	Allogeneic hematopoietic stem cell Transplantation
IFN	Interferon
ICOS	Induced co-stimulator
Ig	Immunoglobulin
IL	Interleukin
IL-2	Interleukin 2
IL-7	Interleukin 7
KIR	Killer cell immunoglobulin-like receptors
MAC	Myeloablative conditioning
MC	Mixed chimerism
MDC	Mixed donor-donor chimerism
MDS	Myelodysplastic syndrome
MHC	Major histocompatibility antigen
MIP	Macrophage inflammatory protein
MRD	Minimal residual disease
MSC	Mesenchymal stem cells
NK	Natural killer
PBMC	Peripheral blood mononuclear cells
PBSCT	Peripheral blood stem cell transplantation
PD	Programmed cell death protein
PMA	Phorbol 12-myristate 13-acetate
PTLD	Post-transplantation lymphoproliferative disease
RIC	Reduced-intensity conditioning
RNA	Ribonucleic acid
TBI	Total body irradiation
TCR	T-cell receptor
Th	T-helper

TIL	Tumor-infiltrating lymphocyte
TNC	Total nucleated cell dose
TNF	Tumor necrosis factor
TRM	Transplantation-related mortality
TSLP	Thymic stromal lymphopoietin
UCB	Umbilical cord blood
UCBT	Umbilical cord blood transplantation
VZV	Varicella-Zoster virus

I. INTRODUCTION

Signs of disease in ancient remains, *e.g.* findings of malaria, tuberculosis and cancer in Egyptian mummies (1), illustrate the integral role diseases have played in human life over past millennia. The disease-mediated impact on the health and life expectancy of human beings is severe, but has been considerably ameliorated by the medical breakthroughs and improved nutrition of recent centuries. Today, effective treatments and vaccines are available for many common diseases, and the knowledge of human illnesses is continuously increasing. However, there are still areas where the extent of medical progress is insufficient. The aim of this thesis was to address a subject found in one of these medical areas, namely allogeneic hematopoietic stem cell transplantation (HSCT) with umbilical cord blood (UCB). This is an effective treatment for several malignant and non-malignant diseases, but limited by severe toxicities and a substantial risk of treatment failure.

The indications for HSCT are heterogeneous, and include several congenital conditions and hematological malignancies (2-4). These diseases all currently lack other satisfactory and effective treatments unassociated with severe toxicity. The rationale behind HSCT is to exchange the affected hematopoietic system of a patient for a healthy one transplanted from a donor. For the inborn diseases, this change of the origin of hematopoiesis is sufficient for a cure. However, the treatment principle is more complex in hematological cancers. In this patient group, the cure is achieved by a combination of chemotherapy-mediated reduction of cancer cells, replacement of the malignantly transformed hematopoiesis and an immunologically mediated graft-versus-tumor (GVT) effect. The donor immune system is here required to exert alloreactivity, which signifies an immune reaction towards recipient cells (5).

HSCT is known to be associated with important limitations. The applicability is limited by the requirement of a donor matched for certain highly polymorphic genes (6, 7). There are also severe morbidity and mortality associated with HSCT (4, 8). UCB is a relatively new source of grafts for HSCT with unique properties that have been used to improve the applicability of HSCT. However, umbilical cord blood transplantation (UCBT) is associated with drawbacks such as poor immune reconstitution and a lack of donor cells for post-transplantation adoptive therapies (9-11). To increase the applicability and success of HSCT and UCBT, it is necessary to increase the knowledge of immune mechanisms involved in stem cell transplantation and of how these mechanisms can be favorably manipulated.

The studies described in this thesis have all been performed at the Center for Allogeneic Stem cell Transplantation (CAST) at the Karolinska University Hospital Huddinge, Stockholm, Sweden. Mentions in the text of local medical practices and CAST are references to this clinic and its practices.

I.1 THE IMMUNE SYSTEM

I.1.i A short introduction to the immune system

The immune system is essential to protect human beings against the microbes and viruses in the surrounding environment. It consists of a complex network of cells and molecules that together combat infectious pathogens. The necessity of the immune system for human survival is dramatically illustrated in individuals with immune deficiencies and human immunodeficiency virus infection (HIV), where the lack of an immune defense permits relatively harmless viruses and microorganisms to cause devastating infections (12, 13). Genetic findings suggest that humans and pathogens have developed side by side, with infection-related mortality driving a process of natural selection that has shaped the immune system in its present form (14). The immune system not only conveys protection against the microbes in the environment; it is also necessary for a peaceful co-existence with the microbial flora in the gastrointestinal tract and on the skin, among other sites (15). The immune system also exercises a degree of protection against the development of cancer. Transformed cells are recognized by the immune system, which can cause their eradication by immune-mediated mechanisms (16, 17). The same system that provides protection against infections and tumors can unfortunately also mediate damage when activated against the incorrect targets. This is the cause of autoimmune diseases such as Crohn's disease (18) and rheumatoid arthritis (19), where normal structures in the bowel and joints are targeted by the immune system. Another instance of incorrect immune activation towards harmless substances is found in allergy (20).

The immune system is a collective term for all the cells, tissues and molecules that co-operate in an immune reaction, the process with which the defense against infection is performed. The immune system is classically divided into two sections, the innate, or native, system and the adoptive system. A schematic illustration of the immune system and its two sections is found in Figure 1.

The first encounter with a pathogen is normally handled by the innate system. Innate immunity comprises the physical barriers of the body, antimicrobial substances produced at the barriers and systemically, antigen-presenting cells (APCs), and effectors cells (20). The most striking characteristic of the innate immune response is the immediate nature of its activation. This is possible due to the inborn, conserved mechanisms used in the innate system to recognize pathogens. The major functions of the innate immunity can be divided into three parts: 1) Keep intruders out. 2) Kill the intruders that do pass the barrier. 3) Raise the alarm for additional help if the situation cannot be easily resolved. An infecting pathogen, *e. g.* a virus, bacteria or fungi, first has to pass one of the body's barriers; the mucosa of the mouth, gastrointestinal tract, genitalia or lungs, and remain unharmed through exposure to the antimicrobial substances excreted there. If a pathogen penetrates the barrier successfully, the next obstacle is the complement system, a set of plasma proteins that function by the

recruitment of phagocytic cells, the induction of phagocytosis of pathogens, and by direct lysis of microbial cells (20). The immune system launches phagocytic cells to clear the pathogens by ingestion and destruction. The phagocytes also produce cytokines to summon other immune effector cells. Intracellular pathogens are difficult to clear by phagocytosis. This type of pathogen can be effectively eradicated by natural killer (NK)-cells, as they have the ability to kill infected cells. This prevents the spread by destroying the reservoirs of intracellular pathogens.

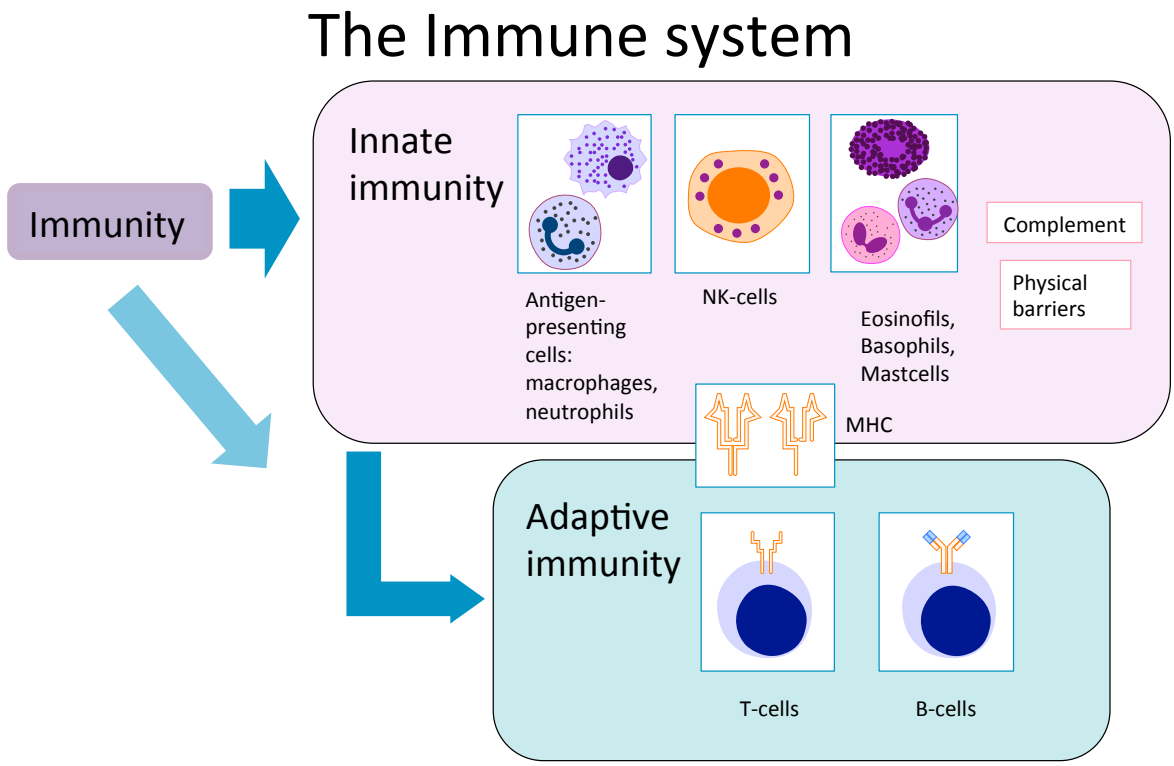

Figure 1. Figure 1: A schematic representation of the immune system's two sections. The MHC molecules form a bridge between the two compartments, as antigens need to be presented on these molecules for activation of the adaptive system.

The second part of the immune system, the adaptive immunity, cannot respond with the same rapidity as the innate system in consequence of the antigen-specific nature of its response. The adaptive immune system is activated by the innate system via APCs or, more rarely, by antigens binding directly to the receptors of adaptive immune cells, which are produced by random gene rearrangement, making every receptor unique (20). The cellular agents of adaptive immunity are the T-cells and the B-cells. Their effector functions include antigen-specific killing of infected cells and microbes, antibody production, and cytokine production. The activation of the adaptive effectors occurs in the secondary lymphoid organs: the lymph nodes, the spleen and the mucosa-associated lymphoid tissues. Pathogens and APCs travel to these tissues to activate antigen-inexperienced T- and B-lymphocytes, which then proliferate and differentiate into effector cells (20). These then travel via the blood to the infection site. The adaptive arm of the immune system also provides immunological memory. This memory function enables the immune system to respond quickly and efficiently to the pathogen if

encountered a second time. Immunological memory is also the mechanism that enables vaccination.

Communication between different sections of the immune system is necessary for successful eradication of pathogens. The molecules that are mainly responsible for the activation of the adaptive system by binding to receptors on adaptive cells are called major histocompatibility antigens (MHC) or human leukocyte antigens (HLA). They function by presenting parts of pathogen-derived proteins (peptides), to T-cells. The T-cell receptor (TCR) can only recognize pathogen-derived peptides if they are presented on a MHC molecule, which means that T-cell-mediated immunity against infection only can be induced by the presentation of pathogen-derived peptides on MHC molecules (20, 21). MHC molecules are categorized into two classes: class I and class II. A **MHC class I** molecule is made up by a α-chain that forms the peptide-binding structure, and a supporting protein called β2-microglobulin (20, 21). The MHC class I molecules are expressed on practically all nucleated cells, and presents peptides derived from protein breakdown in the cytoplasm of cells. The presented peptides reflect the intracellular processes of the presenting cell, and thus also if an intracellular infection is present (20, 21). MHC class I molecules bind specifically to the TCRs of CD8+ cytotoxic T-cells, which have the ability to kill infected cells upon presentation of pathogen-derived peptides on MHC class I molecules (20, 21). **MHC class II** molecules consist of two proteins, a α-chain and a β-chain, which together form the peptide-binding domain of the molecule. This class of MHC is expressed on APCs. The APCs present peptides on MHC class 2 molecules to CD4+ T-helper cells that on activation produce cytokines and provide co-stimulation to propagate the immune reaction (20). The MHC, or HLA, genes are also the most important to match between donor and recipients in HSCT, which will be discussed below.

I.1.ii A presentation of the components of the immune system

The white blood cells are the central actors of the immune system. They all develop from hematopoietic progenitor cells in the bone marrow. A simplified model of their development is shown in figure 2.

The **epithelial cells** of the physical barriers (the skin and the mucosa of the mouth, lungs, gastrointestinal tract, and urinary tract) tightly adhere to each other to prevent the passage of microbes and particles, and produce antimicrobial peptides that can kill certain types of bacteria (20).

The effector cells of the innate immune response can be divided into several categories with distinct function. The **phagocytes**, neutrophil granulocytes, monocytes and macrophages, exert their functions partly by ingesting pathogens, known as phagocytosis. Ingestion is triggered by receptors on the surface of the phagocyte binding to pathogens (20, 21). This group of receptors is called pathogen recognition receptors. This group is further divided into

subgroups such as scavenger receptors, Toll-like receptors, NOD-like receptors, and RIG-like receptors. They recognize constituents of the bacterial cell wall, bacterial flagella, virus RNA and DNA, and other pathogen-associated particles (20). **Neutrophils** are usually the first cell type to respond to signals of local infection. They migrate through the capillary walls to the tissues in response to changes in the endothelial cells of the vessel wall. In the affected tissue, they ingest and destroy microbes, and also partake in the clearing of debris, such as dead cells and microbes (20). **Macrophages** have a similar role, but they also produce cytokines, a type of signal substances crucial to the coordination of the immune system. Macrophages also co-operate with the adaptive immune system by antigen-presentation of ingested pathogens to lymphocytes on MHC class II and by phagocytosis of antibody-covered targets (20).

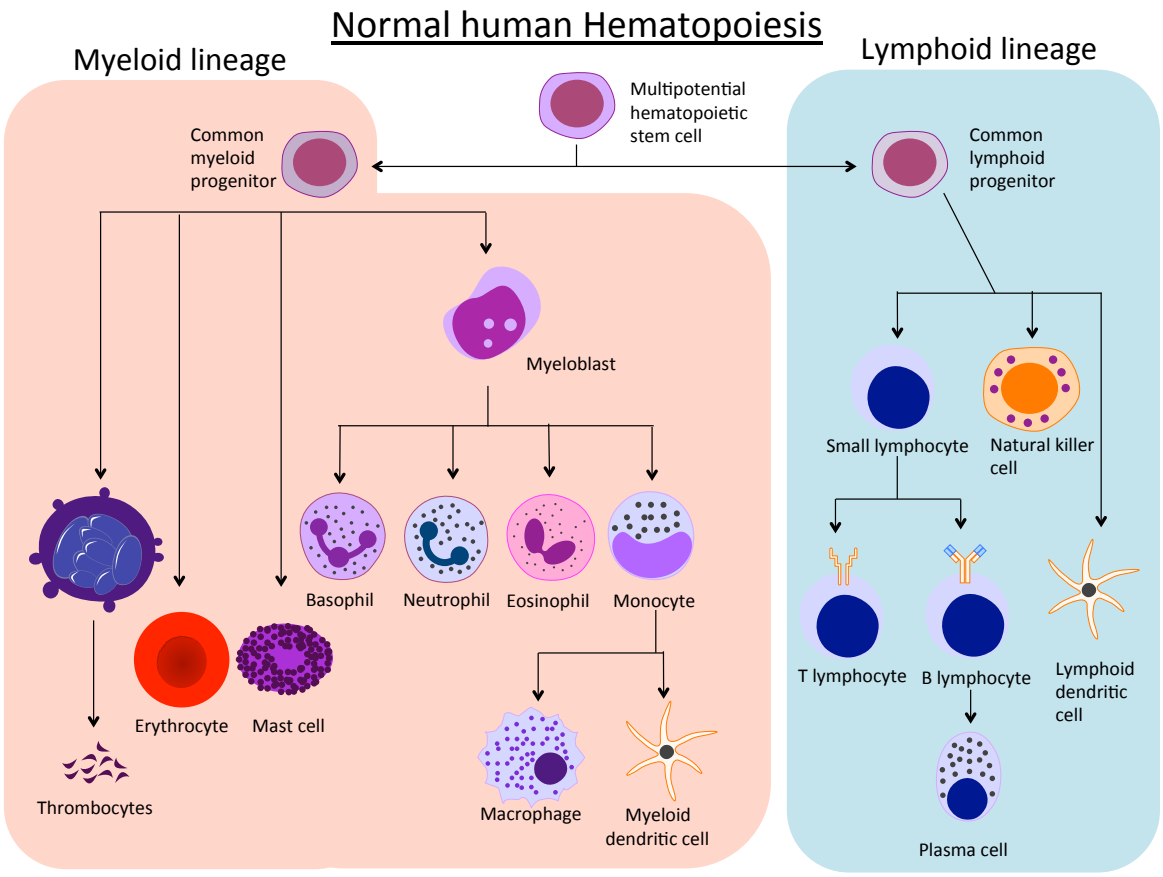

Figure 2. A simplified model of human hematopoiesis.

Antigen-presenting cells are one of the links between innate and adaptive immunity, and exert this function by presenting parts of digested antigens to adaptive immune cells on MHC molecules. The **Dendritic cell** is a professional APC that is found in peripheral tissues, functioning as a sentinel. Dendritic cells are activated upon ingestion of pathogens and responds by producing inflammatory cytokines, and migrating to a lymph node to present the captured pathogen to lymphocytes (20).

The last of the cell types in the innate immune system that will be presented in this brief introduction is the **NK-cell**. This cell is special both in function and mechanism of activation.

The NK-cells express a number of inhibitory and activating receptors, and the resulting activation or inhibition of the cell is determined by the sum of the signals transmitted through the receptors. NK-cells are central in virus defense by their killing of infected cells, thus eradicating intracellular virus reservoirs. They can also recognize and kill certain types of malignant cells by their lack of ligands for the inhibitory receptors (20).

Two main cell types make up the adaptive immune defense: B-cells, and T-cells. The **B-cells** express antigen-specific B-cell receptors able to recognize a variety of pathogen-associated antigen types, such as proteins, polysaccharides and lipids. The receptor genes are randomly rearranged during B-cell development, resulting in an extremely diverse repertoire of antigen specificities (20). After activation, a modified soluble form of the B-cell receptor is excreted as a circulating antibody. The antibodies function by activation of the complement system, by binding to pathogens, and triggering their phagocytosis or destruction, or by neutralizing pathogens by binding to and blocking important molecules on their surface (20).

T-cells are central to this thesis, and their development and function will be described in more detail in the next section. Briefly, the function of T-cells can be divided into two major categories by their expression of the co-receptors CD4 and CD8. The T-cells expressing CD4 are designated helper cells and mainly function through co-ordination of the immune response by cytokine production and co-stimulation of other effector cells, such as macrophages, eosinophil granulocytes and B-cells. The functions of CD8+ T-cells are similar to NK-cells, but are orchestrated through the presentation of foreign peptides on MHC class I molecules. When the T-cell receptor binds to an MHC class I molecule holding the appropriate peptide, they can kill infected or transformed cells.

I.1.iii The development and function of T-cells

T-cells have several diverse functions in the immune system. The broad division of T-cells into CD4+ helper cells and CD8+ cytotoxic cells can be further subdivided according to their expression of other markers and transcription factors. The CD4+ T-cell compartment is highly diverse, and different types of CD4+ T-helper (Th) cells are adapted to optimally respond to different pathogens (22, 23). The subset of the naive CD4+ T-cells is decided by the signals in the surrounding milieu during their priming, and the nature of these signals depends on the infecting pathogen. This enables development of Th subsets suited to infecting pathogens. The best-described Th subsets are the Th1, Th2, Th17 and follicular helper T-cells (20, 23). Regulatory T-cells are also a subset of the CD4+ T-cell family

Th1 cells are described to mainly focus on intracellular pathogens: intracellular bacteria and viruses. They are generated when naive T-cells are primed in the presence of interleukin (IL)-12. They are characterized by expression of a transcription factor named T-bet and production of interferon (IFN)-γ, which activates macrophages to destroy intracellular bacteria (23-25).

Th2 T-cells are important in immunity against extracellular parasites like worms (23). They are also thought to interact with B-cells to induce antibody class switching, especially to immunoglobulin (Ig) E, which is associated with immune responses to parasites (22). Th2 cells are also involved in allergy and asthma (23, 26). The Th2 subset is induced when naive T-cells are primed in the presence of IL-4. They are characterized by expression of the transcription factor GATA-3. Th2 cells mainly produce the cytokines IL-4, IL-5 and IL-13 (23, 27).

Th17 subset is important in immune responses against fungi and extracellular bacteria. They express the transcription factor RORγ that is responsible for the production of IL-17 (23, 28). IL-17 acts via epithelial cells to attract neutrophils to the site of infection (23). RORC also induces the expression of chemokine receptor (CCR) 6, which is important to guide the Th17 cells to the site of infection (29). Th17-mediated immunity is also thought to be implicated in certain autoimmune diseases (30).

T follicular helper cells are located in the lymph nodes in close proximity to B-cell germinal centers. They promote proliferation and differentiation of antigen-specific B-cells. The inducible co-stimulator (ICOS), a classic CD28 family co-stimulatory molecule, has been found to be important for the follicular recruitment of activated T-helper cells in mice (31).

One CD4+ T-cell subset, the **regulatory T-cells**, has very distinct functions from the others subsets. They negatively regulate the activity of other T-cells. They are usually characterized by the expression of high levels of the IL-2 receptor α-chain and a transcription factor called forkhead box protein 3 (FoxP3). Unfortunately, these markers are also found on activated CD4+ effector T-cells, which can make their identification in cell cultures difficult. There are several other markers that also characterize regulatory T-cells, but most are expressed also by other subsets (32, 33).

The **CD8+ T-cell** subset is more homogenous than the CD4+ subset. The CD8+ T-cells are all capable of mediating cellular cytotoxicity, e g killing of cells expressing an MHC class I molecule with a peptide of the correct specificity. They are characterized by the expression of the transcription factor forkhead box protein O1 (FoxO1) (34). Activation of CD8+ T-cells requires a stronger signal than CD4+ T-cells, possibly due to the destructive property of their effector functions. They are activated either by a combination of MCH: TCR signalling and stimulation by CD4+ helper T-cell, or by binding to MHC class II molecules on activated APCs, that also express co-stimulatory molecules. The combination of TCR-mediated stimulation and additional signals in the form of co-stimulation is then sufficient for their activation (22).

The development of the T-cells begins in the bone marrow. They then migrate to the thymus at the stage of progenitor cells. The thymus is an organ located close to the heart, which is specialized in the development and “education” of T-cells (35, 36). Here, the cells are

committed to the T-cell lineage, and the gene rearrangement of the T-cell receptor takes place. This process is called VDJ recombination and comprises the random combination of different versions of three gene segments and additional changes in the junctions connecting the segments (20, 35). The rearrangements enable the production of up to10^{16} different TCRs (20). Two different protein chains make up the TCR (22). In the majority of the T-cells, also called conventional T-cells or TCR αβ T-cells, there is one α-chain and one β-chain. The TCR αβ recognizes peptides presented by MHC molecules (22). A minority of T-cells expresses a TCR with one γ-chain and one δ-chain. These recognize non-typical antigens, like the phospholipids on mycobacteria (37).

During the receptor gene rearrangement, the T-cells begin to express both the CD4 and CD8 co-receptors and are termed "double-positive thymocytes" (CD4+CD8+) (35). After the receptor rearrangement, the double-positive thymocytes undergo a selection process in two steps: positive selection and negative selection. In positive selection, the thymocytes are exposed to self-antigens presented to them by thymic epithelial cells. These epithelial cells are induced by the transcription factor autoimmune regulator (AIRE) to present a majority of the self-peptides that T-cells are likely to encounter in the body (35). The survival of the thymocytes depends on the appropriate binding of their TCR to peptide-loaded MHC molecules. If the TCR fail to bind, or bind too weakly, to MHC: antigen complexes, the thymocyte undergoes neglect-induced cell death, or apoptosis. This is due to thymocyte survival being dependent on TCR-mediated signaling. This process is called **positive selection**, and prevents development of T-cells incapable of MHC-mediated activation (20). After positive selection, the thymocytes express either CD4 or CD8, according to the class of MCH of the MHC-peptide complex they have recognized. CD4 is expressed if the TCR binds to a peptide-bearing MHC class II molecule, and CD8 is expressed if it recognized a peptide-binding MHC class I molecule (22, 35). The T-cells are then termed “single-positive thymocytes”.

Negative selection is a process where thymocytes that bind too strongly to MHC-antigens complexes are eliminated by apoptosis to protect the organism from autoimmunity (22, 35). Negative selection can occur during positive selection: the degree of affinity to self-antigen:MHC complexes is thought to decide whether the thymocyte is positively selected to survive (lower affinity) or negatively selected to become apoptotic (higher affinity) (20). Negative selection can also occur when single-positive thymocytes that have successfully undergone positive selection encounter bone marrow-derived APCs in the marrow of the thymus. These APCs express co-receptors, and the combination of a strong TCR affinity to a self-antigen: MHC complex and co-stimulation also eliminate self-reactive single-positive thymocytes (20). The thymocytes that successfully undergo positive and negative selection leave the thymus as naïve T-cells, ready to partake in an immune response.

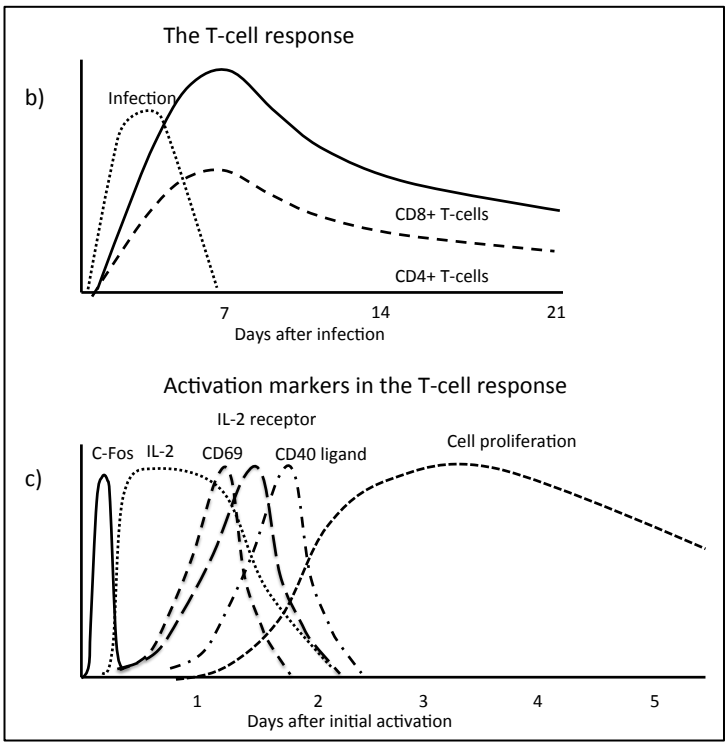

Figure 3. The T-cell immune response. The top panel depicts the kinetics of CD4+ and CD8+ T-cell numbers in an immune response. The bottom panel shows the kinetics of activation markers in T-cells.

During an immune response, naïve T-cells are activated to proliferate, and differentiate into effector cells. T-cell immune reaction follows a distinct kinetic pattern, as is shown in the top panel of Figure 3. During the activation process, different activation markers are up-regulated in a tightly controlled procession (21), as is shown in the lower panel in Figure 3. This enables determination of the activation status of T-cell populations. In a successful T-cell response, T-cell numbers reach a peak after approximately one week. Then, when the infection is resolved, the contraction phase begins. A majority of the effector T-cells undergo apoptosis, as they are no longer necessary to control the infection (38). Activation in itself drives a propensity for apoptosis by up-regulation of receptors for apoptosis induction and changes in gene regulation towards a pro-apoptotic profile (39, 40). Antigen-mediated TCR stimulation, cytokine signaling and co-stimulation counteract activation-induced apoptosis, which means that the T-cells will receive survival signals as long as the immune reaction is actively ongoing, but not when the infection is resolved (21, 38).

A small number of memory T-cells will remain long-term after the infection is resolved. These T-cells are, together with memory B-cells, responsible for immunological memory. The memory T-cells are primed to rapidly proliferate and differentiate into effector T-cells again on a new encounter with the same pathogen. The development of memory T-cells is debated, and several models have been described (41). One model of memory development suggests that naïve T-cells differentiate into effector cells upon activation, and that memory

T-cells are developed out of a smaller subset of the effector T-cells during the contraction phase of the immune response. A selection process based on fitness or avidity have been suggested to decide which effector T-cells that develop into memory T-cells (41, 42).

Another model have suggested that naïve T-cells differentiate directly into a stem memory T-cell subset, and then into central memory T-cells, with capacity of proliferation and trafficking into lymph nodes. Subsequently, a differentiation into effector memory T-cells, a

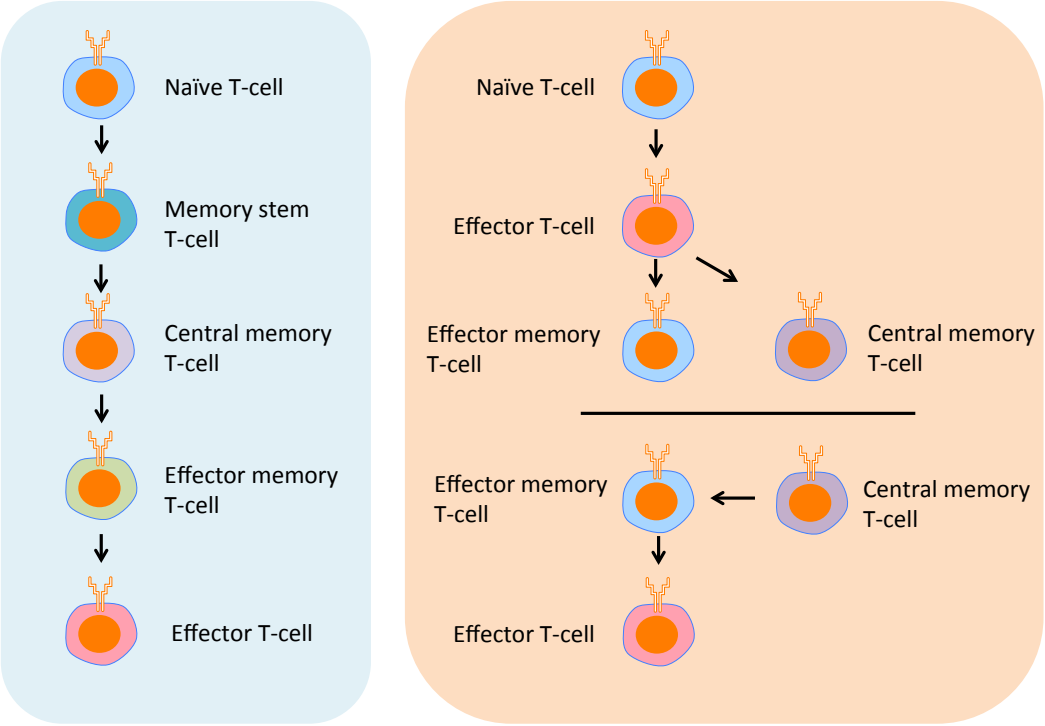

Figure 4. Models of memory development. The first model on the left hypothesizes that naïve T-cells initially differentiate into memory T-cells and later into effector T-cells, due to the finding that effector T-cells have the shorter telomeres. The Second model on the right suggests that memory T-cells develop from a small subset of effector T-cells during the contraction phase of an immune response.

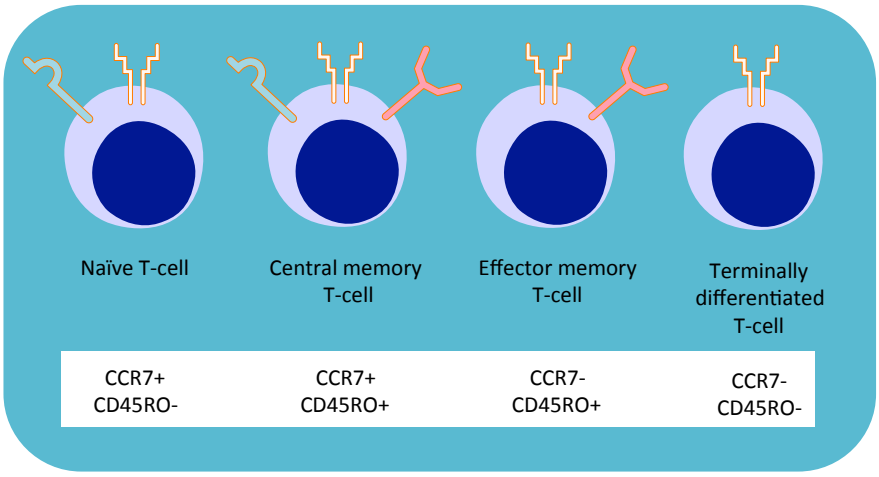

Figure 5. Definition of memory development stages.

memory subset with some effector function, and a capacity for rapid differentiation into effector cells is suggested. This hypothesis is based on the finding of shorter telomeres in effector T-cells than in memory T-cells. This could indicate that effector T-cells have undergone more numerous cell divisions, and is the later development phase (41, 42). Some mechanisms believed to partake in the memory development process, like up-regulation of

the IL-7 receptor α-chain, and the importance of IL-2 signaling during priming, are discussed in the chapters I.1.IV. The two models are pictured in figure 4.

Expression of cell surface markers CD45RO and CCR7 have been used to characterize different subsets of CD3+ T-cells with regard to memory development in this thesis (11, 43, 44). The following definitions were used: CD45RO- CCR7+ were termed naive T-cells, CD45RO+ CCR7+ were considered central memory T-cells, CD45RO+ CCR7- were termed effector memory T-cells, and CD45RO- CCR7- were considered as terminally differentiated memory T-cells. The definitions are pictured in Figure 5.

I.1.iv Cytokines: IL-2 and IL-7

Cytokines are signal molecules essential to hematopoiesis, and to coordination and regulation of immune responses. They have various functions in the production, proliferation and differentiation of hematopoietic cells. Cytokines stimulate, direct and regulate the components of immune reactions to ensure rapid response to pathogens, recruitment of the correct responders, and prevent immune-related damage (22). Cytokine signaling directs immune responses into the correct path by inducing effector differentiation of selected cell types. Disease symptoms and healing may be affected if the path of the immune reaction is not optimal. This is seen in Hansen's disease, where a milder form of the disease is seen in patients that respond predominantly with a Th1 response and macrophage activation, compared to patients with a response dominated by recruitment of neutrophil granulocytes and antibody production by B-cells (45). The effects of cytokines on non-immune cells include induction of marker expression on vessel endothelium necessary for trafficking of plasma proteins and leukocytes, and acute phase protein production in liver cells. There are several categories of cytokines including chemokines, interferons, interleukins and tumor necrosis factors. They are known to have both pro-inflammatory and anti-inflammatory effects (22). As such, cytokines are clinically important mediators in a wide range of diseases, from acute and chronic inflammatory and autoimmune diseases to neurodegenerative disease and the joint disease seen in diabetes and obesity (46-49).

The cytokines that will be in focus in this thesis are IL-2 and IL-7. An introduction into the functions of those two cytokines is found below.

I.1.iv.1 IL-2

IL-2 was identified in 1976, and was described initially as a T-cell growth factor produced by lymphocytes (50, 51). The finding of IL-2 enabled in vitro T-cell culture, which previously had not been possible. IL-2 is secreted predominately by activated CD4+ T-cells, but also by CD8+ T-cells, NK-cells and activated dendritic cells (51). The IL-2 receptor is made up of three distinct subunits, and exist in two different subunit-combinations: a trimeric high-affinity IL-2 receptor containing the α-chain (CD25), β-chain (CD122), and the common γ-chain (CD132, also part of the receptors for several other cytokines), or a low-affinity dimeric

receptor consisting of only the β- and γ-chains (51, 52). The IL-2 receptor α-chain is present as both a membrane and soluble protein (52, 53). Binding of IL-2 to the IL-2 receptor results in signaling through the tyrosine kinases JAK1 and JAK3, and gene regulation through the transcription factor STAT5 (52, 54). The IL-2 receptor is expressed on both T-cells and NK-cells.

IL-2 has various effects in the T-cell and NK-cells compartments: it can promote the generation of effector T-cells. In addition, IL-2 is vital in the priming phase of an immune reaction for the development of long-lived memory T-cells in both the CD4+ and the CD8+ T-cell subsets (52, 55-57). IL-2 is also a central factor in the maintenance of regulatory T-cells (51). In NK-cells, IL-2 up-regulates proliferation and effector functions, such as the induction of lymphokine-activated killer activity (58, 59).

Recombinant human IL-2 has been used in clinical trials as a treatment against cancer with some success since the 1980s. Injection of IL-2 in patients with advanced stage metastatic malignant melanoma and renal cell carcinoma achieved complete remissions in approximately 7% of the patients, and partial responses in an additional 10% of the patients (51, 60). IL-2 has also been studied as a cancer treatment in combination with cellular therapies with, *e. g.* tumor-infiltrating lymphocytes T-cells with response rates around 20-30% (61-63).

I.1.iv.2 IL-7

IL-7 is a homeostatic cytokine that, contradictory to being called an interleukin, is mainly produced by stromal cells (52, 64-66). IL-7 is necessary for the survival and development of T-cells and has an essential role in early T-cell development (67-71). It is produced by the epithelial cells in the thymus, and IL-7 signaling is critical for thymocyte development (72). The necessity of IL-7-mediated signaling in T-cell development is clearly illustrated by the fact that humans with defects in the IL-7 receptor develop a severe combined immunodeficiency characterized by a lack of T-cells, but the presence of NK-cells and B-cells (2, 66, 73). In a study in mice where the expression levels of IL-7 could be controlled by a transgene, it was shown that there are distinct effects of different concentrations of IL-7 upon T-cell development. Only low doses of IL-7 could support TCR-αβ T-cell development, while high doses effectively blocked development at an early intrathymic stage (66, 74).

Circulating serum levels of IL-7 is normally low (0.3–8.4 pg/ml) (64), supporting T-cell development. However, this changes in lymphopenia when the levels can rise considerably as a response to low lymphocyte levels, as has been described in patients with HIV (75). Abnormally high levels can induce the development of lymphoma (76). Experimental data also suggests that different IL-7 levels influence the fate of mature T-cells. Low concentrations of IL-7 provide survival signals, while high concentrations can induce proliferation (69, 77). Different effects of IL-7 on naive CD4+ T-cells compared to CD4+

memory T-cells have also been seen in vitro, with a higher proliferation rate in the memory compartment (78). The induced proliferation has also been seen to differ between CD4+ T-cells and CD8+ T-cells: CD4+ T-cells proliferate significantly slower (79). In animal experiments of injection of IL-7, the increase of CD4+ T-cells was only four-fold as compared to the 14-fold increase in CD8+ T-cells (80).

A central function of IL-7 is its pro-survival effects in memory T-cells. IL-7 is the main cytokine involved in the survival of CD4+ memory T-cells, and is thereby necessary for the maintenance of the CD4+ memory pool (81). Also, the homeostatic cytokines, including IL-7 and IL-15, have been shown to induce proliferation in CD4+ effector memory T-cells, and to a lesser extent also in CD4+ central memory T-cells (82). Umbilical cord blood CD4+ T-cells have a comparatively higher expression of the IL-7 receptor α-chain (CD127) than adult CD4+ T-cells, which might lead to increased sensitivity to IL-7 stimulation in this type of T-cells (83).

IL-7 is also important to CD8+ memory T-cells (70, 84). The IL-7 receptor is essential in memory T-cell development (70, 85). Increased expression of the IL-7 receptor α-chain has been found to be a marker of memory development in CD8+ effector T-cells (86). However, the development of CD8+ memory T-cells has been shown to be possible to achieve in the absence of IL-7 (87). Thymic stromal lymphopoietin (TSLP), whose receptor consists of IL-7Rα and a β-chain specific for this cytokine, may be able to replace IL-7 in CD8+ T-cell memory development (88). IL-2 has been found to increase responsiveness to homeostatic proliferation induced by IL-7 and IL-15 in CD8+ memory T-cells (84), which might indicate that the addition of IL-7 could increase the efficiency of CD8+ T-cell proliferation in cell culture.

IL-7 is a mediator of inflammation by inducing production of the pro-inflammatory cytokines IFN-γ, IL-2, tumor necrosis factor (TNF)-α and IL-4. This has been shown both in vitro and in animal models (89-91). IL-7 has also been shown to be associated with autoimmunity, supporting the hypothesized role of this cytokine in inflammatory responses. There is evidence of an association between polymorphisms in IL-7Rα and an increased risk of multiple autoimmune conditions, such as multiple sclerosis (92), rheumatoid arthritis (93), systemic lupus erythematosus (94), and inflammatory bowel disease (95), indicating that IL-7 is a link in a major pathway underlying these diseases (92, 96).

I.2 ALLOGENEIC HEMATOPOIETIC STEM CELL TRANSPLANTATION

Allogeneic hematopoietic stem cell transplantation is currently the best available therapy for patients with a wide range of diseases such as hematologic malignancies, immunodeficiencies, inborn errors of metabolism and acquired bone marrow failure syndromes (4, 97). In many of these diseases, HSCT is the only potentially curative treatment available (97).

I.2.i A short history of HSCT

The history of HSCT began more than 50 years ago with E. Donnall Thomas's report of a new approach to cancer treatment based on radiation and chemotherapy followed by the intravenous infusion of bone marrow (98). An interest in the effects of irradiation had been awakened after World War II by the threat of atomic bombs. When Jacobson et al. found that mice could survive lethal irradiation if the spleen were protected by a lead foil (99), and Lorenz et al. described a similar protective effect of an infusion of spleen or marrow cells after irradiation (100), the foundation for what is today known as HSCT was lain (101).

During the late 1940s and 1950s, intensive research was conducted into this new concept, but with little success. All recipients of bone marrow died of graft rejection, graft-versus-host disease (GVHD), or opportunistic infection. The breakthrough came in experiments conducted by Professor Thomas in an outbred canine model. He found that most dogs that received irradiation and infusion of littermate marrow had the same problems as humans, but that occasionally a dog became a healthy long-term survivor with marrow cells of donor origin. He and his colleagues then developed canine histocompatibility typing. By the mid-1960s, they could show that most dogs given sufficient irradiation followed by grafts from dog leukocyte antigen-matched littermates survived long-term (102, 103). When histocompatibility testing in humans emerged, studies in humans was again initiated, culminating in the first successful transplantations in the 1970s (103).

During the decades that followed, great advances in several aspects of the procedure have greatly improved the outcomes of HSCT recipients. These developments include better preparatory conditioning regimens and improved immunosuppressive and antimicrobial treatments (97, 101). The arrival of alternative donor sources has also increased the availability of HSCT (6, 7, 104-108). The number of transplants has increased continually, and currently over 50,000 HSCTs are performed annually worldwide (97, 109).

I.2.ii Indications for HSCT

The indications for HSCT are continually changing. Hematological malignancy is still the most common disease group in HSCT recipients, followed by primary immunodeficiency and other congenital hematological disorders. However, solid tumors are also HSCT indications in some transplant centers, and some autoimmune disorders have also been the cause of treatment with HSCT (110). Here follows a concise description of the more common HSCT indications:

Of the hematological malignancies, acute and chronic myeloid leukemia (AML and CML, respectively), acute and chronic lymphatic leukemia (ALL and CLL, respectively), myelodysplastic syndrome (MDS), lymphomas and myeloproliferative diseases, are relatively common HSCT indications. Leukemia is a heterogeneous disease group defined by an infiltration of bone marrow, blood and organs by cancer cells of hematopoietic origin (111). The prognosis and treatments alternatives vary between different leukemia types.

AML and **ALL** are fast-growing undifferentiated leukemias derived from the myeloid and lymphoid cell lineages, respectively (see Figure 2). AML arise from myeloid precursor cells of different maturation stages: approximately 20% of AML cases are found in cells that lack maturation from an early precursor stage, while others are found in later precursor stages such as monocytes, erythroid cells, or megakaryoblasts. ALL is derived from lymphoid precursors cells: B-lymphoblasts and T-lymphoblasts. The B-cell precursor types of ALL are most common. ALL is the most common malignancy in childhood (111). The different types of both ALL and AML differ significantly in many respects, including in prognosis.

CML and **CLL** are malignant diseases of more mature cells of the myeloid and lymphatic cell lineages, respectively. (111). CML is characterized by clonal expansion of myeloid cells driven by a chromosomal fusion-derived gene, BCR-ABL (Philadelphia chromosome), and the progression is divided into different phases: chronic phase, accelerated phase and blast crisis, identified by the amount of malignant blast cells. The phase division is important for prognosis. In the case of CLL, the malignant cells originate from mature B-cells (111, 112). CLL is considered the leukemic manifestation of a type of lymphoma: small lymphocytic lymphoma (111). **Lymphoma** is a group of malignant diseases in lymphocytes, both T-cell and B-cell forms exist (111). Classical lymphomas are characterized by solid lymphoid tumors in different body sites. They are divided into Hodgkin's lymphoma and non-Hodgkin lymphomas. The latter are further classified as indolent, aggressive and highly aggressive, according to the rate of growth. This classification is of great importance for the choice of treatment strategy. HSCT is a treatment option in several of the lymphoma types, in some as a primary treatment, in others after a relapse (110).

MDS is a group of diseases caused by genetic aberrations in hematopoietic progenitor cells, classified as neoplasias (111). **Myeloproliferative diseases** is a collection of disorders characterized by overproduction of hematopoietic cells, e g thrombocytes or erythrocytes, and/or myelofibrosis, with defective bone marrow function and risk for leukemic transformation (111).

The non-malignant diseases that are treated with HSCT include aplastic anemias, primary immunodeficiencies, metabolic disorders and hemoglobinopathies. Aplastic anemias can be divided into congenital and acquired types. **Idiopathic severe Aplastic Anemia** (SAA) is an acquired autoimmune condition where the bone marrow is destroyed by an immune reaction that seem to be driven by T-cells and cytokines such as IL-17, IFN-γ and TNF-α (113). **Fanconi's Anemia** is an example of a congenital aplastic anemia and is caused by mutations in DNA damage repair genes. This disease is characterized by development of AML and solid tumors, bone marrow failure and developmental abnormalities (114). Primary immune deficiencies that require transplantation include **severe combined immunodeficiency** (SCID), where the patient lack B-cells and/or T-cells and/or NK-cells, **chronic granulomatous disease** (CGD) characterized by defective phagocytosis, and hemophagocytic lymphohistiocytosis (HLH) that is a condition with uncontrolled

inflammatory responses and the presence of abnormal phagocytes that engulf healthy bone marrow cells, causing cytopenia. Also, Whiskott-Aldrich syndrome, where the patients suffer from thrombocytopenia; IPEX, where the symptoms include immune-mediated cytopenia, and type 1 diabetes mellitus; and hyper-IgM syndrome, are considered HSCT indications (3). Among the metabolic diseases, **Hurler's syndrome** is the most common HSCT indication. Hurler's syndrome is also known as mucopolysaccharidosis type I, and is classified as a lysosomal storage disorder. **Hemoglobinopathies,** such as Thalassemia, are congenital defects in the hemoglobin genes, resulting in decreased functional hemoglobin levels, damage to the erythrocyte membrane and ineffective erythropoiesis (115).

I.2.iii Sources for hematopoietic stem cell graft

Hematopoietic stem cells (HSCs) are located in the bone marrow and can be harvested for HSCT grafts in different manners. The first approach used for obtaining HSC grafts was to harvest bone marrow manually from the posterior iliac crest with a bone marrow biopsy needle (22, 116). Additional methods for obtaining stem cell grafts have emerged during the 50 years HSCTs have been performed (116). The most common graft in many centers today, especially for adult patients, is the peripheral blood stem cell graft. This type of HSC graft is harvested by apheresis after injections with granulocyte colony-stimulating factor (G-CSF) have induced the HSCs to migrate to the peripheral blood (116-118). Umbilical cord blood, harvested by collecting the blood in the umbilical cord and placenta after childbirth, has also more lately been discovered to be a rich source of HSCs that can be easily collected and cryopreserved as HSCT grafts (22, 116, 119).

I.2.iv Donor selection

The selection of a suitable donor is a crucial step in the transplantation process. Donor type is well-known to impact the outcome of HSCT (120). The selection of donors is made primarily on the basis of the human leukocyte antigens genes, striving for as complete a match between donor and recipients as possible. The HLA genes that are analyzed at the Karolinska University Hospital are HLA-A, -B, -C, -DRB1, -DRB3, -DRB4, -DRB5, -DQA1 -DQB1, -DPA1, -DPB1 (121). The primary donor choice is an HLA-identical sibling, if possible avoiding a female donor to a male recipient, as this has been found to give the best outcome (22, 120). The chance of a 12/12 match is higher in siblings. A syngeneic twin, however, is not an optimal choice for patients with malignant disease, as this donor type is associated with an increased risk of relapse (122). At present, a matched unrelated donor from a donor registry is the second donor choice in many transplant centers, including Karolinska University hospital. This is also the recommended in the European Society for blood and Marrow transplant donor choice algorithm (22). Alternative donors sources, such as UCB and partially HLA-matched relatives are usually the 3rd choice of donor source. These alternative donor sources have more recently been introduced into HSCT. Both have gained ground over the last decade, and are now established in many centers. The results in transplantation with

UCB as a stem cell source, and with bone marrow or peripheral blood from haploidentical donors are comparable to those in transplantation with a matched related or unrelated donor (6, 107, 108, 123, 124). This thesis is focused on UCB, which has been the most commonly used 3rd choice graft at Karolinska University Hospital. Umbilical cord blood transplantation is described in more detail in chapter I.2.IX.

I.2.v The transplantation procedure: preparations, conditioning treatment, post-transplantation neutropenia, engraftment and complications

A schematic overview of the transplantation process is shown in Figure 6.

The transplantation procedure is a complex process including several co-operating clinical specialties. First, the patient needs to be in a good clinical status to increase the chances of treatment success, and decrease the risk for complications. The patients with malignant disease are pre-treated with the aim of as extensive disease control as possible, to decrease the risk of relapse (120, 125). All patients are carefully checked for signs of infection and infection risks, as any current or latent infection will be probable to flare up when the control of the immune system is lifted after the transplantation. A previous study at CAST has shown that even a slightly increased level of C-reactive protein remaining after a recent infection is associated with a significant increase in transplant-related mortality (TRM) in patients receiving a reduced intensity conditioning (RIC) (126). This clearly underscores the importance of optimizing clinical infection risks before HSCT.

In parallel to the procedures above, a donor is selected. The pre-transplant conditioning treatment is then initiated. This is a treatment that includes different types of chemotherapist drugs and sometimes also radiotherapy. The main functions of the conditioning treatment are to achieve disease control and to induce immunosuppression to prevent rejection. Conditioning treatment has also been thought to create necessary space in the bone marrow for the graft, but this is controversial (22).

The conditioning regimens are either myeloablative (MACs), causing sufficient damage to the bone marrow calculated to make autologous hematopoietic recovery unlikely, or milder reduced-intensity conditionings. The MACs are usually cyclophosphamide combined with either busulphan or fractionated total body irradiation (TBI) (4). These are tough treatment regimes, limiting their applicability to younger patients in good clinical condition. The RICs used are usually combinations of fludarabine with a lower dose of cyclophosphamide or busulphan than in the MACs, or with treosulfan (4). Fludarabine and cyclophosphamide can also be used together with a low dose fractionated TBI (4). The choice of protocol is based on underlying disease, previous treatment, clinical status and age. Anti-thymocyte globulin (ATG) treatment is also added to the conditioning regimen in patients with malignant disease receiving a matched unrelated donor transplant or a UCBT, and in all patients with non-malignant disease, to reduce the risk o rejection and to prevent GVHD (4).

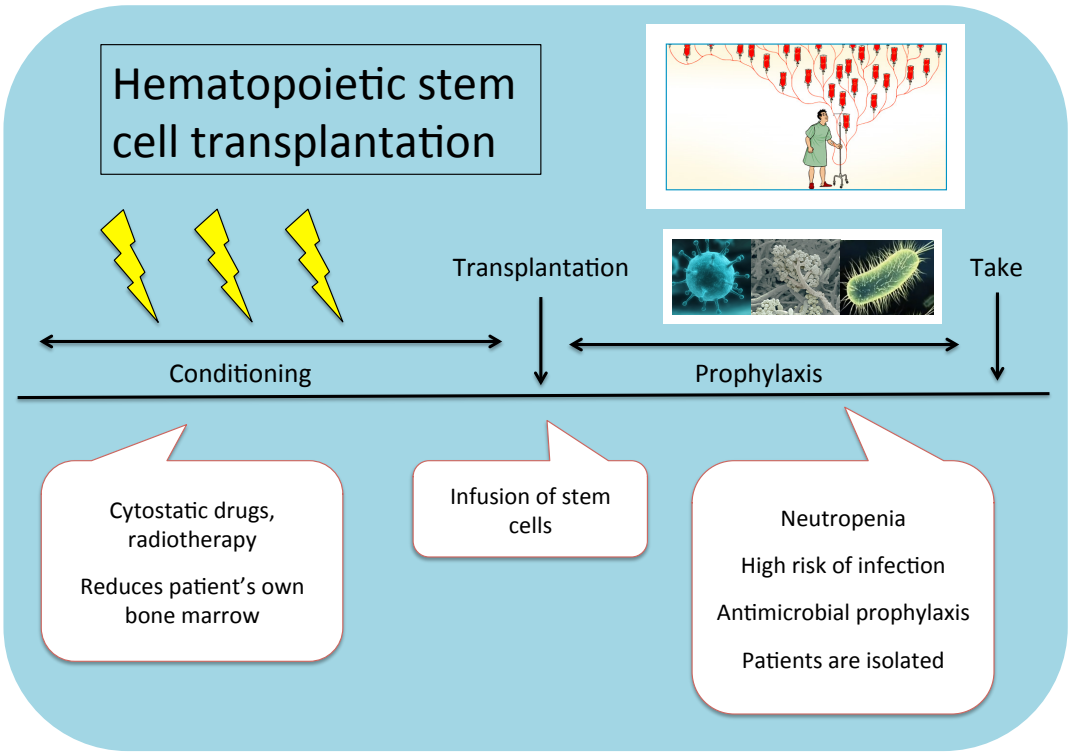

Figure 6. The transplantation process.

The patients are also given immunosuppressive treatment to prevent GVHD. The most common regimes include calcineurin inhibitors, such as cyclosporine A or tacrolimus, in combination with a short course of methotrexate (127-129). The calcineurin inhibitors mediate an inhibition of T-cell function, and methotrexate is used to non-specifically deplete alloreactive cells that are activated soon after transplantation. More recent agents used for GVHD prophylaxis include sirolimus, which inhibits T-cell function by blocking activation of mammalian Target of Rapamycin (130), and mycophenolate mofetil, which causes blockade of T- and B-cell function (22).

The graft is infused after the conditioning treatment is concluded. Then a period of neutropenia follows. The patients either stay in the hospital ward, isolated during daytime, or in home care with special regulations due to their extreme susceptibility to infection during this period (131). During the neutropenia, the damage caused by the conditioning regimen cannot heal properly. This results in mucositis, a painful condition with wounds and blisters in mouth and throat, and diarrhea caused by damage to the mucosa of the gastrointestinal tract. Mucositis resolves at engrafted, when production of neutrophils is resumed.

Engraftment, which is when leukocytes become measurable in peripheral blood after HSCT, is usually a sign of the transplanted stem cells being established successfully in the host. However, in a small percentage of HSCT recipients the emerging cells are autologous and signal graft rejection. Engraftment is defined as a blood neutrophil count of $>0.5\times10^9$/L, and a platelet count above 30×10^9/L. If no engraftment is detected, the graft has failed to be established in the bone marrow. Graft failure is defined as no reconstitution of hematopoiesis

after HSCT. Rejection, where the host immune system manages to eradicate the transplanted cells, is defined as either no detection of donor cells after HSCT or complete loss of donor cells after initial engraftment. These definitions were used in scientific paper I, and are adapted from Nordlander et al (132).

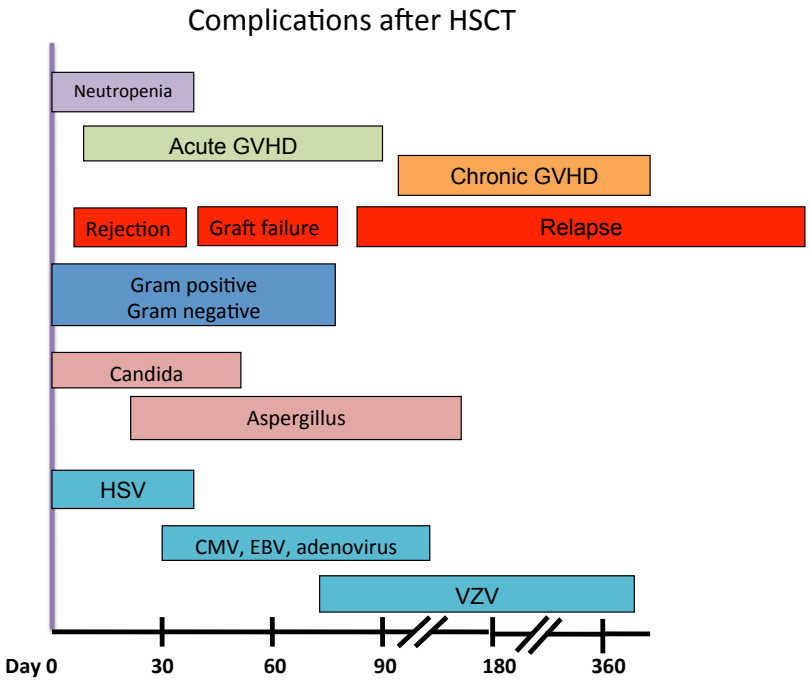

Figure 7. An overview of the common complications after HSCT

HSCT is a difficult treatment, associated with severe complications and even a degree of mortality. The most common categories of complications are infections, GVHD and relapse.

Cancer relapse remains the major cause of failure in HSCT recipients with malignant diseases (4, 133), despite the strong immune-mediated graft vs. tumor effect (GVT) (5). Relapse is also one of the few aspects of HSCT that has not improved during the last decades. This is still an area where additional and improved methods for early diagnosis and treatment are urgently needed. Infectious complications and GVHD will be described more in detail below.

An overview of the complications is found in Figure 7.

I.2.vi Infectious complications after hematopoietic stem cell transplantation

The infectious complications can be divided based on the causative pathogens: bacterial, fungal and viral infections.

Bacterial infections are common in the neutropenic period before engraftment. The most common path of infection is the translocation into the blood of bacteria from the normal endogenous flora in the gastrointestinal tract, mouth, throat and skin. Another common path of infections are via intravascular devices, such as central intravenous catheters. Streptococci,

Staphylococci, Enterococci and E. coli are the most common types of bacteria in neutropenia (22, 134). Later after the transplantation, there is an increased rate of infections with encapsulated bacteria, like Streptococcus pneumonia and Haemophilus influenza. This type of infection primarily affects patients with chronic GVHD, patients that have undergone splenectomy and patients with low blood antibody levels (22).

Fungal infections after HSCT are usually caused either by yeasts, mainly candida, or by molds, most commonly aspergillus. Candida infections dominate in the early parts of the transplant process in consequence of neutropenia and damaged mucosal surfaces. Patients receive prophylaxis against Candida infections, and invasive infections are rare. Mold infections, as is illustrated in Figure 5, become the more frequent of the fungal infections after the first month post-transplantation. Mold infection is a relatively common cause of severe infection and infection-related death after HSCT: 5-15% of HSCT recipients develop a mold infection with apergillus, and 60% of these patients die from the infection (135). Mold infections are most commonly contracted by inhalation of air-borne spores, which makes prevention difficult (22).

Virus infections after HSCT are commonly caused by herpes viruses. The most common of those is cytomegalovirus (CMV). A large proportion of the population is infected with CMV in childhood. The virus becomes latent after the primary infection, and is carried by the infected individual without causing symptoms. Symptomatic reactivation of CMV is only seen in immunosuppressed individuals such as transplant recipients (22). CMV can cause disease in many organs, such as the lungs, the gastrointestinal tract, the meninges and the eyes of an immunosuppressed host, and was one of the more common causes of transplant-related death before the advent of effective treatments (136). Quantitative PCR-based monitoring of CMV reactivation is recommended in all HSCT recipients (136).

Another herpes virus that can cause severe disease in immunosuppressed individuals is Epstein-Barr virus (EBV. Primary EBV infection is common in children and young adults, and causes mononucleosis. Similarly to CMV, EBV is carried in a latent state after the primary infection. EBV reactivation in HSCT recipients it can cause a life-threatening complication called post-transplantation lymphoproliferative disease (PTLD). This is an EBV-driven uncontrolled proliferation of B-cells similar to lymphoma. Monitoring of EBV reactivation using quantitative PCR is recommended in patients with high risk for this complication. The risk factors for PTLD that have been identified are: unrelated and/or mismatched donor; use of T-cell depletion, treatment with ATG; EBV serology mismatch between the donor and the recipient; primary EBV infection, and splenectomy.

Varicella zoster virus (VZV) reactivation is common after HSCT, with a cumulative incidence of approximately 20% in patients at our center (137). VZV is also a member of the herpes virus family, and the cause of chicken pox and shingles. Chicken pox is very common in childhood and represents the primary VZV infection, after which the virus becomes latent.

A later reactivation of VZV results in shingles. Complications of VZV reactivation in HCT recipients include postherpetic neuralgia, disseminated infection, and occasionally even death (137).

Adenovirus is a common virus that mainly affects children, causing respiratory symptoms and gastroenteritis. In HSCT recipients, adenovirus can cause severe infections with disease manifestations such as hepatitis, pneumonitis, encephalitis, colitis and hemorrhagic cystitis, and with considerable morbidity and mortality (138, 139). In a proportion of the transplanted patients, adenovirus appears concomitantly to other infections, such as CMV, EBV and invasive fungal infections, which is associated with poor prognosis (138).

Other important viruses that pose a danger to transplant recipient are human herpes virus 6, which can cause encephalitis in immunosuppressed individuals (22) and Parvovirus B19, that can cause myelosuppression (140, 141). Common infections like influenza and calici virus infection, can also infect transplanted patients, and in them cause more severe symptoms and longer infection duration (142).

I.2.vii Graft-versus-host disease and graft-versus-tumor effects

One major mechanism in HSCT that mediates the cure of hematological malignancy is the immunologically mediated eradication of the remaining malignant cells by the donor immune system. The immunogenicity in the recipient's cancer cells that permits the donor's immune system to identify and destroy them is thought to be induced by the genetic differences between donor and recipient outside the analyzed HLA genes. Evidence of a donor immune-mediated mechanism behind the GVT effect came in the late 1970s, when higher frequencies of relapse after transplantation with syngeneic HSCT than allogeneic HSCT were reported (143). Another milestone report from the early 1990s showed that the incidence of relapse was increased in HSCT with T-cell depleted grafts compared to with T-cell replete grafts. The increased risk of relapse also corresponded to a decreased risk of GVHD (122). The same study also found an increased rate of relapse in patients that had received syngeneic grafts from an identical twin, supporting the theory of an immune-mediated GVT effect driven by genetic differences. When donor lymphocyte infusion (DLI), a type of treatment where bulk donor lymphocytes are infused after transplantation, was shown to induce remission in relapsed malignancies post-HSCT, this formed additional support of an immune-mediated GVT effect (144). The GVT effect is thought to be mainly produced by T-cells (5, 145-147). T-cells could mediate GVT through several mechanisms, including cytokine-mediated effects and cytolytic activity. Cytolytic activity can be mediated either through Fas receptor−Fas ligand-mediated apoptosis or by perforin/granzyme degranulation. CD4+ T-cells appear to predominantly induce apoptosis by Fas receptor−Fas-ligand interaction, while CD8+ T-cells seem to mediate GVT effects mainly by perforin degranulation (146). Data supporting a central role for CD4+ T-cells for the GVT effect in clinical HSCT has been published (148). NK-cells, and other cell subsets, have also been implicated as effectors of

the GVT effect (149). Intense research efforts have been directed against identifying the antigens that drive the immune reaction in GVT, resulting in a growing list of potential candidates. An association between GVHD and GVT has been found in a large number of studies over the decades, suggesting that the GVT effect is closely allied to GVHD and that GVT responses target antigens in a non-tumor-specific manner (122, 150).

GVHD is mediated by a host immune reaction directed towards the tissues of the recipients and can be considered the reverse side of the beneficial alloreactivity of the GVT effect. There is an acute and a chronic form of GVHD that differ in pathogenesis, symptoms and time of debut.

Acute GVHD is usually developed during the first months after transplantation, and most commonly affects the liver, the gastrointerstinal tract, and the skin. The symptoms can be so severe that they directly, or indirectly by subsequent infection, lead to death. The pathology has been attributed to a process that starts with the tissue damage caused by the conditioning regimen. This leads to activation of host antigen-presenting cells and donor T-cells and finally to inflammation and tissue necrosis associated with inflammatory cytokines such as IL-1 and TNF-α (22). Risk factors for GVHD include HLA mismatch between donor and recipient, and female-donor-to-male-recipient HSCT (22). The latter risk is believed to be due to the fact that the new host immune system will not have encountered Y-chromosome gene products (151).

Chronic GVHD usually arise more than three months after HSCT. It is caused, like acute GVHD, by host alloreactivity directed towards recipient tissues, butchronic GVHD is also considered a disease of immune dysregulation with, and shares features with autoimmune diseases such as Sjögren's syndrome, scleroderma, primary biliary cirrhosis and immuncytopenias (22). The deails of the pathogenesis is poorly understood. T-cells and B-cells are believed to play central roles (22, 152). There is an increased rate of mortality in patients with chronic GVHD as well as in acute GVHD, but mainly indirectly, as a result of an increased susceptibility to infection. GVHD treatment is based on immunosuppressive therapy, and the treatment time of chronic GVHD is often long. The immunosuppressive treatment together with the immunosuppressive effects of the chronic GVHD in itself lead to a dramatically increased risk of infection (22). Some major risk factors for chronic GVHD are prior acute GVHD, HLA mismatch, older patient age, previous splenectomy and female-donor-to-male-recipient transplantation (22, 153, 154).

Chronic GVHD is the form of GVHD most firmly associated with GVT effects, observed in the improved long-term survival in patients that do develop this complication. However, extensive chronic GVHD causes severe morbidity and mortality, like the autoimmune diseases that it shares characteristics with. The chances of achieving GVT effect is always balanced by the risk of inducing GVHD in HSCT, and immunosuppressive treatment is at present the only clumsy tool available for manipulating this precarious balance with.

I.2.viii Monitoring of patients after HSCT

Close monitoring of patients after HSCT is of paramount importance for early detection of complications. The monitoring methods available are mainly based on the analysis of peripheral blood samples and bone marrow biopsies. These are assessed for the ratio of donor- and recipient-derived cells (chimerism) and for molecular, cytogenetic or morphological signs of relapse (in patients with malignant disease), signs of abnormalities in the hematopoietic cells in the bone marrow indicating suppression of the graft, signs of organ-specific GVHD and signs of infection. The early detection of these complications enables early treatment, which is associated with better outcome in most of the complications after an HSCT.

Chimerism analysis is a genetic assay used for determination of the percentage of recipient and donor origin in hematopoietic cells. This assay allows the assessment of immunological interactions between the donor and recipient immune systems. Chimerism analysis enables assessment of successful engraftment, and can also provide the physician with early indications of graft rejection (22, 155-158) and impending relapse in patients with malignant disease (159, 160).

Chimerism analysis was performed on samples from blood and bone marrow after transplantation in all the UCBT recipients to monitor engraftment and to facilitate early detection of relapse. Analysis was performed regularly at short intervals initially, up to 3 months, then at 6, 9, 12, 18, and 24 months, and thereafter annually. At Karolinska University Hospital, chimerism analysis is performed to evaluate donor and recipient chimerism in CD3+, CD19+, and CD33+ cells enriched from peripheral blood using immunomagnetic beads (Dynal, Oslo, Norway) (160). Two methods of analysis have been used in this thesis for chimerism analysis. One method used was based on polymerase chain reaction (PCR) amplification of variable numbers of tandem repeats, using minisatellites (160), while the other comprised a real-time PCR analysis based on single nucleotide polymorphism (7).

Complete donor chimerism (DC) was defined as >95% donor cells. Mixed chimerism (MC) was defined as <95% but >5% donor cells. Mixed donor-donor chimerism (MDC) was defined as a DC after double cord blood transplantation (DCBT) with no remaining recipient cells, but with a proportion of both the donors present.

Bone marrow biopsies were performed for histopathology analysis of relapse of malignant disease, and to a lesser extent to assess the engraftment of donor hematopoiesis. The bone marrow samples were also sent for cytogenetic analysis and flow cytometry to assess return or persistence of malignant cells. This assessment is called minimal residual disease (MRD).

I.2.ix Umbilical cord blood transplantation (UCBT)

Umbilical cord blood has emerged as a valid alternative graft source in allogeneic HSCT in the 25 years that have passed since the first successful UCBT (104, 161). Grafts are obtained

by collecting the blood from the umbilical cord and placenta after childbirth. The collected blood is cryopreserved without any previous cell separation, to preserve as many cells as possible, in appointed cord blood banks (104). Transplantation with UCB as a graft source leads to successful hematopoietic engraftment and yields clinical results comparable to those seen in HSCT with other stem cell sources (7, 104, 106, 124). Interest in this mode of HSCT has increased during the last decades, and UCBT has been implemented internationally. The unique advantages with UCB as a graft source are appealing: the most striking merit of this graft type is the permissibility for HLA-mismatch, which is not seen with adult graft sources. This permissibility greatly increases the chance of finding a donor for patients lacking an HLA-matched related or unrelated donor (9, 104). Other advantages with UCB include rapid availability of the frozen grafts from umbilical cord blood banks and comparatively risk-free collection of grafts with regard to the donor (9, 104). There are, however, also drawbacks to UCB as a graft source: UCBT is associated with delayed engraftment and poor immune reconstitution, especially during the initial months after transplantation. During this period, UCBT recipients have an increased risk for severe infections compared to recipients of transplantations with other graft sources. The grafts are comparatively expensive to obtain, and the costliness of the UCBT procedure is also increased by the complicated post-transplant period. The unavailability of donor cells for post-transplant therapy such as DLI and stem cell boosters is another major drawback (11, 104, 162)

I.3 IMMUNOTHERAPY

I.3.i Anti-cancer effects of the immune system

The concept of an immune surveillance function where the immune system locates and eradicates cancer cells, have been discussed for more than 50 years, and supportive evidence have been difficult to obtain. A recent review, however, has stated several arguments in support of the existence of such a function. One of the arguments was that both animals and humans with immunodeficiency have increased risk of cancer development. Mice that lack adaptive immune cells and mice that lack the ability to produce IFN-γ have an increased risk for both spontaneous and carcinogen-induced tumor development (163, 164). Humans with primary immunodeficiency and HIV have also been reported to have increased incidences of cancer (17, 165, 166). Organ transplant recipients, who are treated with immunosuppressive drugs, are also more prone to cancer development (17, 167). The quantity and quality of the immune cell infiltrate found in tumors has been shown to be an independent prognostic factor for outcome (17, 168-174). Cancer cells also selectively accumulate mutations to avoid infiltration of immune cells and a consequent immune-mediated destruction of malignant cells (17, 175, 176). Lymphocytes bearing the NK-cell receptor NKG2D receptor can find and eliminate premalignant cells (17, 177). Collectively, this indicates that the immune system plays a role in preventing cancers from developing. To harness this anti-cancer effect and focus it in engineered therapies are the main goals in immunotherapy.

I.3.ii Approaches in immunotherapy

Harnessing of the anti-cancer capacity of the immune system for cancer treatment has been attempted with promising results. In essence, cancer immunotherapy can be defined as manipulations of the immune system to achieve an increased activity toward tumor cells. Current approaches in immunotherapy are directed toward a wide range of different mechanisms of the immune system. Studies focused on the inhibition of T-cell development checkpoint molecules like cytotoxic T-lymphocyte-associated protein (CTLA)-4, programmed cell death protein (PD)-1, or PD-ligand 1 have produced interesting clinical and pre-clinical data (178-182). Intravenous interleukin treatment, as discussed in chapter I.1.IV.1, has been tried with both IL-2 and IL-7, among other substances, with promising results. Treatment with IL-2 has been found to induce durable cancer regression in a small portion of patients with advanced malignant melanoma and renal cell cancers (60). Treatment using cultured tumor-infiltrating lymphocytes, with and without parallel administration of intravenous IL-2, has also induced tumor regression in patients with very advanced cancers (61, 62). Treatment with IL-7 in patients with refractory malignancies resulted in increased T-cell levels, and possible anti-tumor effects in one out of 16 patients (183). Bi-specific T-cell and NK-cell engager antibodies (BiTEs and BiKEs) are other relatively new approaches in immunotherapy. These antibodies allow the in vitro coupling of effector and target cells, activating the effector cells while simultaneously marking out the target cells for destruction (184, 185). Adoptive cell therapy (ACT) is another branch of immunotherapy and comprises infusion of different kinds of in vitro manipulated cells. This includes treatment with T-cells transfected with tailored chimeric antigen receptors (CARs) directed towards cancer targets, such as CD19, a treatment modality that have lately astounded the research community (186, 187). Cultured tumor-infiltrating lymphocytes (TILs) (184, 185, 188) is another promising type of ACT. Jointly these approaches underscore the versatility and unprecedented possibilities of the immunotherapy field. Donor lymphocyte infusions is currently the most common ACT in the HSCT setting. The major indication for DLI treatment is relapse of hematological malignancy. The benefits of DLI are most pronounced in patients with relapsed CML and low-grade lymphoid malignancies (144, 189). DLI treatment has also been shown to be of benefit against relapse after HSCT in other hematological malignancies, especially if the treatment was initiated due to molecular signs of relapse rather than overt hematological relapse (144, 190, 191). Whether the results warrant the toxicity (mainly GVHD) in acute leukemia and MDS is arguable (189, 192-195). Virus-specific T-cells from a haploidentical third party donor for treatment of post-transplantation viral complications have also generated good clinical results (196, 197).

I.3.iii Immunotherapy in UCBT

One considerable drawback to UCB as a graft source is the lack of conventional DLI, which is a consequence of the low cell doses of UCB grafts and ethical considerations that excludes approaching the donor after the initial donation (10, 11, 198-200). As the number

of performed UCBTs continues to grow, it becomes increasingly urgent to find alternatives to DLI for UCBT recipients. Several approaches have been tried to develop alternatives to conventional DLI in the UCBT setting (10, 11, 198-200). One of those is the non-specific culture of T-cells from an aliquot of the original UCB graft (10, 11, 198, 200). This strategy has the advantage of yielding a product that shares characteristics with conventional DLI. To produce a product with non-selected, polyclonal T-cells is a logical step as antigens that drive the GVT effect are unknown. There is a high probability of producing cultured T-cell populations that contain alloreactive T-cell when all T-cell clones are equally stimulated. Other approaches that have been tried include bulk CD4+ T-cells (201) and expanded T-cells with single or multiple pre-determined specificities (199, 202). Some of the engineered products described in the literature have combined specificities for several viruses and known leukemic antigens in the same cell population (199).

The culture protocols used in T-cell based ACTs usually rely on expansion induced by artificial TCR/co-receptor stimulation combined with further activation and modulation by cytokines (10, 198, 200). The choice of cytokines is instrumental in creating a cell product well suited to its purpose. In this thesis, focus has been on the cytokines IL-2 and IL-7. As described above, these cytokines have several characteristics that single them out as potential candidates for effective cell culture in the UCBT setting. This will be discussed further in the results section.

The developmental and functional phenotype of adoptively infused T-cells has been found to have a considerable impact of the efficacy and longevity of the treatment. Preclinical ACT studies of in mice and non-human primates have shown that central memory T-cells persist longer in vivo, and have greater antitumor efficacy, than effector memory T-cells (203-205). Also, a study performed in mice has indicated that a Th17-polarised CD4+ T-cell population is the most effective ACT in malignant melanoma (206). Another candidate for ACT is a relatively recently described T-cell memory phenotype, termed memory stem T-cells. They are described to have a distinct phenotype, are thought to be long-lived, have the ability to self-renew, and to have a high degree of plasticity of differentiation, *e. g.* into potent effectors (41, 207, 208). Other in vitro studies has shown that effector memory T-cells have superior potential to acquire and exert cytolytic functions and cytokine production in vitro (209, 210). These partly conflicting reports indicate that there is more to learn before the optimal T-cell phenotype or ACT can be determined. They also show that the memory status of the cultured cells is important for the clinical outcome of the treatment. However, products with a high proportion of central memory T-cells, or possibly memory stem T-cells, could prove to be most long-lived, and thereby possible also most effective. Different strategies to induce the requisite phenotype will then need to be developed. Strategies that have been suggested include aiming for a short culture time, keeping cytokine levels as low as effectively possible and attempting artificial enforcement of a more naïve phenotype by induction of CD28 or telomerase expression (211).

I.4 AN INTRODUCTION TO THE METHODS USED IN THE THESIS WORK

I.4.i Flow cytometry

Flow cytometry was used in scientific papers III and IV of this thesis, and is a method for analyzing the properties of a particle, in the present case, a cell. The great advantage of this method is that it allows the assessment of properties of a single cell in a sample. Flow cytometry can be used to determine the presence and relative amount of molecules on the extracellular surface of the cell, or inside the cell, in the cytoplasm and the cell nucleus (212). This provides information on the cellular phenotype, or characteristics, and can also determine if a cell is alive, apoptotic or dead. Analysis of cell cycle characteristics can also be performed. The method allows the user to categorize cells into different subsets, which can yield information regarding what type of cells there are in the sample, if they are alive, if they are producing and secreting different signaling molecules, and if they have effector substances stored in granules or expressed on their surface.

Flow cytometric analysis is based on the use of marker-specific antibodies coupled to fluorochromes, which allows the detection of the targeted marker in the flow cytometer. The flourochrome is essentially a type of dye, a molecule that accepts light at a certain wavelength; and then emits the energy at another wavelength. This process of emission is known as fluorescence (212).

Fluorescence is utilized in flow cytometry by letting a laser beam hit the flourochrome in a controlled and predictable fashion and then detecting the emitted fluorescence. The laser beam is scattered at a forward and at a ninety degrees angle to allow assessment of the basic cellular characteristics, such as size, nuclear complexity and cytoplasmic granularity (212). During data aquisition the cells are passed through the laser beam in a thin stream forcing the cells into a single file. This single-cell stream is obtained by hydrodynamic focusing, where the sample stream is injected into a faster flowing stream of sheath fluid, most often phosphate-buffered saline (212). This enables analysis of a single cell at a time. The number of channels for fluorescence detection of the flow cytometer used, which can vary from 3 or 4 up to 18 (213, 214), and determines the complexity of single cell information that is possible to analyze in a single sample.

The analyses of the present study have been focused on the study of mainly T-cells, but also to a lesser extent NK-cells and B-cells. Phenotype, cytokine production, degranulation and viability have been studied with this method. The markers used and their properties are found in Table 1.

Table 1

Marker	Cell type	Characteristics and area of use
CD3	T-cells	Expressed by all T-cells, used for identification
CD4	T-helper cells	Co-receptor expressed on all T-helper cells
CD8	Cytotoxic T-	Co-receptor expressed on all cytotoxic T-cells
CD14	Monocytes	Expressed on monocytes and macrophages
CD16	NK-cells	NK-cell marker
CD19	B-cells	Expressed by B-cells, used for their identification
CD25	T-cells	IL-2 receptor α-chain. Expressed on T-regs.
CD28	T-cells	Co-activating receptor on T-cells, mostly on naive
CD45RA	T-cells	Found on naive T-cells
CD45RO	T-cells	Marker of memory identification, see definition
CD56	NK-cells	Expressed by NK-cells, used for identification
CD69	T-cells	Early activation marker in T-cells
CD85j	NK-cells	Inhibitory NK-cell receptor for MHC class I
CD94	NK-cells	NK-cell receptor, expressed by some T-cells
CD95	T-cells	Receptor for induction of apoptosis
CD107a	T-cells	Marker of degranulation
CCR7	T-cells	Marker of memory identification, see definition
IL-2	T-cells	Cytokine, T-cell activation
IL-10	T-cells	Anti-inflammatory cytokine
IL-17a	T-cells	Effector cytokine of the CD4+ T helper 17 subset
IFN-γ	T-cells	Cytokine produced by T- and NK-cells. Activates macrophages.
TNF-α	T-cells	Pro-inflammatory cytokine
TCR αβ	T-cells	TCR made up by one α-chain and one β-chain
TCR γδ	T-cells	TCR made up by one γ-chain and one δ-chain
TCR Vγ9	T-cells	One of the γ-chain subtypes of the TCR γδ
TCR Vβ 1-3, 5, 7, 8, 11-14, 16, 17, 20, 22	T-cells	TCR β-chain subtypes, used for the assessment of polyclonality
HLA class I A3	All nucleated cells	MHC class I of type A3, here used for separation of two donors in a DCBT recipient

I.4.ii T-cell stimulation assay

The T-cell stimulation assay was used to analyze T-cell function in scientific papers III and IV. Peripheral blood mononuclear cells from blood samples and thawed cultured UCB T-cells, in scientific paper III and IV, respectively, were re-suspended in complete medium with substances causing non-specific T-cell activation to assess the T-cells ability to respond to mitogens. The substances used were ionomycin calcium salt and phorbol 12-myristate 13-acetate (PMA). To assess the cytokine production upon activation, cellular cytokine secretion was inhibited by adding GolgiStop® or Brefeldin A, in scientific paper III and IV, respectively. Cells were subsequently incubated at 37° C with 5% CO_2 for 16 hours. The cells were then stained for the markers of interest and analyzed by flow cytometry.

I.4.iii NK-cell stimulation assay

NK-cell function was analyzed in paper III. Peripheral blood mononuclear cells from blood samples were resuspended in medium with GolgiStop® and incubated with PMA, or K562 cells at a ratio of 1:1, for 4 h at 37°C in 5% CO2. This was done to enable assessment of the NK-cells capability to respond to appropriate stimulation. K562 is a myeloid leukemia cell line of the erythroleukemia type, derived from a 53 year old female CML patient in blast crisis (215). K562 cells are easily killed by natural killer cells (216) as they lack the MHC complex required to inhibit NK-cell activity (217). After incubation, cells were harvested and stained for flow cytometric analysis.

I.4.iv Luminex

Luminex analysis was used to determine the cytokine content of culture supernatants at the end of UCB T-cell culture in scientific paper IV. At the end of culture, concomitant with cryopreservation of the cultured T-cells, supernatant was collected and analyzed for: eotaxin, granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), IFN-α2, IFN-γ, IL-1a, IL-1b, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-15, IL-17, IFN-γ-inducible protein -10, monocyte chemotactic protein-1, macrophage inflammatory protein (MIP)-1a, MIP-1b, tumor necrosis factor (TNF)-a, and TNF-b with the Milliplex Map Human cytokine/chemokine—premixed 26 Plex from Millipore. The Luminex analysis is based on beads with defined spectral properties that are conjugated to protein-specific capture antibodies. Addition of the beads to samples results in the target protein binding to the capture antibodies. Then additional antibodies bearing a fluorochrome are added before the sample is analyzed with a Luminex® detection system. By monitoring the spectral properties of the beads and the amount of fluorescence, the concentration of one or more proteins can be determined.

I.4.v UCB T-cell culture and obtaining samples for culture

For the study presented in scientific paper V on the culture of UCB T-cells for potential clinical use as ACT, an aliquot was obtained from the UCB grafts thawed for transplantation at CAST. Five percent of the total volume of every UCB graft has been collected since 2007. In total, cultures have been performed for 40 CB units (31 patients, 33 UCBTs). The expansions were given consecutive numbers as they were collected. The median total nucleated cell count in the aliquot was 60.5 million (range 20-154).

Non-clinical units were used for the studies of the effects of adding IL-7 to the culture protocol that are presented in scientific paper IV. The UCB was then obtained from healthy volunteers undergoing cesarean section at the maternity ward at Karolinska University Hospital Huddinge. All donors provided informed consent before donation as approved by the ethics committee at the Karolinska Institute (DNR 2007/4:10). Mononuclear UCB cells For the cultures, either the aliquot of the clinical UCB graft was taken at thawing, or non-clinical cryopreserved mononuclear UCB cells were thawed, according to the protocols in scientific paper IV and V, respectively. T-cells were positively selected with anti-CD3+/CD28+-covered paramagnetic beads. The beads also function as artificial APCs and mediate an activation of the T-cells. The selected T-cells were cultured at a concentration of 3 × 10^5 cells/mL in complete medium1640 RPMI with 10% pooled human AB-serum, 100

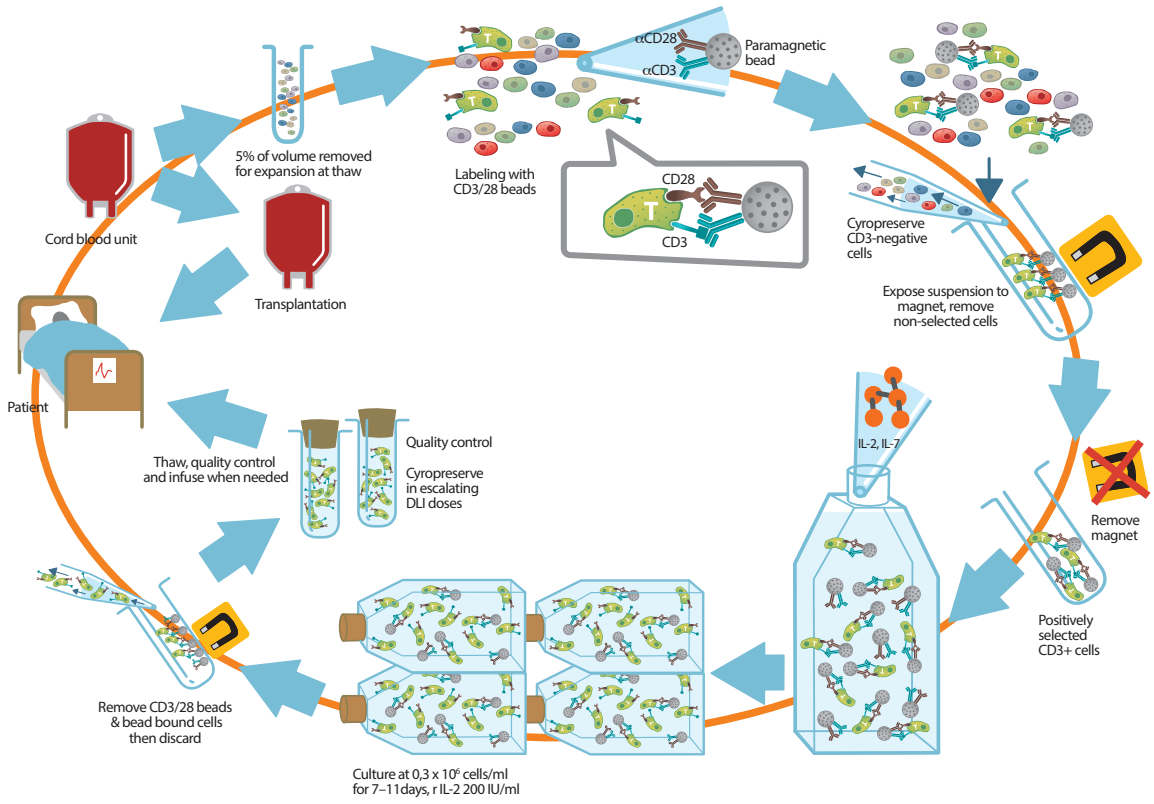

Figure 8. The UCB T-cell culture process.

IU/mL penicillin G, 100 mg/mL streptomycin, 0.25 mg/mL amphotericin B and 2mM L-glutamine. Recombinant IL-2 was added in varying concentrations (0–600 IU/mL) and IL-7 (20ng/mL) was added according to protocols in scientific papers IV and V (Table 2). Cell culture was performed at 37°C at 5% CO_2. Viable cells were counted regularly and resuspended to maintain the concentration of living T-cells at 3×10^5 cells/ml. At the end of culture, T-cells were harvested and remaining beads were removed by magnetic separation. Expanded UCB T-cells were then cryopreserved. The culture process is illustrated in Figure 8.

II. AIMS

The aims of this thesis work were to achieve a greater understanding of the UCBT process, and to improve the treatment opportunities available to recipients of UCB grafts. This patient group lacks the possibility of treatment with additional donor cell after the transplantation, which is available for recipients of grafts from adult stem cell sources. This lack of donor-derived cellular therapies results in a severely limited treatment arsenal against relapse of hematological malignancies and graft failure in UCBT recipients. This work was conceived in response to the urgent need for novel treatment approaches in UCBT. We aimed to:

1. Evaluate clinical outcome and risk factors for complications after umbilical cord blood transplantation, and to analyze the development of donor chimerism in umbilical cord blood recipients.

2. Develop clinically safe expansion protocols of donor T-cells from umbilical cord blood, and to characterize them in order to treat rejection or relapse of the underlying disease after umbilical cord blood transplantation. Also, we aimed to assess of the safety and outcome of treatment with the cultured cells.

III. RESULTS AND DISCUSSION

The five articles presented in this thesis were performed with the intention of reaching a greater understanding of the UCBT process, and of developing novel adoptive cell therapies for patients that have undergone UCBT.

The first aim was to comprehensively study the results of UCBTs performed at our clinic. This type of retrospective single-center analysis is of significant value, and not only for quality control purposes. The compiled single-center data, when compared to international results, can help to identify factors that can be improved in local medical practice. The elucidation of local factors influencing treatment outcome is another important point in favor of retrospective single-center studies. Factors such as climate parameters, affecting both the panorama of infectious pathogens (218), and patients' behavior with regard to *e. g*. exercise and sun exposure, can necessitate adaptations in medical practice. Differences in diagnostic procedures and availability of specialist consultants can also be of importance for the patient outcome. These factors cannot always be predicted or extrapolated from international experience. Thus, analysis of single-center clinical results can provide guidance in clinical choices and pinpoint how local factors interact to create unexpected effects. However, the comparative smallness of our center necessitates longer data collection times, which complicates the analysis by adding time-dependent issues such as changed medical practices and variable follow up time. The small number of annual UCBTs also resulted in inclusion of heterogeneous patient materials in the studies.

The results of scientific papers I and II have been useful in guiding decisions about the transplant procedure at CAST. The results of scientific paper I led to a discontinuation of the use of mesenchymal stem cells (MSCs) as engraftment support in UCBT, and the dosage of ATG has been lowered for recipients of DCBT as a result of the findings in scientific paper II.

A large part of the work presented in the first three scientific papers is focused on chimerism development after UCBT. Our center has a tradition of frequent chimerism analysis after HSCT, in three or more cell lineages, creating a unique foundation for comprehensive chimerism analysis. We wanted to exploit this by performing in-depth studies of the dynamics of chimerism development in UCBT recipients. In scientific paper I, chimerism development was analyzed in all the patients that had been transplanted with UCB at CAST. Scientific paper II focused on chimerism development in patients that had undergone DCBT. In this study we described two patients with long-term mixed donor-donor chimerism (MDC) after DCBT with two unrelated and partly HLA-mismatched UCB grafts, a very unusual occurrence. The co-existence of two transplanted immune systems of unrelated origin in the same host is fascinating. We wanted to further explore and characterize this interesting chimerism state, which led to the performance of the study presented in scientific paper III. We here studied whether the immune phenotype and functionality differed in these two

patients compared to UCBT recipients with only one donor system, and how the two transplanted units differed from each other.

The second aim was to develop alternative adoptive cell therapies for the UCBT setting. The research group in which this thesis was performed has for several years been researching alternatives to conventional DLI for UCBT recipients, and some of this work was published prior to the present project (11). The chosen strategy was to culture UCB T-cells from an aliquot of the UCB graft, using CD3/CD28-stimulation with antibody-covered paramagnetic beads and IL-2. The work presented in scientific paper IV was performed in attempting to optimize the protocol by adding IL-7 to IL-2 in the culture medium. We also wanted to study the biological effects of IL-7 in combination with IL-2 in UCB T-cells.

The cultured cell products attained using this method have also been used for clinical treatment in four patients. The main aim of this study was to assess the safety of the cell preparations, and the secondary aim was to identify potential benefits of the treatment. The results are presented in scientific paper V.

III.1 CLINICAL OUTCOME AFTER SINGLE AND DOUBLE UMBILICAL CORD BLOOD TRANSPLANTATION (SCIENTIFIC PAPERS I AND II)

The studies presented in scientific papers I and II were both performed as retrospective single-center analyses of the clinical outcome of UCBT. The study in scientific paper I included 50 patients that had received a UCBT at CAST, from 2001 to 2010. Patients undergoing re-transplantations with a second single or double cord blood graft were not included in the study. The patient characteristics were heterogeneous as all UCBT recipients were included. There were 30 children and 20 adults. Eighteen of the patients, all children, had non-malignant diseases, while the remaining 32 had different hematological malignancies. They received conditioning treatment with six different combinations of chemotherapy and radiotherapy, the majority of which were full myeloablative regimens. All patients received ATG. The donor-recipient HLA-matching varied: a full match of six out of the six assessed HLA antigens was achieved in only five patients. The remaining grafts were matched on 3 to 5 out of six HLA antigens. The heterogeneity of the patient group complicated the interpretation of the results. Additionally, the status of UCB as a third choice graft source is likely to have influenced the results of the study. This means that other options had been exhausted before a search for a suitable UCB graft was initiated, resulting in delayed transplantation even though UCB grafts are more rapidly available. Thus, the time from diagnosis to transplantation was longer than what is usual for patients at CAST (median 11 months (range 2-148) compared to 9,5 months (range 1-318), personal communication Prof Mats Remberger). The third choice status of UCBT also resulted in a comparatively high percentage of patients with advanced malignant disease (63%) (4). As a consequence, this patient group had an inherently poor prognosis.

The overall survival of the whole group was 43% at 5 years. Multivariate analysis of factors

that could influence survival revealed that increasing age (as a continuous variable, hazard ratio (HR) 1.04; P=0.001), acute GVHD grades III and IV (HR 3.43, P<0.001), and treatment with MSCs (HR 2.66, p<0.027) were significantly associated with decreased 5-year survival. Factors that were significantly associated with a lower 5-year survival in the preliminary univariate analysis, but were no longer significant in the multivariate analysis, were malignant disease (p = 0.03) and DCBT (P = 0.05). As the majority of both the recipients of DCBT and the patients with malignant disease were adults, these associations could have been partly attributable to age.

As expected, adult patients with malignant disease had the lowest 5-year overall survival (OS, 24%). Conversely, the group with the highest 5-year OS was patients with non-malignant disease, all children (72%). The low 5-year overall survival in adults with malignant disease was partly due to a high relapse rate and partly to a high rate of transplant-related mortality (TRM).

The strong negative correlation between age and survival might be partly attributable to the fact that 18 out of 30 of the children had non-malignant diseases, associated with better prognosis than malignancies. Children and younger adults are known to have a better immune reconstitution after HSCT, better thymus function, and a superior capacity to recover from strenuous treatments such as HSCT (219). A recent study in mice has suggested that age-related involution of the thymus can be reversed by forced expression in thymic epithelial cells of the transcription factor Forkhead box protein N1 (FoxN1) (220). This is of course experimental data, but if the concept can be taken into a clinical setting, this strategy for improving thymic function would be of considerable interest.

As is often seen in UCBT, the children in this study received higher doses of total nucleated cells (TNC) and CD34+ cells/kg patient weight than the adult patients. They also received comparatively better-matched grafts. A high cell dose and a well-matched graft are known to positively influence outcome (9, 221, 222).

None of the five patients with malignant disease that had received a reduced-intensity conditioning were alive at the close of the study, while the 5-year overall survival was 33% in patients with malignant disease that had undergone a myeloablative conditioning (p = 0.05). The patients in the RIC-group were all older than 50 years, and thus part of this association could be attributable to the negative correlation between age and survival. Due to the low number of patients in the RIC-group (only 5, compared to 27 patients in the MAC-group) this should be interpreted with caution.

The negative impact of higher age, acute GVHD grades III-IV and malignant disease on survival have been previously described in both UCBT and in HSCT in general (4, 223, 224). However, the association with MSC treatment has not been described previously. Treatment with MSCs was given to nine patients, either infused with the UCB graft as engraftment support (n = 7) or given as treatment for GVHD or hemorrhagic cystitis (n = 2). Of these nine patients, only one remains alive. This patient developed a life-threatening

EBV-associated PTLD three months after UCBT that was unresponsive to conventional treatment. The patient's life was saved by successful treatment with an experimental therapy devised in the research group: EBV-specific cytotoxic T-cells from a haploidentical related donor (197). The 5-year survival of the patients that had received co-infusion of MSCs for engraftment support was assessed separately in multivariate analysis to reduce the heterogeneity of the groups. Even when the patients that had received MSCs as treatment for severe conditions were excluded, there was a significant negative association with 5-year overall survival ($p = 0.04$).

Our research group has performed additional mechanistic studies of MSC treatment after UCBT, and found that treatment with MSCs was significantly associated with reduced T-cell receptor excision circles (TREC) levels, indicating a poor thymic output (225). The reduced TREC levels can have several explanations. Reports have indicated that MSCs influence thymus function via effects on the thymic epithelial cells (226, 227). Bone marrow MSCs from a mismatched allogeneic donor could be speculated to have a negative effect on thymic positive selection of naive T-cells from the graft (225). A percentage of intravenously infused cells is known to be trapped in the lungs. This has been shown for both MSCs and stem cell grafts (98, 228). Interactions between the MSCs and graft lymphocytes in the lungs could be another explanatory mechanism. MSCs are known to have immunosuppressive properties and have been shown to have a positive effect in the treatment of steroid-refractory GVHD (229, 230). The immunosuppression mediated by MSCs seems to be potent and may increase the risk of infectious complications and possibly also relapse in certain groups of HSCT recipients. A recent study of risk factors for pneumonia-related death in HSCT recipients at CAST have reported a significant association between MSC treatment and an increased risk of death by pneumonia (231). The etiology was invasive mold infection in a substantial portion of the cases. Increased adenovirus-associated mortality after MSC treatment has also been reported in the literature (232). In vitro studies have shown that MSCs inhibit stimulation-induced proliferation and IFN-γ production of virus-specific T-cells in vitro, which might correlate with increased risk of virus infections in vivo (232).

Jointly these findings have resulted in a discontinuation of the use of MSCs as engraftment support in UCBT at CAST. Also, an increased caution is exercised before MSC treatment is used in patients with a high risk of severe infection, especially invasive fungal infections. Anti-mold prophylaxis should be given to all patients receiving MSC after HSCT.

These results regarding negative effects of MSC treatment do not, however, stand unchallenged. Three previously published studies of MSCs as engraftment support in UCBT have not found negative associations between MSC treatment and patient survival (233-235). A fourth study even found that co-infusion of MSCs could improve engraftment (236). The studies were small, including seven, nine and 13 patients, respectively. Result comparisons were performed between the included patients of the four studies and either

historical controls (n = 2), or a cohort of contemporary controls, approximately numbering 3:1 to the number of patients (n = 2). The median follow-up time varied from 8 months to 6.8 years. No increased infection-related mortality was reported in any of the studies, and only one study reported infection-related deaths, at an expected level (233). The heterogeneity of the studies and the limited material could possibly have disguised a negative association between MSC infusion and overall survival or increased infection-related mortality. One important parameter that differed between the studies was the use and dosage of ATG. The patients included in one study did not receive any ATG, and in the remaining three studies equine ATG was used, while rabbit ATG was used in the studies included in this thesis. If the different use of ATG had any impact on the outcomes is difficult to know. ATG exerts a significant immunosuppression by depletion of T-cells (8), potentially to an increased degree in UCBT, and might increase the impact of MSC-mediated immunosuppression. TREC levels have, as far as it has been possible to ascertain, not been analyzed in the patients included in the four studies mentioned above, or in any other similar study, preventing a comparison of thymic output after MSCs between the studies. The main focus of the trials evaluating MSCs as graft support in UCBT was safety, with additional interest in incidence of GVHD, and speed and probability of engraftment. Even though survival and rates of infection were studied as well, this might have increased the risk of obscuring a possible correlation.

The incidence of relapse in patients with hematologic malignancies was 29% at 1 year and 40% at 5 years, which is higher than in HSCT in general at our center (4), but not unexpected in a group of patients with advanced malignancies, such as the group studied here (6, 124, 237, 238). Transplant-related mortality was 16% at 100 days and 30% at 1 year, which is similar to what has been reported in the literature (105, 106, 221, 239), but comparatively high when compared to results from HSCT with other stem cell sources in our center (4). Possible contributing factors to the high transplant-related mortality include the high percentage of patients with advanced malignancies, long diagnosis-to-transplantation times, and the use of high-dose ATG (8).

The incidence of acute GVHD grades II to IV was 36% at 180 days, of which 16% was grades III and IV. These figures are consistent with previously published data (105, 106, 239). In multivariate analysis, factors associated with an increased incidence of acute GVHD grades II to IV were major ABO blood group antigen mismatch (HR, 2.61; p = 0.05), and MAC (HR, 4.17; P = 0.047).

The definition of major ABO mismatch was adapted from Blin et al (240) where ABO mismatch in HSCT was defined and categorized as minor, major, or bidirectional. Minor ABO incompatibility (*e. g*. from a type O donor to a type A, -B, or -AB recipient) is characterized by the ability of donor B-cells to produce anti-recipient isoagglutinins. Major ABO-incompatible HSCT (*e. g*. from a type A, -AB, or -B donor to a type O recipient) is characterized by the presence of preformed anti-donor isoagglutinins. In bidirectional ABO

incompatibility (*e. g*. type A donor to a type B recipient), a combination of both major and minor mismatches is present.

The finding of increased incidence of GVHD in patients transplanted with ABO-mismatched grafts is partly supported by previous studies, but mainly in recipients of a minor ABO-mismatched graft after transplantation with stem cells from adult graft sources (241). There is also, however, contradicting results in the literature: several studies exploring risk factors of GVHD have not found any such correlation (241, 242). Specifically, a study was performed in 503 UCBT recipients to assess if ABO mismatch increased the risk of GVHD in the UCB setting. No such correlation was found, and the authors speculated that the naivety of UCB T-cells might be protective against triggering of GVHD against ABO-related antigens (243). There is no obvious explanation for the discrepancy between the results published there and the results in scientific paper I, but differences in graft handling, use of ATG, or other transplant practices might have influenced the results. In the reporting center of the study discussed above, ATG is used only in a third of the patients, namely those that have received less than two cycles of chemotherapy during the three months prior to transplantation (239), which might have been of importance to the outcome.

The study presented in paper I also explored chimerism development in this patient group and these results are discussed under the Chapter III.2.

In summary, the findings of scientific paper I showed that treatment with MSCs was associated with a lower 5-year survival after UCBT. This finding caused MSC infusion to be discontinued as engraftment support in this context. The low survival rate of adult patients with malignant disease in this study is an incentive to explore other alternative graft sources in these patients. The poor outcome in adult patients with malignant disease that received a RIC was justifies increased consideration in the choice of conditioning regimen for this patient group, though the patient number was low and the results should be interpreted with caution.

The scope of the study presented in scientific paper II was limited to patients that had been transplanted with DCBT. DCBT recipients treated at CAST between 2004 and 2008 were included, in total 12 patients. The main aim of the study was to explore chimerism development after DCBT, leading to exclusion of five patients due to early re-transplantation (n = 2), early death (n = 2) and inability to separate the two cord blood units with chimerism analysis (n = 1). Of the seven remaining patients, five had received a MAC and two had received a RIC. All patients were treated with ATG, at a total dose of 8 mg/kg.

Three patients out of seven developed acute GVHD, in two of the cases classified as grades III–IV. Four patients of seven died during the study, due to GVHD (n = 1), relapse (n = 2) and PTLD (n = 1). The rate of infectious complications was relatively high, with five cases of CMV infection, one case of varicella-zoster infection and one case of EBV-associated PTLD. Possibly, this could be a consequence of the high dose of ATG administered to

recipients of DCBT. Consequently,the ATG dose used in this setting have been reduced at our clinic.

The study of chimerism development of the patients in scientific paper II is described in Chapter III.2.

III.2 CHIMERISM DEVELOPMENT AFTER UMBILICAL CORD BLOOD TRANSPLANTATION (SCIENTIFIC PAPERS I, II AND III)

Chimerism development was a major focus of the studies described in scientific papers I and II. We aimed to thoroughly describe the dynamics of chimerism development in UCBT recipients, and to attempt to elucidate the underlying mechanisms. The frequent and comprehensive chimerism analyses performed routinely at CAST for all HSCT recipients provided a solid platform from which to launch such studies.

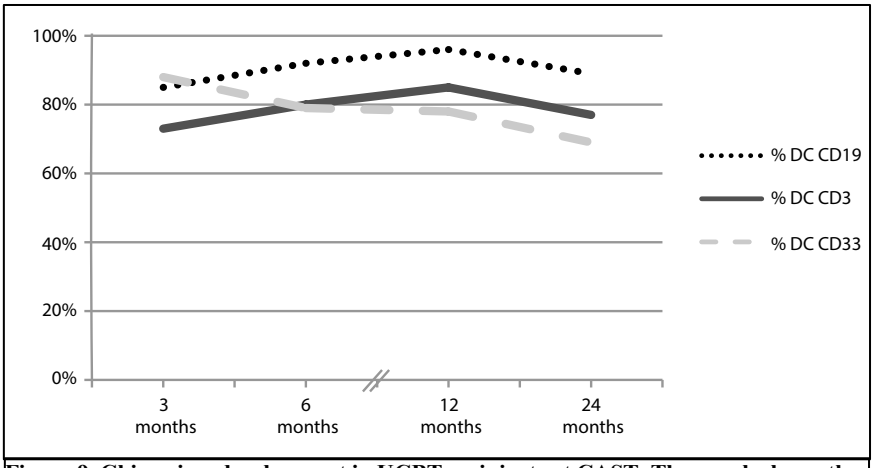

Figure 9. Chimerism development in UCBT recipients at CAST. The graph shows the percentage of patients with donor chimerism in the patient group. The cell lineages are plotted separately.

In the study presented in scientific paper I, we compiled the chimerism data for the CD3+, CD19+, and CD33+ cell lineages at set time points: 3, 6, 12 and 24 months after UCBT. We found that complete donor chimerism in all three cell lineages was reached in 22 patients out of 40 assessable at 3 months (55%). It should be noted that the proportion of patients with DC at a given time point, especially at the earlier time points, was limited by a lack of data, in one or more of the cell lineages, in a number of patients due to technical difficulties or low cell counts. The number of patients with DC in a given lineage varied over time, as is illustrated in Fig 9.

Median time to DC differed between the three cell subsets: 35 days for the CD19+ cell lineage, 28 days for the CD3+ cell linage, and 21 days for the CD33+ cell lineage. This could be a reflection of the dynamics of post-UCBT immune reconstitution (162).

In multivariate analysis, TBI-based conditioning was associated with DC for all cell lineages: in the CD19+ cell lineage, HR 2.93 ($p = 0.002$); in the CD3+ cell lineage, HR 3.16 ($p = 0.003$); and in the CD33+ cell lineage, HR 3.49 ($p = 0.001$). All patients who received TBI-based conditioning achieved DC in all cell lineages, while only 69-72% of the patients treated with chemotherapy-based conditioning reached DC in the different cell lineages (P=0.02-0.04). Additionally, the median time to DC was also significantly shorter

in patients that had received TBI-based conditioning than in patients pre-treated with only chemotherapy in the CD19+ cell lineage: 28 days vs. 56 days (P=0.04), and in the CD33+ cell lineage: 21 days vs. 28 days (P=0.03).

TBI-based conditioning treatment is mainly given to patients with ALL or lymphoma, who are usually heavily treated with chemotherapy prior to HSCT. This may partly explain the high probability and rapid development of DC in this patient group. Chemotherapy can cause chronic bone marrow damage resulting in chronic disruption of hematopoietic function (244). This can impair the host's capacity to react against the transplanted immune system, as indicated by a decreased risk of rejection in heavily pretreated patients (245).

The association between conditioning containing TBI and an increased probability of DC that have been described here is supported by the results of a subsequent study at CAST investigating long-term mixed chimerism after HSCT with stem cells from adult graft sources in patients with non-malignant disease (246). Patients with long-term mixed chimerism were less likely to have received TBI prior to HSCT than the DC controls. However, in a recent study of chimerism development in children after UCBT, no significant association was seen between conditioning containing TBI and development of DC (247). One possible partial explanation to these contradictory results is that all the patients in the latter study were children, who are known to have a superior capacity of recovery after chemotherapy (248, 249). Another study comparing myeloablative conditionings with cyclophosphamide and busulphan or cyclophosphamide and TBI to RIC with fludarabine and low dose TBI did not find any significant difference in either the proportion or kinetics of DC development for the three conditioning regimens (250). Our analysis compared all TBI-containing conditionings regardless of classification as RIC or MAC to all conditionings containing only chemotherapy, hence these data are difficult to interpret in relation to the ones presented in scientific paper I. Two other differences between the studies might have influenced the disparate findings. The patients included in the TBI group of scientific paper I almost exclusively suffered from either ALL or lymphoma, while only approximately 30% of the two TBI groups in the other studies were diagnosed with these diseases. This study also analyzed T-cell chimerism and total leukocyte chimerism, while our analysis included T-cell, B-cell and myeloid cell chimerism, which might also have influenced the findings.

There were factors associated with a significantly increased rate of DC development in univariate statistical analysis that was not significant in the subsequent multivariate analysis: malignant disease for DC in the CD19+ (P=0.012) and CD33+ (P=0.001) cell lineages, acute GVHD grades II to IV for DC in the CD3+ cell lineage (P=0.01), and higher CD34+ cell dose for DC in the CD19+ cell lineage (P=0.01). Higher cell dose has been found to associate with development of DC previously (250).

The association between acute GVHD and an increased proportion of DC in the CD3+ cell lineage is consistent with previously published data in UCBT and in HSCT with adult stem

cell sources (247, 251). This might be partly due to the role of alloreactive T-cells in establishing and maintaining engraftment after HSCT. Strong donor T-cell alloreactivity is thought to mediate immune-mediated destruction of recipient cells (250). However, strongly alloreactive donor T-cells also cause an increased risk for acute GVHD. Conversely, strong alloreactivity in recipient T-cells can cause graft rejection if not sufficiently controlled by the conditioning and immunosuppressive treatment (251). Mixed chimerism in the CD3+ cell lineage is considered more likely if the alloreactivity of donor T-cells is weak, since it can be thought to permit persistence of recipient T-cells and development of tolerance (251, 252). An association between mixed T-cell chimerism and relapse has been debated, with some reports supporting this correlation, while others have failed to find such an association (247, 250, 251, 253).

The results of scientific paper I showed that a higher rate of transplant-related mortality was associated with DC in the CD3+ cell linage (P=0.024). This might be a secondary to the association with GVHD, which is negatively associated with survival, as discussed in Chapter III.1. Jointly, available knowledge indicates a central role for T-cell chimerism in the transplant outcome.

The association between malignant disease and DC is also consistent with previously published data after HSCT with UCB, bone marrow transplantation and peripheral blood stem cell transplantation (PBSCT) (158, 247). Patients with malignant disease have undergone more extensive pre-treatment, essentially reducing the risk of rejection and decreasing the likelihood of MC development (245).

A proportion of patients retained stable MC over time. MC is thought to reflect immunologic tolerance between donor and recipient (156, 251, 254-256). (It is, however, important to separate stable mixed chimerism, which is discussed here, from an increasing recipient chimerism heralding rejection or relapse in malignancy.) Several factors can be speculated to have contributed towards development of tolerance in this study: the low cell dose leading to slow engraftment, creating the opportunity for tolerance development and/or the fact that all the patients received ATG (8, 105, 222). Tolerance in this setting is an interesting immunological phenomenon as it necessarily is a consequence of two genetically different immune systems existing side by side. This co-existence could be speculated to result in an increased immune diversity, leading to a resulting joint immune system recognizing a wider spectrum of antigens. Tolerance can also be speculated to be accompanied by an impaired general immune reactivity, as it reflects low donor-recipient reactivity (156, 257). As discussed above, there is an ongoing debate regarding if mixed T-cell chimerism is a risk factor for relapse in malignant disease. Stable mixed chimerism has been found to associate with low rates of GVHD (158, 246, 247, 250, 251, 253), consistent with a state of tolerance. This could theoretically lead to increased relapse risk since GVHD and GVL are known to be associated (5, 258, 259). Mixed chimerism has not been described in the literature to cause an increased risk for infectious complications, as far as

has been possible to ascertain. One study has reported increased MC development and an increased risk of infections after alemtuzumab compared to after ATG, but the treatment itself rather than the mixed chimerism state was considered to cause the increased susceptibility to infections (260). A study performed at CAST with the aim of exploring the clinical outcomes of patients with long-term MC found that the risk of blood stream infection seemed to be lower in those patients compared to controls with donor chimerism (246).

Mixed donor-donor chimerism, where persistence of both donors transplanted in DCBT is seen in the absence of remaining recipient cells, was the main focus of scientific paper II. MDC can be considered a subtype of complete DC, as no recipient cells remain, but can also be defined as a kind of MC, as the immune system is composed of cells from two genetically different host origins. This is a relatively new phenomenon, as double UCB graft transplantation is more recent HSCT method. The number of reported cases is small as the majority of previously published studies have reported that sustained hematopoiesis after DCBT is almost exclusively derived from only one of the donated units (261-264).

The results presented in scientific paper II showed that four of the seven patients had complete DC in all cell lineages in chimerism analysis one month after DCBT. Out of those four, two had a MDC in all three cell subsets. One patient showed high levels of recipient cells in both T-cells and myeloid cells in peripheral blood, indicating a threatening rejection. However, in the bone marrow, 90% of CD34+ cells were of donor origin. He received cultured umbilical cord blood T-cells, and his further clinical history is described in Scientific Paper V and in chapter III.3.

At four months after UCBT, three out of five assessable patients had complete DC in all cell lineages, and one of them still retained a mix of both donors present in all cell lineages. One of the patients with MDC one month after UCBT died of a severe acute GVHD at 3 months, still retaining this chimerism status. A fourth patient presented with 20% percent recipient cells in the B-cell compartment. The remaining 80%, and 100% of both the CD3+ and CD33+ cells, were of mixed donor origin, with a proportion of each donor in all cell lineages. A full MDC developed in this patient at six months.

The two patients that developed MDC at four and six months after DCBT, respectively, retained this chimerism pattern until the end of this study, at 25 and 35 months after transplantation, respectively. Both patients were then in good clinical condition.

Finding two patients with long-term MDC was unexpected, and of particular interest due to the scarcity of previously reported cases. At the time when papers II and III were written, only three patients with MDC had been described. Five additional patients have since then been reported (263, 265-272). The follow-up times for the eight reported patients were 12 months (2 patients), 16 months (2 patients), 24 months (3 patients), and 28 months (1 patient), respectively. As both patients were doing clinically well and had long-term stable MDC at 25 and 35 months, this finding provided a unique opportunity to study this

chimerism pattern further.

Existing knowledge remains insufficient to predict which patients will develop persistent mixed donor chimerism after DCBT. The two patients studied here did not differ from the other patients regarding the given cell dose, percentage of viable cells or HLA-matching.

Neither of the two patients developed GVHD. This indicates that the state of tolerance in recipient-donor MC was likely to exist also in MDC. It is difficult to determine if the absence of GVHD is a consequence of the tolerance in MDC, or if the long-termed mixed chimerism is allowed to persist due to the absence of GVHD. It has been reported that patients with MC have reverted to complete DC after development of GVHD (251, 273). The GVHD then signals the breaking of the tolerant state.

The development of long-term MDC in our material might have been facilitated by the use of high dose ATG, 8 mg/kg. This high dosage was selected with regard to the HLA disparity found in the majority of UCBTs. A high dose of ATG is associated with a delayed development of donor chimerism after HSCT (8, 274). In the present study, the high ATG dose in combination with the low T-cell dose in the graft and the naivety of the UCB T-cells could have caused high levels of ATG to remain after the transplantation, causing an unwanted degree of in vivo T-cell depletion. This in vivo T-cell depletion could have permitted tolerance and consequent MDC development. In support of this supposition, T-cell depletion in major HLA-mismatched bone marrow infusion have been described to cause tolerance and mixed chimerism in animal models (275). This is one explanatory model for the findings of scientific paper II. However, further studies were considered necessary to explore the causes and immunological consequences of this phenomenon.

We continued the investigations into the properties of MDC in scientific paper III. The two patients with MDC were there compared to control patients with single-unit DC after DCBT or UCBT. The patients were male, 20 and 30 years old. The underlying diseases that had led to transplantation were AML and B-cell lymphoma. They both received MAC. As stated above, neither developed GVHD.

The phenotypical and functional characteristics of the immune systems of the MDC patients were studied by flow cytometry. This revealed that the frequencies of T-, B-, NK-cells and monocytes did not differ markedly between MDC patients and single unit DC controls. There were also no differences in ‘dim’ and ‘bright’ NK-cell subset frequencies, or in memory B cells. However, the frequencies of the TCR-γδ T-cells and FoxP3+CD25+ possible regulatory T-cells (there are difficulties in separating activated effector T-cells and regulatory T-cell based on these two markers (33)) were lower in the MDC patients compared to the control group.

Expression of the cell surface markers CD45RO and CCR7 was used in this thesis to characterize different subsets of T-cells, as discussed in chapter I.1.iii (11, 43, 44). CD45RO- CCR7+ were termed naive T-cells, CD45RO+ CCR7+ were central memory T-

cells, CD45RO+ CCR7- were effector memory T-cells, and CD45RO- CCR7- were considered terminally differentiated memory T-cells. The T-cell maturation profile of the MDC patients was discovered to be more naive than that of the single unit DC controls: the frequencies of naïve cells were noticeably higher, and the frequencies of effector memory T-cells lower.

To further investigate the immunological properties of MDC, a comparison of the cord blood units within each of the patients was also performed, to identify potential individual phenotypes. Retrospective chimerism data revealed a fluctuation of the two units over time (Fig. 10). This could be due to immunological processes or to random variations in cell trafficking in the peripheral blood. To examine the two units separately within each patient we performed a flow cytometric typing assay with anti-HLA-A3 in one of the patients, and a PCR-based chimerism analysis for the other patient, to enable further analysis. This revealed that both patients had a major and a minor unit, as regards the proportion of circulating cells. The minor unit in both patients displayed a higher prevalence of NK cells.

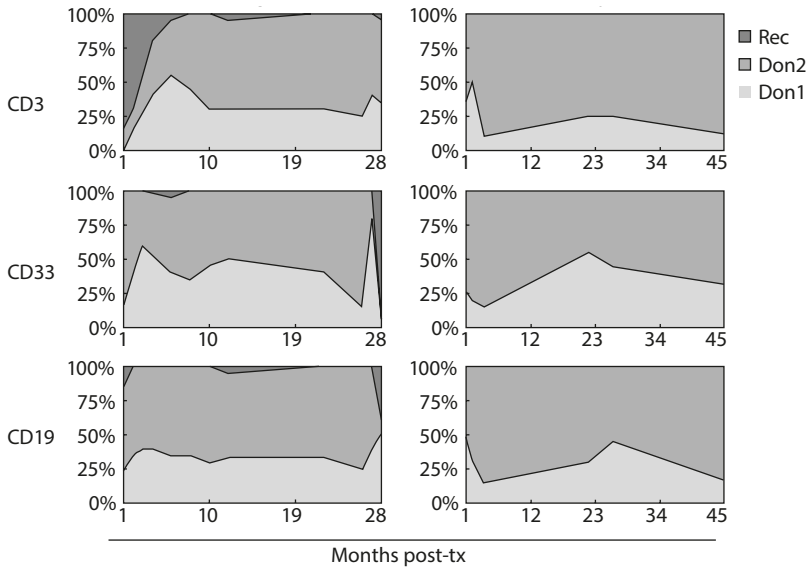

Figure 10. Chimerism development in the two patients with MDC. The figures shows the chimerism results for the recipient and the two donors in the CD3+, CD19+ and CD33+ cell linages. The panels on the left pictures the chimerism results of the patient with AML, while the panels to the right present the results for the patient with lymphoma.

The patient with AML was not included in further analyses due to disease relapse (seen as an increase of CD33+ cells of recipient origin in Fig. 10, panels on the left side). Hence, the following flow cytometry typing assays were performed in a single patient. In this remaining patient, the minor unit had lower frequencies of memory B-cells and TCR-γδ T-cells, but higher frequency of possible regulatory FoxP3+CD25+ T-cells.

To study the TCR repertoire of the two cord blood units, a flow cytometry typing assay for 15 different Vβ subsets was performed. This indicated polyclonal patterns of TCR β-chain usage, and was comparable between the two units.

Differences in the T-cell maturation profile of the two cord blood units was investigated by flow cytometry of the expression of CCR7 and CD45RO. The minor unit displayed lower frequencies of both central memory and effector memory T-cells, and higher frequencies of naïve T-cells compared to the major unit.

We further analyzed T-cell and NK-cell function, using stimulation assays, in the remaining patient to further investigate immune cell function in MDC. Result comparison was performed between the MDC patient and the control patients with single unit DC, and between the two UCB units in the MDC patient. Peripheral blood mononuclear cells (PBMCs) were stimulated with ionomycin and PMA, and analysis of cytokine production was performed by flow cytometry. The frequency of T-cells producing IFN-γ, TNF-α and IL-2 was lower in the MDC patient, while the proportion of T-cells producing IL-17 was comparable to the single unit controls. When the two cord blood units were compared to each other, we found that the T-cells of the major unit tended to produce more IFN-γ, TNF-α and IL-17. In contrast, the minor unit had slightly higher frequencies of IL-2-producing cells.

NK-cell function was assessed by stimulation of PBMCs with PMA or K562 cells, followed by flow cytometric analysis. The frequencies of NK-cells responding to PMA with CD107a up-regulation, a measure of degranulation, and IFN-γ production, were comparable between the MDC patient and the single unit controls. However, when stimulated with K562 cells lacking MHC class I molecules, a higher percentage of NK-cells in the patient with MDC up-regulated CD107a compared to NK-cells of control patients with single unit chimerism. The NK-cells of the separate units in the MDC patients both responded with up-regulation of CD107a and IFN-γ production in response to stimulation with mitogen and K562 cells, but the majority of the NK-cells that up-regulated CD107a were found in the major unit. This finding suggests that even though there is a state of tolerance in the MDC system, the potential of an NK-cell response per se is not impaired.

We could not detect any obvious functional advantages or disadvantages of MDC in the two patients included in the study, compared to the single UCB unit DC controls. We found that the MDC patients had fewer effector memory T-cells and TCR-γδ T-cells, and a more naive T-cell phenotype compared to the control patients. NK-cell function was seen to be comparable between the MDC patient available for further analyses and controls, with a suggestion of increased NK-cell reactivity against MHC-deficient targets in the MDC patient. NK-cells are regulated partly by inhibitory and activating killer cell immunoglobulin-like receptors (KIRs) that recognize certain HLA-C molecules (276). When NK-cells are confronted with allogeneic target cells that do not express inhibiting ligands, NK-cell alloreactivity may occur. The donor units of the two patients with MDC were HLA-C matched, which might induce mutual NK-cell tolerance. Some support for this hypothesis was found when studying HLA-C matching in the DCBT recipients with a single prevailing unit in scientific paper II. The units used in those patients were not

matched for HLA-C, with the exception of one patient. A plausible reason to why this patient did not develop MDC, despite receiving HLA-C matched cord blood units, could be the extremely high inter-unit difference in CD34+ numbers (22 to 1) and TNC (3 to 1) in favor of the prevailing unit.

The importance and consequences of HLA-C matching in HSCT has been discussed in other contexts. HLA-C/KIR mismatch between donor and recipient has been shown to positively influence the GVT effect in haploidentical HSCT (277), although there are some contradictory reports in unrelated HLA-mismatched HSCT (278, 279). In UCBT, HLA-C/KIR mismatch has been associated with less relapse as well as better survival (280). Moreover, a lower relapse risk has also been reported for DCBT compared to single UCBT, regardless of HLA-C (281).

The dynamics of chimerism development after DCBT, with special focus on single unit dominance, have attracted widespread interest. Potential predictive factors for unit dominance have been studied and debated over several years and results have been contradictory. Parameters suggested to be predictive have included: HLA-matching, total nucleated cell dose, CD34+ or CD3+ cell doses, viability, ABO typing, gender match and order of unit infusion. All these have been found to correlate with unit dominance in one or several studies, but none have been uniformly shown to predict which unit will prevail (239, 263), and, thus, no consensus has been reached. The CD3+ T-cell dose in the graft, and the amount of CD8+ T-cells, in one study especially naïve CD8+ T-cells, have been found to predict which unit eventually prevails after DCBT (261-263, 271). The increased risk of GVHD and reduced risk of relapse described in DCBT compared to single UCBT might indicate that immune reactions between the units and between the donors and the recipient, are more pronounced after DCBT and might play a role in single unit dominance (263). In vitro data supporting a graft-versus-graft immune interaction for unit dominance has been published previously. In this study, in vitro assays showed that the cord blood mononuclear cells from the dominant unit were able to develop a more pronounced cytotoxic activity than the mononuclear cells of the minor unit in a mixed lymphocyte reaction setup (264). Another mechanism for unit dominance that has been suggested is CD8+ T-cell mediated rejection of the losing graft by the prevailing unit (262, 271). This is supported by in vitro findings showing that CD8+ memory T-cells of the dominant unit produces IFN-γ if challenged by cells from the non-engrafting unit, but not when challenged with third party cells (262). No allogeneic response was detected in the CD8+ memory cells of the non-engrafting unit against cells from the dominant unit. In patients with mixed chimerism, no alloreactivity-induced IFN-γ production was triggered in the CD8+ memory T-cells of any of the units (262). Other groups have suggested that the potential NK-cell alloreactivity of one unit might contribute to unit dominance (277, 280). However, there is at present still no consensus regarding factors with predictive value for unit dominance.

It is difficult to identify factors that influence development of MDC due to the scarcity of cases. We proposed in scientific papers II and III that high-dose ATG and HLA-C match might facilitate development of MDC. The in vivo T-cell depletion caused by high-dose ATG, and possible NK-cell tolerance between units due to HLA-C match could increase the chances of inter-unit tolerance. T-cells reconstitute more slowly after DCBT compared to HSCT with adult hematopoietic cell sources, and the T-cell numbers in UCB is also significantly lower. High-dose ATG may have too large an impact in the DCBT setting (8, 162, 263). A recent meta-analysis of chimerism development after DCBT collected the published cases of MDC and summarized the present state of knowledge (263). Excluding the possible causes of MDC discussed in this thesis, few other factors have been suggested to influence development of this chimerism pattern. One factor proposed to be important in MDC development is the type of immunosuppression used, and the timing of its discontinuation. Immunosuppressive treatment can have bearing on whether a tolerizing milieu will arise in the host to facilitate MDC development.

The findings presented in scientific papers II and III did not show that this MDC confers any obvious advantages in immunity. The immune system in the patients with MDC seemed to have a more naïve immune profile than single UCB unit chimerism controls, which could be an implication of impaired immunological memory formation. However, MDC did not seem to entail any obvious clinical disadvantages, as two of the three long-term survivors in scientific paper II showed this chimerism pattern. One of the patients with MDC is still doing well today, many years after transplantation, and is the only one still alive of the 12 DCBT recipients included. Of note, the patient material is small and the results must thus be interpreted with caution.

III.3 CULTURE OF UMBILICAL CORD BLOOD T-CELLS FOR USE AS DONOR LYMPHOCYTE INFUSIONS (SCIENTIFIC PAPERS IV AND V)

The second aim of this doctoral project was to develop adoptive cell therapies in the UCBT setting. Donor lymphocyte infusion is the most common form of ACT after HSCT, and is an important treatment for relapse of hematological malignancy. The lack of conventional DLI is a considerable drawback of UCBT, and several methods to develop alternatives in vitro have been evaluated (10, 11, 198-200). We attempted to address this issue in the studies presented in scientific papers IV and V. We chose to culture bulk T-cells from an aliquot of the original graft (10, 11, 198, 200) to imitate conventional DLI as closely as possible. Other approaches that have been attempted previously are discussed in chapter I.3.iii.

The culture protocols used in T-cell based ACTs usually rely on expansion induced by artificial TCR/co-receptor stimulation combined with cytokines (10, 198, 200). A clinical trial using activated and expanded conventional DLI, prepared with a very similar method as used here, has shown promising results regarding anti-tumor efficacy with only a

moderate risk of GVHD (282). This is in accordance with reports showing that cultured T-cells are less alloreactive than un-manipulated T-cells (283, 284). A low risk of inducing GVHD would be beneficial in a UCB DLI product, but the decreased alloreactivity might also implicate a decreased GVT effect.

The choice of cytokines is of major importance in the production of a purpose-fitted cell product. The study of UCB T-cell culture for putative ACT was initiated in the research group previous to the work described in this thesis, using a protocol relying on anti-CD3/-CD28-covered paramagnetic beads and high-dose IL-2 (11). IL-2 was the only cytokine available in clinical-grade preparations for T-cell expansion at the start of this project, and is comprehensively studied in this setting (11, 285). The initial feasibility study published by our group on UCB T-cell culture showed that the use a 5% aliquot obtained at transplantation from a clinical UCB graft yielded sufficient numbers of T-cells for the cryopreservation of DLI and could be performed in accordance to good manufacturing practice (GMP) regulations. The cultured T-cells had an activated phenotype, with a majority of the cells having characteristics of central memory and effector memory T-cells. There was a polyclonal usage of β-chains in the cultured cell population, indicating a diverse TCR repertoire. In functional assays, the cultured UCB T-cells responded normally to non-specific T-cell stimuli and to allogeneic cells (11).

Additional cytokines have become available in clinical grade preparations, making he choice of cytokines for in vitro T-cell culture increasingly complex. A culture protocol relying on artificial TCR stimulation and high levels of IL-2 has inherent drawbacks, like significant up-regulation of apoptosis markers (10). Also, T-cells are prone to activation-induced apoptosis after strong TCR stimulation, and this tendency is more pronounced in UCB T-cells (286). The massive proliferation during the in vitro culture could also limit the proliferative potential of the cell product in vivo (211). However, one of the benefits of UCB in this context is the comparatively longer telomeres associated with T-cells of this origin compared to adult T-cells (287), which might indicate an increased mitotic capacity. Another issue that needs to be addressed is the risk of cytokine deprivation in vivo, resulting in anergy or apoptosis, due to the much lower cytokine levels in vivo compared to in vitro (10, 198, 211).

The addition of IL-7 was explored in scientific paper IV as a step toward a more functional, long-lived UCB DLI product. The rationale for this was that 1) IL-7 had become available in clinical grade preparations, 2) several of the unique characteristics of IL-7 were speculated to decrease the risk for apoptosis and exhaustion in the cultured cells. IL-7 is a homeostatic cytokine required for the survival and development of T-cells (67-71). We wanted to explore the possibility of using both IL-2 and IL-7 in the cultures, to enable a decrease of the IL-2 levels. A lower IL-2 level might lessen the risk of cytokine deprivation, while the addition of IL-7 could have anti-apoptotic and proliferative effects. Also, IL-2 has been found to increase responsiveness to homeostatic proliferation induced

by IL-7 and IL-15 in CD8+ memory T-cells (84). This suggests that the combination of IL-2 and IL-7 could increase the efficiency of CD8+ T-cell proliferation in cultures. CD4+ UCB T-cells have a comparatively higher expression of the IL-7 receptor α-chain (CD127) than adult CD4+ T-cells, possibly making them more responsive to IL-7 (83).

Culture protocol Cytokine content in media		
Condition nr.	**IL-2 (IU/ml)**	**IL-7 (20 ng/ml)**
1	-	-
2	-	+
3	50	-
4	50	+
5	100	-
6	100	+
7	200	-
8	200	+
9	400	-
10	400	+
11	600	-
12	600	+

Table 2. Culture protocol used in scientific paper IV.

The effects of IL-7 were explored in the presence of different levels of IL-2. The culture protocol is found in Table 2. After the culture of four UCB units under the 12 conditions in Table 2, certain trends were clearly visible in the proliferation rates of the cultured cells. We chose to continue with culture of further UCB units in only the four culture conditions with the most interesting preliminary results (C5, C6, C11, and C12). The choice was based on (a) the comparatively high IL-2 concentrations used in the protocol for UCB T-cell culture previously published (15) (C11 and C12), and on the lower level of IL-2 that combined with IL-7 resulted in the highest fold expansion (C5 and C6). We compared C5 with C6, C11 with C12, and C6 with C11. The latter comparison was made to study differences between the protocol that we have used in the previous studies (C11) and the condition with the best preliminary results (C6).

We found several phenotypic differences between cells grown with identical levels of IL-2 but in the presence or absence of IL-7. To begin with, the median fold expansion was higher in T-cells cultured with both IL-2 and IL-7, compared with T-cells cultured with IL-2 alone. This was statistically significant in C6 compared to C5 ($P=0.0078$), in C12 compared to C11 ($P=0.0098$), and in C6 compared to C11 ($P=0.0098$). Increased proliferation induced by IL-7 in UCB T-cell culture has been reported previously (198). Here, the effect was striking, and allowed an almost 85% reduction of the level of IL-2 without impacting on the end-of-culture cell numbers.

The CD4+/CD8+ T-cell ratio was higher in cultures exposed to IL-7, reflecting a comparatively higher percentage of CD4+ T-cells and a lower proportion of CD8+ T-cells, than in their counterparts cultured with IL-2 only (Figure 11a). Our previously published study showed a low CD4+/CD8+ ratio in UCB T-cells cultured with high-dose IL-2, lower than in both peripheral blood and in fresh UCB (15). Similar results have been published previously in a study with similar set up (10). Here, IL-7 was seen to skew the ratio towards that seen in peripheral blood. This could be partly due to the fact that IL-7 is critical for

CD4+ memory-cell survival, but less ubiquitous for the sustenance of CD8 + memory T-cells (70, 81). Also, the relatively high concentration of IL-7 used here could have led to preferential expansion of CD4+ T-cells, as CD4+ T-cells have been shown to require higher IL-7 concentrations for effective proliferation compared to CD8+ T-cells, an effect mediated by Lck (66).

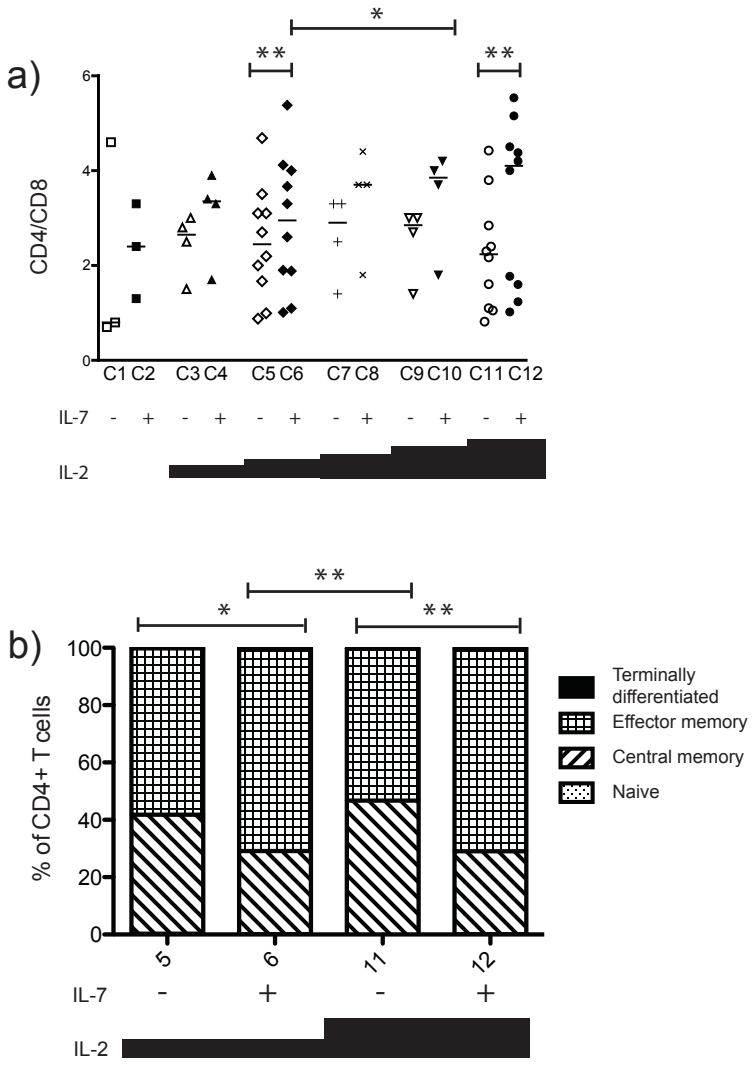

Figure 11. a) shows the CD4+/CD8+ T-cell ratio of the cultured T-cells. One dot represents one culture condition in one culture.
b) The median percentage of different memory development phenotypes are shown for conditions 5, 6, 11 and 12.

The developmental and functional T-cell phenotype has been found to impact of the efficacy and longevity of the adoptively infused cells, as was discussed in chapter I.3.I. Central memory T-cells have been shown to have superior efficacy and persistence (203-205), while effector memory T-cells have been shown to have superior potential to acquire and exert effector functions in vitro (209, 210). Though both memory subsets may have advantages in adoptive therapy, central memory T-cells might preferable due to the importance of in vivo persistence of transferred cells. New strategies for inducing the requisite phenotype will be needed, and short culture time and minimal cytokine exposure have been suggested in the literature (211).

With this in view, the need to assess the memory phenotype of cultured cells was evident. Thus, the frequency of different memory T-cell phenotypes was analyzed based on the expression of CD45RO and CCR7, as described in Chapter III.2. Addition of IL-7 skewed the CD4+ T-cell compartment toward higher frequencies of effector memory T-cells at the expense of central memory T-cells (Figure 11b). This was statistically significant for C6 vs. C5 ($P = 0.0137$), C12 vs. C11 ($P = 0.0039$) and C6 vs. C11 ($P = 0.0098$). There were no significant differences for these two memory phenotypes in the CD8+ T-cell population.

The CD4+ T-cell findings are in contrast to a previous report of UCB T-cell expansion with IL-7 (10 ng/mL) and a single set concentration of IL-2 (100 IU/ mL). There, a majority of the T-cells maintained a naive phenotype, there defined as CD45RA+ CD62L+ T-cells, after 14 days of culture (48). This might indicate that different levels of IL-7 produce different effects in UCB T-cell cultures. Previous studies have shown that low doses of IL-7 induce T-cell development, while high doses can block development at an early stage. Also, low concentrations of IL-7 were found to provide survival signals, while higher concentrations enabled proliferation (66, 69, 77). This indicates dose-dependent effects exercised by IL-7 in different concentrations, and suggests that further studies titrating the optimal IL-7 dose might be useful. Both CD4+ and CD8+ central memory T-cells are able to differentiate into effector T-cells in response to homeostatic cytokines such as IL-7 (42, 82, 84). However, whereas a large proportion of CD4+ T-cells differentiate into a late-stage effector phenotype in response to homeostatic cytokine signaling, CD8+ T-cells react more heterogeneously, resulting in a mixed cell population of effector T-cells, effector memory and central memory T-cells, and even naïve T-cells upon exposure to homeostatic cytokines (84). This might partly explain the shift from central memory to effector memory T-cells in the CD4+ compartment. Another possible explanation to this shift is that CD4+ memory T-cells have a higher proliferation rate than naïve CD4+ T-cells in response to IL-7 (78). This finding could be of importance for the potential longevity of the cultured cells in vivo, as a study with adoptive transfer of CD8+ T-cells in macaques have demonstrated that central memory, but not effector memory, CD8 + T-cells persist in vivo after transfer (203). As the optimal memory T-cell phenotype for ACT is yet unknown, further studies are necessary to determine how to further engineer the UCB T-cell cultures in the future.

The addition of IL-7 decreased the frequency of CD69+ T-cells compared to cultures without IL-7 in C12 versus C11 and in C6 versus C11. CD69 is considered an early activation marker in T-cells (288). Lower frequencies of CD69+ cells in the presence of IL-7 might reflect different activation kinetics in UCB T-cells cultured in the presence of this cytokine. See also Figure 3 for an overview of T-cell activation markers.

There were no significant differences in AnnexinV or 7-Aminoactinomycin D staining, the markers for apoptosis and cell death used in this study. This finding was unexpected, as IL-7 is known to have anti-apoptotic effects by maintaining the length of telomeres and inhibiting pro-apoptotic signals (81, 289-292). A previous study with a similar set up using anti-CD3/-CD28 beads, IL-2 and IL-7 (10 ng/ml medium, half of the concentration used in scientific paper IV) showed that the combination of IL-7 and IL-2 resulted in a higher percentage of viable cells compared to IL-2 only (198). The pro-survival effects of IL-7 have, as discussed above, been found to be of a dose-dependent character (66, 69, 77). Another factors that can influence the anti-apoptotic effects of IL-7 is intensive TCR stimulation which inhibits the signaling through the IL-7 receptor, lessening the pro-survival effects of IL-7 in adult blood T-cells (291). Also, prolonged IL-7 signaling has been shown to induce proliferation, production of IFN-γ and finally IFN-γ-triggered cell

death in adult naive CD8+ T-cells. However, intermittent (not continuous) IL-7 stimulation supported the survival and quiescence of CD8+ T-cells (290). Homeostatic TCR stimulation was found to protect against apoptosis in this study by interrupting IL-7-mediated signals, naive CD8+ T-cells with insufficient TCR affinity for self-ligands underwent apoptosis in the presence of homeostatic levels of IL-7 (290). It could be speculated that the yield of viable UCB T-cells at the end of culture in our system could be increased if intermittent and/or lower levels of IL-7 was applied.

Many strategies have been explored for engineering ACT products to induce longevity and efficacy in vivo. Short culture time and different cytokine combinations to maintain a comparatively naïve phenotype have been tried, aiming to preserve proliferative capacity and enable the cultured cells to home to secondary lymphoid organs, (211). The recent introduction of chimeric antigen receptors (186, 187), which can retarget T-cell specificity, is a strategy to decrease the need for extensive proliferation in vitro in ACT settings with a single target antigen. Other approaches include strategies to improve engraftment, and thereby, the longevity of infused cells by influencing host parameters. Decreased immunosuppression can be applied to avoid compromising the functional activity of infused cells, and pre-infusion lymphoablation can decrease the risk of rejection and the competition for cytokines for the adoptively transferred cells (211).

Functionality of the cultured UCB T-cells is also fundamental in evaluating the product's potential to exercise effects in vivo. We performed T-cell stimulation with ionomycin and PMA followed by intracellular cytokine staining (TNF-a, IFN-g, and IL-2) and extracellular staining for the degranulation marker CD107a. In the CD4+ compartment, we found a tendency toward increased polyfunctionality, defined as the expression of two or more of the assessed factors, in culture conditions with added IL-7. There were trends toward higher frequencies of cells producing 3 or more factors ($p = 0.063$, due to the number of included units, $n = 4$, this was the lowest achievable p-value) at the expense of cells producing two factors, and a single factor in C6 vs. C5. In CD8+ T-cells there was a trend toward increased polyfunctionality with a higher frequency of cells producing 3 or more factors in T-cells cultured with IL-7 in C6 vs. C11 ($p = 0.063$). The results are shown in Figure 12.

We then grouped culture conditions based on culture with or without IL-7, regardless of the IL-2 level: C5 and C11 were grouped and compared to a group consisting of C6 and C12. We then found an increased frequency of T-cells producing 3 or more factors in IL-7-cultured CD8+ T-cells cultured in the presence of IL-7 ($p = 0.027$), indicating increased polyfunctionality. See Figure 12.

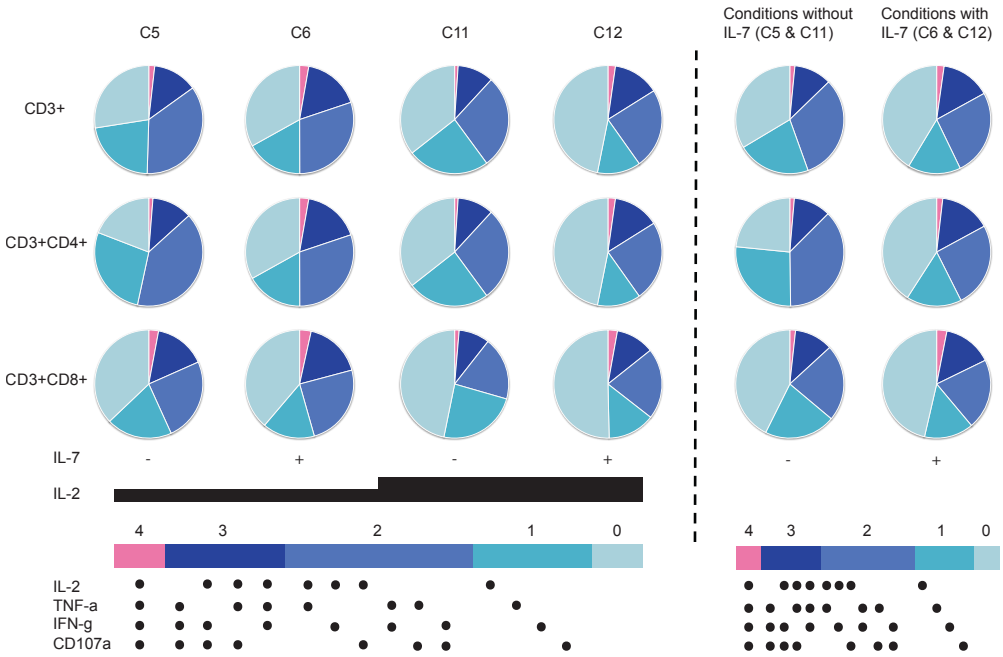

Figure 12. Median frequency of 5 experiments is presented for C5, C6, C11, and C12 (left), and for cultures grouped according to the absence (C5 and C11) and presence (C6 and C12) of IL-7 (right) for total CD3 + , CD3 + CD4 + , and CD3 + CD8 + cells positive for 0, 1, 2, 3, or 4 of the factors. The bottom graph shows the different combinations of factors included in the analysis.

It is encouraging that the observed polyfunctionality was present in both the CD8+ and the CD4+ T-cell compartment in cells cultured according to the potential new expansion protocol (C6), with IL-7 in addition to low-dose IL-2. This might imply a better chance of long-lasting immune responses, as previous reports have found that immune defense against infection is superior in mice with more polyfunctional T-cells (293), and that the in vitro functionality is superior in polyfunctional T-cells regarding the level of cytokine production and degranulation, and the magnitude of CD40L expression (294). Also, IL-7 treatment has been shown to augment the number of high-quality polyfunctional pathogen-specific CD8 T-cells of a non-exhausted phenotype in mice (295), supporting our observation of an increase of polyfunctionality in cells cultured with IL-7. Polyfunctionality is thought to be associated with increased efficacy in a potential therapy situation, as polyfunctional T-cells have been shown to be more effective in preclinical studies of ACT of viruses and malignancies (296, 297).

In scientific paper V we present the results of clinical treatment with the cultured UCB DLI in four patients. They received this treatment due to increasing recipient chimerism (n = 2), MRD (n = 1) or graft failure (n = 1). The first three patients received units cultured according to the first protocol, where only high levels of IL-2 was used (C11); while the last patient received cells cultured with the lower dose of IL-2 combined with IL-7 (C6). The patients were aged 17-52 years, two were females and two were males. They suffered from Pre-B ALL, AML and paroxysmal nocturnal hemoglobinuria with suspected development of MDS, respectively. The first patient received a DCBT, while the remaining three patients underwent single UCBT. All the patients received myeloablative conditioning

treatments. The first patient received 4 doses of cultured UCB DLI with a cell dose ranging from 5×10^3 to 5×10^5 CD3+ cells/kg patient weight. The second patient received three doses, 1×10^5 - 1×10^6 CD3+ cells/kg, and the third and fourth patients each received one dose of 1×10^5 CD3+ cells/kg patient weight. The low starting dose used in the first patient was chosen in consideration of the possibly increase of toxicity risk due to 1) the activated phenotype of the cells and 2) the presence of HLA mismatches.

There were no acute adverse reactions observed at infusion of UCB DLI. We did notice positive clinical events that may indicate benefit of the treatment in two of the patients: Patient 3, treated with UCB DLI because of mixed chimerism, had decreased levels of recipient cells in both the CD19+ cell lineage and, after an initial rise, also in the CD3+ cell lineage, in temporal association with the infusion (Figure 13). Patient 4 received UCB DLI because of the recurrence of detectable MRD (Figure 13). After a single UCB DLI dose, no MRD was detectable for several months. It should be noted that the MRD was already negative just before infusion. This was not known at the time, due to the inherent delay of test results. Unfortunately, the patient had a hematological relapse later without any preceding molecular signs of disease recurrence. It is debatable whether the patient would have benefited from additional UCB DLI infusions following the first, to prevent relapse. Due to the small material, and the variability of chimerism development and transformation of a detectable MRD to overt relapse, there is no certain causal association between the positive developments perceived in the patients and the UCB DLI treatment. There were no signs indicating possible benefit of the treatment in the two remaining patients.

We were unable to track the infused cells as they were derived from the original UCB graft, preventing detection with chimerism analysis. Additional manipulation of the cells enabling tracking was judged inadvisable. However, if cell tracking could be used in the future, this could help us to assess associations between UCB DLI treatment and clinical symptoms indicating effects. Also, to track the in vivo presence of the transferred UCB DLI cells could be of great interest, since the longevity of the cells is uncertain (as related to the risks of cytokine deprivation and exhaustion after in vitro culture discussed above) (10, 11, 211). There are non-toxic and efficient cell labeling systems that could be considered in the future for further studies (298).

A known treatment risk with conventional DLI is the induction and exacerbation of GVHD (189-192, 195). A study performed at our center has shown that 34% of the DLI recipients included did develop acute GVHD (190). It can be speculated that this risk increases with treatment with activated lymphocytes, due to the lower activation threshold in activated cells. However, there are data indicating that cultured and in vitro activated T-cells are actually less alloreactive than untreated T-cells (283, 284). A clinical study of activated conventional DLI treatment did not show increased GVHD incidence (299). In the present study, we saw no certain GVHD in the included patients. One patient (patient 2) had symptoms consistent with acute GVHD, but these symptoms predated the UCB DLI

infusions and could have been caused by an adenovirus infection the patient was suffering from at the time. Unfortunately, because of the patient's poor status, the diagnosis could not be clarified.

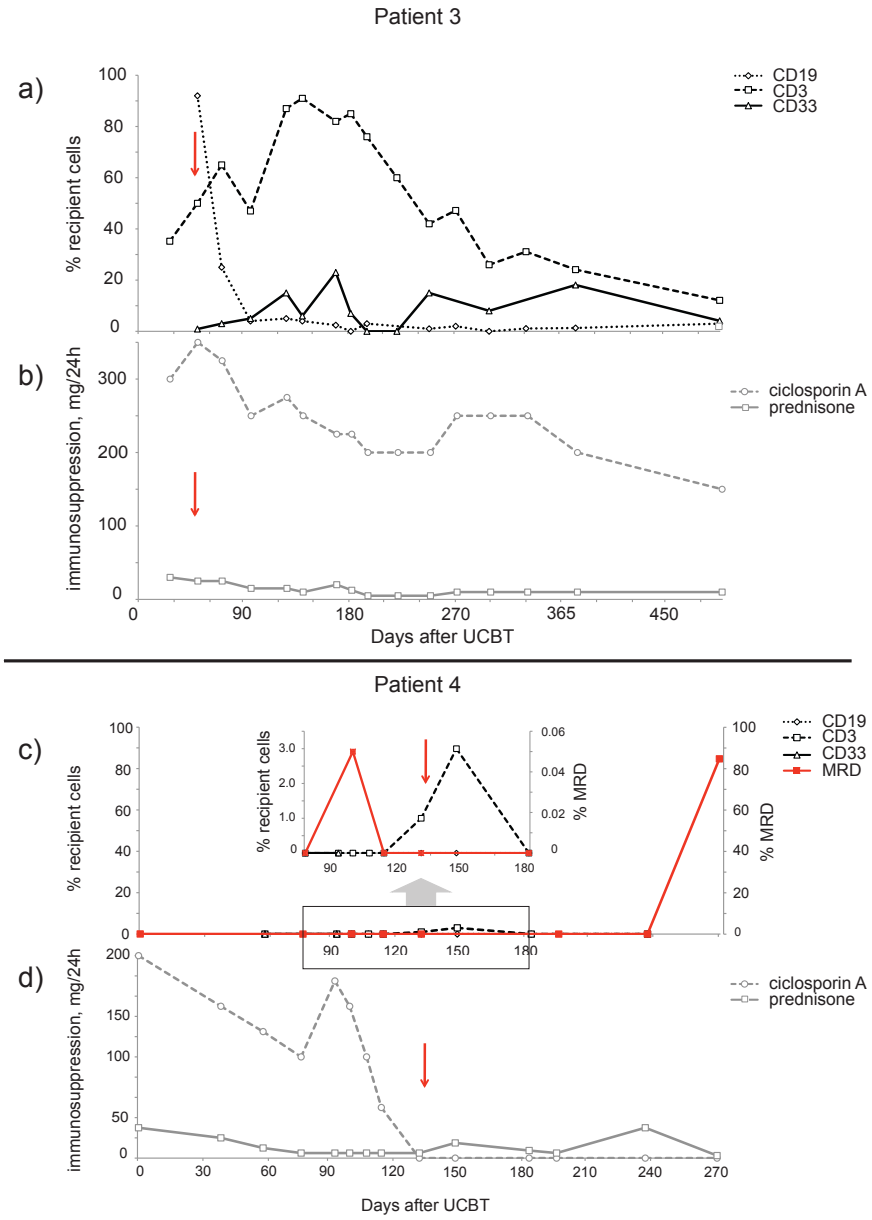

Figure 13. Chimerism development and immunosuppressive treatment of Patient 3 and Patient 4. a), c) Chimerism development the CD19+, CD3+ and CD33+ cell lineages in peripheral blood, presented as percentage of recipient cells. Development of MRD is shown for patient 4 in c). Inset in c) shows the time between day +75 and day +180 to illustrate the variations of recipient chimerism and MRD. b), d) Immunosuppressive treatment. Arrows show when UCB DLI were given.

As discussed above, the cultured T-cells in all the UCB DLI products had an activated memory T-cell phenotype. The CD4+/CD8+ T-cell ratio differed between the units used in the study in scientific paper V. It was found to be higher in the units given to patients 3 and 4. However, with only 4 patients, no conclusion can be drawn regarding the in vivo effects

of phenotypical differences between units.

In summary, we have found that the chosen culture strategy produces adequate cell numbers for an alternative ACT to conventional DLI in UCBT recipients, regardless of which of the cytokine combinations was used. IL-7 confers a proliferation advantage and allows the IL-2 concentration to be lowered below one fifth of the concentrations used previously. We observed changes in the phenotype of the cultured cells when IL-7 was added: a higher CD4+/CD8+ ratio and an increased proportion of effector memory T-cells in the CD4+ compartment were the most striking differences. There was also a tendency toward increased polyfunctionality in T-cells cultured in the presence of IL-7.

When patients were treated with the cultured UCB DLI products, possible benefits were noted in two patients: one patient who had received UCB DLI cultured with the high concentration of IL-2 only, and another receiving UCB DLI cultured with low dose IL-2 and IL-7. The treatment with UCB DLI was observed to be safe and feasible. Possible benefits of UCB DLI were seen, but no certain causal association can be found in such a small material. A larger study is necessary before any conclusions can be drawn regarding the effect of cultured UCB DLI infusions.

IV. CONCLUDING REMARKS AND FUTURE PROSPECTS

The discussion regarding the choice between UCB and an HLA-haploidentical relative for alternative donor transplantation will be continuing in the future. The results presented in this thesis have shown that the results in UCBT for adult patients with malignant diseases at our center were poorer than anticipated. Thus, it is of great importance to improve transplantation outcomes in this patient group. Lately, the use of allelic HLA-matching in UCBT has been found to improve outcomes (221), and as the cord blood banks have a large number of units stored, this could be applied to reach better clinical results in adult patients. Haploidentical HSCT using a new type of GVHD prophylaxis with post-transplantation cyclophosphamide is currently being implemented in many centers. This method could prove to be a valid alternative, as recently published studies have reported promising results (6).

In scientific papers II and III, we presented data regarding two patients with long-term MDC. We described some unique characteristics of the lymphocytes of the two patients with MDC that distinguished them from patients with single UCB unit chimerism. The two units that jointly form the immune system in MDC also seemed to have different characteristics. We also found two possible predisposing factors for MDC development: high dose ATG and inter-donor HLA-C matching. These data are novel and interesting, but the scarcity of the material makes it necessary to interpret the results with caution. As very few reports regarding this chimerism state have been published, little additional information can be found in the literature. With regard to his current paucity of knowledge the causes and consequences of MDC must still be considered as unclear. As cases accumulate over time, there will be opportunity to study this condition in-depth and reach a better understanding of the underlying mechanisms.

Results presented in this thesis and in the literature jointly indicate that the procedures used for culture of ACT products in UCBT can be further enhanced. Continued studies of the relative in vivo effects of different memory and effector phenotypes are warranted. New strategies for the culture of UCB T-cells should be investigated, to design T-cell products that are well adapted to in vivo demands. Different cytokine combinations, and different cytokine concentrations also merit further studies in order to improve the cultured products. Artificial manipulations to induce a more naïve phenotype, such as induction of telomerase expression, and of the expression of homing markers that enable migration to secondary lymphoid tissues, are potential strategies. Labeling the cultured products to enable tracking of the infused cells could be another valuable approach to improve evaluation of treatment effects. Another interesting future perspective could be to introduce new concepts into UCB ACT, like CARs or post-infusion activating BiTE antibodies. A small number of studies of CARs in the UCB settings have been published with promising results.

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