NOVEL IMMUNOTHERAPEUTICAL STRATEGIES IN ALLOGENEIC HEMATOPOIETIC STEM CELL TRANSPLANTATION

Mantas Okas
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Mantas Okas, MD

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To my family
ABSTRACT

Allogeneic hematopoietic stem cell transplantation (allo-HSCT) is the most potent immunotherapeutic measure used in clinical practice. This thesis deals with three problems related to allo-HSCT. Firstly, often no suitable donor is available for patients who need allo-HSCT and the use of umbilical cord blood (UCB) as a source of stem cells is one solution. The feasibility of this alternative is examined in paper I, and drawbacks of this approach are addressed in papers II and III. Secondly, allo-HSCT is an established therapy for hematological malignancies and a complementary treatment for metastatic solid tumors. A very important effector mechanism of allo-HSCT, the graft-versus-tumor (GVT) effect, comes hand in hand with the allogeneic reaction of the donor immune system to the host: so-called graft-versus-host disease (GVHD). GVHD is also one of the most fatal complications after allo-HSCT, which poses an immediate problem for successful management of the patient. Papers II and IV contribute to the studies of GVT and its differentiation from GVHD. Lastly, morbidity and mortality due to infections are substantial after allo-HSCT, and Epstein-Barr virus-associated post-transplant lymphoproliferative disease (EBV PTLD) is a condition for which effective treatment is sometimes lacking. Paper III presents a novel therapeutic approach to this problem.

In paper I, we compared the outcomes of allo-HSCT when using a human leukocyte antigen (HLA)-mismatched unrelated donor (MM URD) or UCB. We showed that UCB recipients had a significantly higher probability of survival than MM URD recipients and, consistent with reports from others, engraftment was significantly delayed after UCB transplantation (UCBT). Our results have led us to continue with UCB as a stem cell source for patients lacking an HLA-identical donor.

One of the limitations of UCBT is the lack of subsequent access to the donor, which limits the possibility of the recipient receiving a donor lymphocyte infusion (DLI) for a threatening rejection or relapse of the underlying malignant disease. In paper II, we described a clinically feasible procedure for in vitro expansion of CB-derived T-lymphocytes for use as DLI. We also presented an analysis of the phenotype and repertoire of the expanded T cells and demonstrated their capacity to produce cytokines and to proliferate in response to stimulation.

UCBT is also associated with an increased risk of severe viral infections after transplantation. In paper III, we presented a novel approach for rapid isolation of EBV-specific cytotoxic T-cells (CTLs) from a haplo-identical donor. The EBV CTLs were administered to a female patient with a life-threatening EBV PTLD without complications, and they persisted in the patient and contributed to the clearance of the disease.

Allo-HSCT has not been described previously for prostate cancer. In paper IV, we showed a GVT effect in a prostate cancer patient after HSCT. We were also able to demonstrate the presence of functional prostate-specific CTLs in the patient, which were dormant in the female donor. These cells may be important in mediating a GVT effect.

In conclusion, the studies presented in this thesis demonstrate how a strong collaboration between clinical and basic research can lead to increased knowledge and possible treatment modalities for patients undergoing HSCT. The data presented may be of importance for further development of adoptive immunotherapy protocols for infections and malignancies after HSCT.
LIST OF PUBLICATIONS

I. Olle Ringdén, Mantas Okas, Michael Uhlin, Mehmet Uzunel, Mats Remberger, Jonas Mattsson
Unrelated cord blood and mismatched unrelated volunteer donor transplants, two alternatives in patients who lack an HLA-identical donor
Bone Marrow Transplantation 2008 Nov; 42(10): 643-8

II. Mantas Okas, Jens Gertow, Mehmet Uzunel, Helen Karlsson, Magnus Westgren, Klas Kärre, Olle Ringdén, Jonas Mattsson, Michael Uhlin
Clinical expansion of cord blood-derived T cells for use as donor lymphocyte infusion after cord blood transplantation
Journal of Immunotherapy 2010 Jan; 33(1): 96-105

III. Michael Uhlin, Mantas Okas, Jens Gertow, Mehmet Uzunel, Torkel Brismar, Jonas Mattsson
A novel haplo-identical adoptive CTL therapy as a treatment for EBV-associated lymphoma after stem cell transplantation
Cancer Immunology, Immunotherapy 2010 Mar; 59(3):473-7

IV. Michael Uhlin*, Mantas Okas*, Helen Karlsson, Jens Gertow, Lars Henningsohn, Olle Ringdén, Klas Kärre, Victor Levitsky, Jonas Mattsson
Increased frequency and responsiveness of PSA-specific T cells after allogeneic hematopoetic stem-cell transplantation
Transplantation 2009 Feb 27; 87(4):467-72
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<td>Activation-induced cell death</td>
</tr>
<tr>
<td>AIRE</td>
<td>Autoimmune regulator protein</td>
</tr>
<tr>
<td>ALL</td>
<td>Acute lymphoid leukemia</td>
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<tr>
<td>Allo</td>
<td>Allogeneic</td>
</tr>
<tr>
<td>AML</td>
<td>Acute myeloid leukemia</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
</tr>
<tr>
<td>ATG</td>
<td>Anti-thymocyte globulin</td>
</tr>
<tr>
<td>AUC</td>
<td>Area under the curve</td>
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<tr>
<td>B-cell</td>
<td>Bursa fabricii dependent lymphocyte</td>
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<tr>
<td>BM</td>
<td>Bone marrow</td>
</tr>
<tr>
<td>BO</td>
<td>Bronchiolitis obliterans</td>
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<tr>
<td>BPH</td>
<td>Benign prostate hypertrophy</td>
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<tr>
<td>Bu</td>
<td>Busulfan</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of Differentiation</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony forming unit</td>
</tr>
<tr>
<td>CLG</td>
<td>CLGGLTLMV</td>
</tr>
<tr>
<td>CML</td>
<td>Chronic myeloid leukemia</td>
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<tr>
<td>CMV</td>
<td>Cytomegalovirus</td>
</tr>
<tr>
<td>CT</td>
<td>Computed tomography</td>
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<tr>
<td>CTL</td>
<td>Cytotoxic T lymphocyte</td>
</tr>
<tr>
<td>Cy</td>
<td>Cyclophosphamide</td>
</tr>
<tr>
<td>CsA</td>
<td>Cyclosporine A</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell</td>
</tr>
<tr>
<td>DLI</td>
<td>Donor lymphocyte infusion</td>
</tr>
<tr>
<td>EBER</td>
<td>Epstein Barr virus encoded small nonpolyadenylated RNA's</td>
</tr>
<tr>
<td>EBNA</td>
<td>Epstein-Barr virus nuclear antigen</td>
</tr>
<tr>
<td>EBV</td>
<td>Epstein-Barr virus</td>
</tr>
<tr>
<td>EFS</td>
<td>Event free survival</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence activated cell sorting</td>
</tr>
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<td>FasL</td>
<td>Fas ligand</td>
</tr>
<tr>
<td>Flu</td>
<td>Fludarabine</td>
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<tr>
<td>G-CSF</td>
<td>Granulocyte colony stimulating factor</td>
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<tr>
<td>GAG</td>
<td>SLYNTVATL</td>
</tr>
<tr>
<td>GLC</td>
<td>GLCTLVAML</td>
</tr>
<tr>
<td>GMP</td>
<td>Good manufacturing practice</td>
</tr>
<tr>
<td>GnRH</td>
<td>Gonadotropin releasing hormone</td>
</tr>
<tr>
<td>GVHD</td>
<td>Graft versus host disease</td>
</tr>
<tr>
<td>GVL</td>
<td>Graft versus leukemia</td>
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<tr>
<td>GVM</td>
<td>Graft-versus-malignancy</td>
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<tr>
<td>Gy</td>
<td>Gray</td>
</tr>
<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
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<tr>
<td>HLA</td>
<td>Human leukocyte antigen</td>
</tr>
<tr>
<td>HSCT</td>
<td>Hematopoietic stem cell transplantation</td>
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<tr>
<td>Hsp</td>
<td>Heat shock protein</td>
</tr>
<tr>
<td>HSV</td>
<td>Herpes simplex virus</td>
</tr>
<tr>
<td>HTLV</td>
<td>Human T lymphotropic virus</td>
</tr>
<tr>
<td>IE</td>
<td>Immediate-early</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IV</td>
<td>Intravenously</td>
</tr>
<tr>
<td>KLK-3</td>
<td>Kallikrein related peptidase</td>
</tr>
<tr>
<td>LAK</td>
<td>Lymphokine-activated killer</td>
</tr>
<tr>
<td>LCL</td>
<td>Lymphoblastoid cell line</td>
</tr>
<tr>
<td>LFS</td>
<td>Leukemia-free survival</td>
</tr>
<tr>
<td>LMP</td>
<td>Latent membrane protein</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
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</tr>
<tr>
<td>MDS</td>
<td>Myelodysplastic syndrome</td>
</tr>
<tr>
<td>Mel</td>
<td>Melphalane</td>
</tr>
<tr>
<td>MFI</td>
<td>Mean fluorescence intensity</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>miHA</td>
<td>Minor histocompatibility antigen</td>
</tr>
<tr>
<td>MLC</td>
<td>Mixed lymphocyte culture</td>
</tr>
<tr>
<td>MM</td>
<td>Mismatched unrelated donor</td>
</tr>
<tr>
<td>URD</td>
<td>Mismatched unrelated donor</td>
</tr>
<tr>
<td>MMF</td>
<td>Mycophenolate mofetil</td>
</tr>
<tr>
<td>MRD</td>
<td>Minimal residual disease</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger-RNA</td>
</tr>
<tr>
<td>MTX</td>
<td>Methotrexate</td>
</tr>
<tr>
<td>MUD</td>
<td>Matched unrelated donor</td>
</tr>
<tr>
<td>NCBP</td>
<td>New York Cord Blood Program</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer cell</td>
</tr>
<tr>
<td>OS</td>
<td>Overall survival</td>
</tr>
<tr>
<td>P10</td>
<td>FLTPK KLQCV</td>
</tr>
<tr>
<td>P9</td>
<td>KLQCVLH</td>
</tr>
<tr>
<td>PAP</td>
<td>Prostatic acid phosphatase</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cells</td>
</tr>
<tr>
<td>PBSC</td>
<td>Peripheral blood stem cell</td>
</tr>
<tr>
<td>PC</td>
<td>Prostate cancer</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PET</td>
<td>Positron emission tomography</td>
</tr>
<tr>
<td>PSA</td>
<td>Prostate specific antigen</td>
</tr>
<tr>
<td>PSMA</td>
<td>Prostate specific membrane antigen</td>
</tr>
<tr>
<td>PTLD</td>
<td>Post-transplant lymphoproliferative disease</td>
</tr>
<tr>
<td>RAK</td>
<td>RAKFQLL</td>
</tr>
<tr>
<td>RIC</td>
<td>Reduced intensity conditioning</td>
</tr>
<tr>
<td>SC</td>
<td>Stem cells</td>
</tr>
<tr>
<td>T-cell</td>
<td>Thymus-dependant lymphocyte</td>
</tr>
<tr>
<td>TAM</td>
<td>Transplant associated microangiopathy</td>
</tr>
<tr>
<td>TAP</td>
<td>Transporter associated with antigen presentation</td>
</tr>
<tr>
<td>TBI</td>
<td>Total body irradiation</td>
</tr>
<tr>
<td>Tcm</td>
<td>Central memory T-cell</td>
</tr>
<tr>
<td>TCR</td>
<td>T-cell receptor</td>
</tr>
<tr>
<td>Tde</td>
<td>Terminally differentiated effector T-cell</td>
</tr>
<tr>
<td>Tem</td>
<td>Effector memory T-cell</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming growth factor</td>
</tr>
<tr>
<td>Th</td>
<td>T helper cell</td>
</tr>
<tr>
<td>Th1</td>
<td>T helper type 1</td>
</tr>
<tr>
<td>Th2</td>
<td>T helper type 2</td>
</tr>
<tr>
<td>Tm</td>
<td>Memory T-cell</td>
</tr>
<tr>
<td>TIL</td>
<td>Tumor-infiltrating lymphocyte</td>
</tr>
<tr>
<td>TMEC</td>
<td>Thymic medullar epithelial cell</td>
</tr>
<tr>
<td>Tn</td>
<td>Naive T-cell</td>
</tr>
<tr>
<td>TNC</td>
<td>Total nucleated cells</td>
</tr>
<tr>
<td>Treg</td>
<td>Regulatory T-cell</td>
</tr>
<tr>
<td>TRM</td>
<td>Transplant related mortality</td>
</tr>
<tr>
<td>TRM</td>
<td>Transplant-related mortality</td>
</tr>
<tr>
<td>UBM</td>
<td>Unrelated bone marrow</td>
</tr>
<tr>
<td>UCB</td>
<td>Umbilical cord blood</td>
</tr>
<tr>
<td>VOD</td>
<td>Veno-occlusive disease</td>
</tr>
<tr>
<td>VZV</td>
<td>Varicella zoster virus</td>
</tr>
</tbody>
</table>
1 INTRODUCTION

1.1 IMMUNE SYSTEM

1.1.1 General remarks

Homo sapiens and other species have evolved in parallel with pathogens, which have continuously posed danger to their survival (1). Studies of defence mechanisms against infectious agents led to the initial accumulation of knowledge about the immune system. Observations that individuals, having survived an infection once, do not usually get infected again on repeated exposure, has led to the term immunity (from Latin immunis, meaning not being subject to munus (obligation), in this context not succumbing to disease). The first systematic scientific investigations in this field are attributed to Edward Jenner and his studies on the protective effects of inoculation with cowpox to prevent smallpox, which he published in 1798, “An Inquiry..” (2).

From the perspective of defence against infection, the immune system can be viewed as a constellation of structures and processes that serve to prevent entry and allow recognition and elimination of the invading pathogen. The immune system functions in close association with other organs and systems of the human body (the circulatory, nervous, respiratory, and digestive systems).

Apart from this, the immune system has an important role in defence against malignancy, by recognizing and eliminating the transformed cells (3). The immune system can also react and respond to non-infectious agents in the environment, and thus participate in the development of allergic conditions or react to self-antigens and induce autoimmunity (4, 5). Further on, I provide a brief overview, based on commonly accepted theories and findings on the immune system (6, 7). Aspects relevant to this thesis are highlighted by additional discussion.

1.1.2 Development of immune cells

The cells of the immune system originate from hematopoietic stem cells (HSCs), which in humans (after birth) reside in the bone marrow (BM). HSCs give rise to common progenitor cells that differentiate further into precursors of immune cells. From the lymphoid progenitor, bursa fabricii (B)-lymphocytes partially mature in the BM, then enter the circulation and complete maturation in peripheral lymphoid organs: the lymph nodes, spleen, mucosal and cutaneous lymphoid tissues. Thymus-dependent (T)-lymphocyte precursors leave the BM, mature in the thymus, and only then populate secondary lymphoid organs. Secondary (peripheral) lymphoid organs are important for survival of the developed lymphocytes and for initiation and perpetuation of the adaptive responses. The cells of the innate immune system enter the bloodstream in the mature state or they mature upon entering the tissues.
1.1.3 Components of the immune system

The immune system is usually divided into innate and adaptive. The innate immunity serves the first line of defence and consists of cellular and biochemical defence mechanisms that are in place even before entry of an infectious agent, and can therefore rapidly respond to it. The innate immune system consists of several major components: physical and chemical barriers, phagocytes (macrophages and neutrophils), natural killer (NK) cells, and blood circulating inflammatory mediators (complement, cytokines, and others).

The innate immunity is sometimes called non-specific. This refers to the facts that it reacts to different pathogens in a similar manner and does not mount an immunological memory. Still, the innate arm of immunity possesses mechanisms for specific discrimination between self and non-self, a most important decision that has to be made before initiating a response.

In contrast to the innate immune system, the adaptive system becomes activated only in the presence of an antigen that is specific to its effectors, and it reacts more vigorously on repeated exposure to the same antigen. Thus, it is also called the acquired immune system. The substances that induce and are targeted by this type of immune response are called antigens. The lymphocytes and their secreted products - antibodies - are the main components of the adaptive immune system.

The common feature of both T- and B-lymphocytes is the possession of an antigen-specific receptor, which is unique for each cell and is formed through somatic gene rearrangement. A vast diversity of different receptors allows the possibility of effective antigen recognition. T-cells and B-cells are cellular effectors of the adaptive immune system. T-cells eliminate infected and transformed cells by direct killing while B-cells mature into plasma cells, which secrete immunoglobulins (antibodies) - the effector molecules of humoral adaptive immunity.

Cytokines are polypeptide mediator molecules in the immune system. They can be produced by various cells of the immune system and other systems, and bind to specific cell surface receptors, which are selectively expressed by subsets of cells. Signaling through cytokine receptors regulates important cellular functions such as differentiation, proliferation, and activation. Cytokines can be viewed as a way of communication between different players of the immune system and their production is usually carefully regulated. Examples of cytokines are interleukins, interferons, colony stimulating factors, growth factors, and chemokines.

1.1.4 The adaptive immune response

Cluster differentiation antigen (CD) 4-positive T-cells are primarily involved in shaping the type of the adaptive immune response; they were originally called T-helper (Th) cells. Th1 and Th2 cells were described according to cytokine profiles and polarization of the immune response. Th1 cells favor development of phagocytic responses by
producing interferon gamma (IFN-γ), which stimulates macrophages, effectors of the innate arm. Th2 cells produce interleukins (IL) -4, -5, and -13, which act on mast cells/eosinophils and thus promote allergic inflammation. More recently, Th17 cells producing signature cytokine IL-17 have been described. Their exact role remains to be defined; at present, immunosuppressive and regulatory properties have been suggested (8). In the context of transplantation, these cells have been implicated in the pathogenesis of graft-versus-host disease (GVHD) (9). T regulatory (Treg) cells are other CD4+ lymphocytes with putative roles in suppression of the immune response as well as establishment and maintenance of tolerance. They are identified by a specific combination of phenotypic markers (CD25+, FoxP3+) and they produce IL-10 and transforming growth factor (TGF)-β (10).

CD8+ T-cells have a mainly cytotoxic function (cytotoxic T-lymphocytes, CTLs or T-killer cells) and they lyse cells that display antigens derived from intracellular pathogens or endogenous transformed proteins. CTLs bear granules containing perforin, which aids pore formation in the target cell membrane, and granzymes, which are released into the target cell and trigger its apoptosis. T-cells can also induce cell apoptosis by crosslinking Fas with the Fas-ligand (FasL) on the target cell. T-cells also secrete IL-2 and interferon (IFN)-γ, which perpetuate the cytotoxic response and inflammation.

B-cells are also effectors of the adaptive immune system. When they encounter antigen, they give rise to plasma cells which produce immunoglobulins (antibodies), the humoral effector molecules of the adaptive response. As discussed below for T-cells, the concept of memory applies to the humoral effector arm also.

1.1.5 Development and differentiation of T-cells

T-cell progenitors arise in the bone marrow and migrate to the thymus for further maturation. In the thymus, naive T-cells are generated, each getting a unique, single antigen-specific T-cell receptor (TCR) due to somatic rearrangement of the gene segments. TCR exists in two forms. Approximately 10% of T-cells bear the rearranged γ:δ TCR and are localized in epithelial layers and lymphoid organs. T-cells bearing the rearranged α:β TCR have been most extensively studied and play an important role in the immune system. The development and function of α:β T-cells is discussed below.

After gene rearrangement, T-cells undergo a selection process in the thymus consisting of two steps, positive and negative selection. According to the avidity (strength of interaction) hypothesis, double-positive (CD4+ CD8+) T-cells, which are able to establish a weak interaction with their TCR towards an HLA:self peptide complex, are positively selected, receive survival signals, and progress into single-positive state (either CD4+ or CD8+). Cells that are not able to establish such an interaction die by apoptosis. In this step, T-cells recognize HLA:peptide on thymic cortical epithelial cells. In the negative selection step, T-cells interact with thymic medullary epithelial cells (TMECs) or thymic APCs. According to the current understanding, a nuclear protein AIRE is present in TMECs and induces expression of peripheral tissue antigens.
in these cells. Thymic APCs also get an opportunity to display the peripheral antigens by engulfing apoptotic bodies from TM ECs. The knowledge on involvement of AIRE in selection processes is currently based on experimental data in mice (11). T-cells, which bind to HLA:peptide on TM ECs or APCs with strong avidity, get deleted ("negatively selected") from the repertoire. In this way, reactivity against self-antigens is prevented and central tolerance is established (11, 12).

T-cells that have left the thymus and have not yet met their antigen are termed naïve (Tn). Such T-cells recirculate to the lymph nodes, where, when an inflammatory process is ongoing, antigenic peptides are displayed in a complex with HLA molecules by professional APCs. If a naïve T-cell recognizes a peptide:HLA complex, signaling through TCR occurs and serves as "signal I" in the T-cell activation cascade. In order to become properly activated, naïve T-cells are dependent on co-stimulation by molecules such as CD80 and CD86. These are expressed on professional APCs, bind to CD28 on T-cells, and deliver the second signal in the activation cascade. Additional signals are delivered by cytokines through cytokine receptors expressed on the cell surface. Altogether, these events trigger clonal expansion (division of a cell bearing one type of antigen receptor) of T-cells and cause them to acquire effector functions (13, 14). Armed effectors, in contrast to the naïve ones, are not dependent on co-stimulation. This allows direct elimination of infected or malignant target cells by clonally expanded T-cells without the need for a professional antigen presenting cell (APC)-mediated activation at this step.

Upon elimination of antigen and termination of inflammation, the expanded clonal pool is contracted, and T-cells die by apoptosis. However, upon repeated challenge with the same antigen, the specific response is mounted faster and eliminates antigen more efficiently. This is termed immunological memory and it is a property of the adaptive immune system. This enhanced response occurs due to T-cells that have developed following the initial response and remain in the organism; they are termed memory T-cells (Tm). Two subsets of Tm are recognized: central memory (Tcm) and effector memory (Tem). CD8+ memory cells have a lower activation threshold and can respond more vigorously to a repeated antigenic stimulus (15).

Thus, at least 4 different subsets of T-cells with different phenotypic and functional properties can be identified: naïve (Tn), central memory (Tcm), effector memory (Tem), and terminally differentiated effectors (Ttde). Co-expression of surface markers CD45RA and CD45RO together with expression of CD62L (L-selectin) and CCR7 are used to identify these subsets. Several different models for this identification have been proposed (16-18). In our studies, we have chosen to define Tn as CD45RO- CCR7+, Tcm as CD45RO+ CCR7+, Tem as CD45RO+ CCR7-, and Ttde as CD45RO- CCR7- (Figure 1).
Figure 1. T-cell subpopulations defined by expression of phenotypic markers. Tn - naïve, Tcm - central memory, Tem - effector memory, Ttde - terminally differentiated effector T-cells.

CD62L and CCR7 are homing molecules that allow the entry of T-cells into the lymph nodes. Both CD62 and CCR7 are expressed in Tcm and Tn subsets. Tem and Ttde subsets lack expression of these homing molecules, indicating their commitment to migrate to the tissues. Freshly isolated Tem cells, and not the Tcm cells, readily possess the cytolytic machinery and kill targets in vitro (19, 20).

Lineage differentiation of T-cell subsets is a subject of debate. The asymmetrical division model suggests that upon antigen challenge, Tn cells divide into different daughter cells that have distinct phenotype and fate to become either short-lived effectors or long-lived memory cells (21). The linear differentiation model postulates that Tn cells differentiate into effector cells upon encountering antigen, and later on, following contraction of the pool, Tem and Tcm cells prevail from effector cells that do not undergo activation-induced cell death (19, 22-25). IL-7 and IL-15 are the cytokines involved in homeostatic maintenance of CD8+ memory T-cells (26).

1.1.6 Histocompatibility and antigen presentation to T-cells

1.1.6.1 Antigen presentation

Most of the nucleated cells of the body express histocompatibility antigens on their surface. These antigens were first identified in murine transplantation experiments and were named the major histocompatibility complex (MHC) (27). In humans, proteins encoded by MHC genes are named human leukocyte antigens (HLAs), as they were first described in lymphocytes (28, 29). MHC genes are present in all vertebrates, and in humans they are located on chromosome 6. The MHC locus is divided into three regions: class I, class II, and class III. Class I genes code for HLA molecules that are expressed on all nucleated cells and present peptides to CD8+ T-cells. HLA molecules encoded by class II genes are mainly expressed on cells with antigen presentation capabilities (DCs, macrophages, and B-cells) and present peptides to CD4+ T-cells. Class III genes code for complement proteins, cytokines, and chaperone proteins (30) and do not code for HLA molecules.
The class I molecule is a heterodimer composed of a single membrane-spanning heavy chain and a soluble light chain, β-2 microglobulin (β2m). The latter is encoded not by the MHC locus but on chromosome 15. The determination of HLA:peptide tertiary structure was a starting point for important insights into HLA:peptide:TCR structural interactions (31). The heavy chain is divided into three domains, α1, α2, α3, encoded by separate exons. The highest degree of polymorphism is encountered in the α1 and α2 domains, consisting of two α-helices and 8 β-sheets. Together they form a cleft where peptides, most often 8–10 amino acids (aa) long, bind. Peptides presented on class I molecules come from intracellular (endogenous and pathogenic) cytosolic proteins, which are ubiquitinated, degraded by proteasomes, and actively transported into the endoplasmic reticulum (ER) by transporter-associated proteins (TAPs). There, peptides are loaded onto class I molecules; both bound peptide and associated β2m are required for stable expression of the HLA on the cell surface.

The class II molecules are composed of an α-chain and β-chain, with two domains in each. The peptide-binding groove is formed between the α-1 and the β-1 domain and, in contrast to the groove of class I molecules, is open at the ends, which allows longer peptides (of 13–25 aa) to bind. The peptides presented on these HLA molecules are most commonly derived from endocytosed proteins that are proteolytically degraded in the endosomal compartment and loaded onto HLA in a TAP-independent fashion.

Consequently, it is most often antigenic peptides from intracellular pathogens and endogenous proteins that are processed through the class I pathway, while peptides from the extracellular milieu are processed through the class II pathway. Mechanisms that allow circumvention of these classical presentation routes have also been described. APCs can present peptides derived from extracellular proteins on class I molecules, a process called cross-presentation. This allows APCs to mediate activation of CD8+ T-cells (32). Intracellular proteins can be presented on class II molecules as a result of, for example, autophagy, when organelles of the cell are degraded in autophagolysosomes. This pathway has also been implicated in tumor antigen presentation (33).

1.1.6.2 Histocompatibility

In the human MHC class I region, the α-chains of classical (HLA-A, -B, and -C) and non-classical (HLA-D, -E, -F, -G, and -H) molecules are encoded. Classical molecules present antigens to CD8+ T-cells. In the class II region, HLA-DR, -DP, and -DQ molecules are encoded and they present antigens to CD4+ T-cells. Besides being polygenic (several loci in one individual), these regions are also highly polymorphic (several isoforms of same gene). MHC alleles are inherited together as a haplotype and are co-dominantly expressed. This generates a large variety of possible combinations, which gives the capacity to present a large amount of different antigens and at the same time to create an individual pattern of co-expression of molecules on the cell surface (34). As of April 2010, over 4,000 different human MHC alleles have been identified. The most polymorphic ones are HLA-B from class I (1,543 alleles) and HLA-DRB1 from class II (762 alleles) (35, 36).
Non-classical HLA class I molecules have a more conserved structure. They also present antigens; however, the repertoire of these antigens is narrower than that of classical HLA molecules. HLA-E presents peptides derived from the leader sequence peptide of HLA class I molecules (37) and from heat shock proteins (38). HLA-E and HLA-G have been found to suppress the cytolytic activity of NK cells (37, 39, 40).

1.1.6.3 HLA and allogeneic hematopoietic stem cell transplantation

The pioneering attempts at allogeneic organ and hematopoietic stem cell transplantation were performed without paying attention to HLA barriers (41, 42). The poor initial results were improved in this sense when our understanding of histocompatibility antigens started to improve (27, 28), and syngeneic transplants were performed with more encouraging results (43-45). In allogeneic hematopoietic stem cell transplantation (allo-HSCT) only about a third of all patients have a possibility of a sibling donor. The current gold standard of HLA matching in unrelated-donor allo-HSCT is a so-called 10/10 match, when HLA-A, -B, -C, DRB1, and DQ alleles are taken into account (46). The first methods used to test for histocompatibility were mixed lymphocyte culture (MLC) and cytotoxicity assays. Later on, HLA typing was performed using serological techniques such as leukoagglutination and microtoxicity assays. Currently, genomic HLA typing is performed, employing PCR-based sequence-specific typing techniques. The nomenclature of HLA has been influenced by historical developments in the field where letters defining HLA molecules reflect the order of their discovery. A common nomenclature has been accepted, and if the name of the locus is followed by an asterisk, it is an indication that genomic typing has been used. Serological techniques are still in use for screening purposes due to the low cost, and if the antigens are typed this way the asterisk is absent. In genomic typing, different levels of precision are defined. Low-resolution or 2-digit typing (referring to the number of digits following the asterisk) identifies broad families of alleles and corresponds to the result of serological typing. High-resolution or 4-digit typing identifies individual alleles within each serotype (47).
Altman et al have pioneered the use of HLA multimeric complexes for staining T-cells (48). This groundbreaking idea is based on joining several HLA:peptide complexes together and thereby increasing the avidity of interaction between such a multimeric complex and a T-cell, which possesses a TCR specific for the HLA:peptide complex (49). The original study has used tetrameric complexes, where recombinant MHC class I protein was produced in E. coli, with a biotinylation site in the heavy chain, and four such biotinylated proteins were linked to a fluorochrome-tagged streptavidine. Tetramers can be detected by flow cytometry (48) or by fluorescence microscopy in situ (50). Complexes with higher valences (pentamers, octamers) are also in use (51, 52) (see Figure 2). Commercially available pentamers have the structural advantage, where all peptide:HLA complexes point at the same direction and thereby increase binding avidity. Multimeric HLA class II are used to detect CD4+ T-cells, the TCR:HLA interaction is weaker in this case and higher valence complexes seem to play an important role in facilitating binding (53). Apart from detection of antigen specific cells, HLA-multimers were employed for depletion of alloreactive cells (54) and deletion of autoreactive cells (55) in animal models. These applications have taken advantage of the observations, that crosslinking of TCRs by multimers induces apoptosis of T-cells (51, 56, 57). In the context of specific CTL selection for further amplification or adoptive transfer, this might be a limiting factor. Interestingly, it was demonstrated, that by alternative assembly method of multimeric complexes the apoptosis induction could be reduced (58). The authors speculated, that apart from influence of valency of the complex, this assembly method could take the advantage of crosslinking more distant TCRs, as demonstrated by earlier experiments (59).
findings remain to be validated, but the reagents are available at good manufacturing practice (GMP) grade. Another modification of the multimers, named streptamers, has been produced and can be advantageous because of possibility for dissociation of the complex from selected cells and availability at the GMP grade (60).

1.2 ALLOGENEIC HEMATOPOIETIC STEM CELL TRANSPLANTATION

1.2.1 Historical developments

Hematopoietic stem cell transplantation (HSCT) is an established procedure used in the treatment of hematological and oncological diseases, and, more recently, autoimmune diseases. Based on donor origin, HSCT can be (1) autologous, when the patient receives his/her own HSCs that were cryopreserved earlier; (2) syngeneic, when HSCs come from a genetically identical individual, i.e. a monozygotic twin; or (3) allogeneic (allo-HSCT), when the donor is not genetically identical, e.g. a sibling or someone unrelated. Autologous HSCT is used as a way of rescuing the patient after a high-dose cytostatic treatment in hematological and/or oncological disease, and is being explored for re-setting the immune system in autoimmune conditions (61). Allo-HSCT relies not only on the toxic effect of cytostatic treatment of the tumor, but also provides the immunotherapeutic graft-versus-tumor (GVT) effect, which is closely associated with alloreactivity. It is an established treatment method for hematological malignancies and is being explored for use in patients with solid tumors. Also, allo-HSCT is routinely used for the treatment of non-malignant hematological disorders, e.g. aplastic anemia, severe combined immunodeficiency disorder, thalassemia, and as enzyme replacement for inborn errors of metabolism. This thesis focuses on immunotherapy in the context of allo-HSCT.

There are reports on attempts to infuse bone marrow for treatment of anemia and leukemia extending back to the end of the 19th century. Later, confrontation with bone marrow aplasia due to radiation came during the Second World War and resulted in studies of radiation effects, which were important for further developments in the allo-HSCT field (62-65). In 1957, Edward Donnal Thomas and co-workers demonstrated for the first time that intravenous infusion of BM resulted in a transient engraftment of infused HSCs in man. In 1990, E.D. Thomas shared the Nobel Prize in Physiology or Medicine with J.E. Murray “for their discoveries concerning organ and cell transplantation in the treatment of human disease”. The finding that BM transfusion was feasible was confirmed by others (66, 67). However, the early results were poor, with most of the patients being transplanted in terminal condition with advanced and therapy-resistant disease. Still, “immunological reactions” were noticed in individuals who had engrafted (68). Today, this reaction - characterized by skin rash, weight loss, and diarrhea - is recognized as graft-versus-host disease (GVHD). The major breakthrough came with identification of the MHC and HLA antigens (28, 29). Soon it also became evident that allo-HSCT could cure both malignant and non-malignant diseases (69, 70).

Initially, the term bone marrow transplantation (BMT) was used for the procedure due to the sole use of BM as the source of stem cells; the term HSCT was introduced after demonstration that HSCs could be retrieved from peripheral blood (71). In Sweden, the
first allo-HSCT was performed in our unit at Huddinge Hospital in 1975 (72). Intensive research in the field, the accumulation of medical knowledge, and developments in pharmaceuticals over the years have allowed the development of allo-HSCT into a successful therapeutic measure and have extended its indications.

1.2.2 An overview of the transplant procedure

As mentioned earlier, allo-HSCT can be performed with the aim of treating both malignant and non-malignant conditions. Upon consideration of a patient for transplant, the search for a donor is initiated by first conducting an HLA typing of siblings, and if no HLA-identical sibling is found, the search is continued in national and international donor registries. Infusion of HSCs is preceded by conditioning therapy, where radiation and/or chemotherapy agents are employed to create space for the stem cells, to suppress responses of the host immune system against the graft, and, in the case of malignancy, to attack the tumor. Immunosuppressive treatment is started before transplant in order to counteract rejection of the transplant by the host, and to prevent allogeneic reactions toward the host from the graft. In almost all cases, HSCs are administered through a central intravenous line. After transplantation, patients are subjected to a period of marrow aplasia, where infectious complications, bleeding, anemia, and other medical conditions arise and are taken care of. Upon engraftment, when the medical status of the patient allows, he/she is discharged and monitored on an outpatient basis for possible early and late complications of allo-HSCT. Immunosuppression is tapered, with initiation and speed of tapering being dependent on a number of factors, like indication for transplantation, donor type, results of post-transplant monitoring and others. In successful allo-HSCT cases, a state of tolerance is eventually established, eliminating the need for lifelong administration of immunosuppressives - which contrasts with organ transplantation.

1.2.3 Sources and procurement of stem cells

In adults, HSCs are mostly found in BM (1-2%), as compared to 0.2% in the peripheral blood. They are mainly characterized by their expression of CD34, although evidence for CD34-negative HSC precursors has also been obtained (73, 74). BM is most often obtained by aspiration from the posterior iliac crest under general or spinal anesthesia (75), and it was originally the main source of stem cells for allo-HSCT. Granulocyte-colony stimulating factor (G-CSF) was shown to induce mobilization of HSCs into the peripheral blood (71), and transplantations from such “peripheral blood stem cells” (PBSCs) were performed (76-78). Currently, PBSCs are the main stem cell source used, mainly due to the possibility of avoiding anesthesia and retrieving more HSCs, which may speed up engraftment after allo-HSCT. Compared to BM, no differences in acute GVHD and survival have been noted, while cGVHD is increased in patients receiving PBSCs (79-81). In children with leukemia who have an HLA-identical sibling donor, bone marrow is preferred as stem cell source, because a retrospective study found that PBSC increased the risk of cGVHD and mortality (82). However, using unrelated donors whom all are adults, there is no difference in acute or chronic GVHD or outcome, whether bone marrow or PBSC is given (83). For BMT, a cell dose of $>3 \times 10^8$ TNC/kg is desirable and for allo-HSCT using PBSC a dose of $>4 \times 10^6$ CD34+ cells/kg needs to be used, where a dose of $>6 \times 10^6$ CD34+ cells/kg needs to be
used in patients with malignant disease. In patients with severe aplastic anemia, it was found that those receiving a nucleated cell dose <3 x 10^9/kg had an increased risk of rejection (84). In patients with leukemia receiving allo-HSCT from HLA-identical siblings, a CD34 cell dose <3 x 10^6/kg was associated with an increased risk of mortality and patients receiving a CD34+ cell dose >6 x 10^6 cells/kg had a decreased probability of relapse (85). In patients receiving PBSC, leukemic patients receiving a CD34 cell dose >6 x 10^6/kg had a decreased probability of relapse. A decreased probability of relapse was also seen in identical twin transplants receiving a high nucleated marrow cell dose (86). In current studies to date, no increase was detected in the risk of promoting development of malignancy in donors due to G-CSF administration (87). The third source of HSCs is umbilical cord blood (UCB). See separate section for a more detailed overview of transplantation from this stem cell source. Fetal liver cells have also been used as a source of stem cells, mainly in intrauterine transplantation (88).

1.2.4 Tissue typing

Identification of genes and proteins responsible for histocompatibility and alloreponses has allowed the development of allo-HSCT into a safer procedure. At the same time, this source of knowledge serves as an important platform for exploitation of the possibilities of immunotherapy. This is further emphasised in this thesis by the use of recombinant HLA multimers, for example. For an overview of MHC/HLA, see separate section.

In a search for related donors, HLA typing of family members is performed. This not only allows identification of a potential donor, but it also confirms the genotype of the patient, which is useful if a search for an unrelated donor has to be initiated. In most cases, serological level HLA-A, -B, and -DR typing can discriminate between paternal and maternal haplotypes in the patient and a potential sibling donor, thus confirming the identity of the whole gene set - a 12/12 match. A haploidentical donor is a donor with one shared haplotype. Transplantation procedures using such donors usually include T-cell depletion from the graft with an add-back of depleted T-cells post-transplant. Roles for NK-cell mediated reactivity and for Tregs have been implicated in these settings (89, 90).

In cases where a related donor is not available, a search in unrelated donor registries is initiated. Currently, a 10/10 match (HLA-A, -B, -C, -DRB1,-DQB1) is a “gold standard”, while a 6/6 match (HLA-A, -B, -DRB1) is the lowest matching level generally acceptable. However, in a recent analysis of 3,857 transplants where patient-donor pairs were high-resolution typed for HLA-A, -B, -C, -DRB1, -DQB1, -DQA1, -DPB1, and -DPA1, an 8/8 match (HLA-A, -B, -C, -DRB1) was the minimum level associated with the highest survival (91). In cases where GVH reactivity is solely a complication, i.e. transplants for non-malignant conditions, a 12/12 match can be sought.

In cases of mismatch, recipient alleles may be missing in the donor (reactivity of donor T-cells is evoked against the recipient, “mismatch in the GVHD direction”) or vice versa (“mismatch in the rejection direction”). The risk of these complications increases with the number of mismatches (46). Class II mismatches are associated with an
increased risk of acute GVHD (92). In contrast, patients grafted with a DRB1 allele mismatch showed an improved survival according to data from Karolinska university hospital (93). There is no consensus on the effect of individual HLA mismatches on outcome or complications of allo-HSCT (94, 95). It was recently reported that single mismatches at HLA-B or -C appeared to be better tolerated than single mismatches on HLA-A or -DRB1 (91). Some retrospective multicenter studies have shown that HLA-C mismatch is associated with worse outcome (96). However, at the Karolinska university hospital, we have not found that HLA-C mismatch is associated with a worse outcome (97).

1.2.5 Principles of conditioning

Conditioning therapy aims to eradicate malignant disease, to prevent rejection of the graft, and to create a space for the engraftment of HSCs. Two major groups of conditioning regimens exist, the myeloablative and the non-myeloablative ones. Highly toxic myeloablative regimens consist of high doses of chemotherapy with or without radiation. The most common protocol uses total body irradiation (TBI) of 10 Gray (Gy) together with cyclophosphamide (Cy) 60 mg/kg on two days, which was originally developed by the Seattle group and called the original “Seattle” or “Cy/TBI” protocol (75, 98). Several modifications of Cy dose and also administration of irradiation in fractions (fTBI) have been introduced (99, 100). TBI can be replaced with Busulfan (Bu) 4mg/kg for four days - the “Bu/Cy” protocol (101). It is often used for myeloablation of children, since they are more sensitive to effects of radiation. The initial Bu/Cy protocol included Cy 50 mg/kg for four consecutive days. Because of too much toxicity, another protocol where Bu was combined with Cy 60 mg/kg for two consecutive days was introduced (102). A randomized trial by the Nordic BMT Group on transplant recipients with leukemia found Bu/Cy to increase the risk of cGVHD, bronchiolitis obliterans (BO), and alopecia (in comparison to Cy/TBI) (103). Blood monitoring of Bu AUC values may reduce the toxicity, and liposomal formation of Bu may improve the results (104, 105).

Higher intensity of the myeloablative regimens has been tried and resulted in less relapse, but higher TRM (106, 107). Non-myeloablative (NM) or reduced-intensity conditioning (RIC) regimens have been developed with the alloreactivity, safety, and accessibility for a less fit patient in mind. Here, the GVH/GVT and not the conditioning regimen are expected to perform the eradication of recipient hematopoiesis and to control the malignancy. Employment of such less toxic regimens has extended the use of allo-HSCT to older patients and to the ones with co-morbidities (108-111). The protocols can be loosely separated into two groups. The first ones (NM) employ low-dose irradiation (e.g. 2Gy TBI) together with fludarabine (Flu) and immunosuppressives such as mycophenolate mofetil (MMF) and cyclosporine-A (CSA) (109, 112-114). In the RIC group, Flu is the main component in combination with other chemotherapeutics such as Cy, Bu, melphalan (M el), and immunosuppressive regimens (108, 114, 115). It has to be mentioned that a plethora of different regimen variations exists in the clinic, most of which are center-specific and complicate comparison and evaluation of transplantation results.
1.2.6 Pre- and post-transplant immunosuppression

Immunosuppressive treatment is started before transplantation and continued afterwards, in order to prevent rejection and GVHD. In allo-HSCT situations, clinical tolerance develops and immunosuppressive regimens are usually discontinued within 3 months to one year for malignant diseases and within one to two years for non-malignant diseases (116). Immunosuppressive treatment is often shorter in related transplants than in unrelated transplants. The Seattle protocol is a combination of methotrexate (MTX) and CsA, and is one of the most common protocols used today (117, 118). In UCB transplants, where the marrow-depressing properties of MTX are to be avoided, a combination of steroids and CsA is most common (119). Other drugs used in combinations are sirolimus (rapamycin) (120, 121), tacrolimus (120, 122), and MMF (109). The common mechanism of action of these agents is inhibition of T-cell proliferation; different agents use different pathways to achieve it and differ in their profiles of side effects (123). Since T-cells are recognized as crucial mediators of GVHD (124, 125), besides the pharmacological agents, depletion of T-cells from the graft or in vivo with anti-T-cell antibodies, such as OKT-3 and anti-thymocyte globulin (ATG), is used. The regimens reduce GVHD, but the risk of relapse and rejection is also increased (126-129).

1.2.7 Transplantation procedure, supportive care, and follow-up

The graft is retrieved from a banking institution or after donation at a local facility, processed with thaw, wash, and quality-control steps, and delivered to the ward. In cases of ABO mismatch, red cells are depleted. At the time of infusion, reactions similar to transfusion reactions can develop. After allo-HSCT the patient is usually neutropenic, which is typically expected to last for around 14 days. Patients receiving RIC usually have a less extended, sometimes barely noticeable neutropenic period. Prolonged neutropenia is associated with the use of UCBT (130). Practices and standards in supportive care are important for the success of the procedure (131, 132). There is a significant center effect and different centers have different outcomes after allo-HSCT. Generally, larger centers with longer experience have better survival than centers that have started more recently and have little experience (133). Accreditation efforts are being undertaken by the Joint Accreditation Committee (ISCT & EBMT; www.jacie.org) with an aim to ensure quality control and the safety of the patient (134). Patients are usually kept under variable degrees of isolation - from “oxygen bubbles” to reversed isolation rooms during the pancytopenic phase. At our institution, home care during the pancytopenic phase has resulted in lower requirement for parenteral nutrition, reduced GVHD, and improved survival (135). The follow-up of transplant patients on an outpatient basis is of great importance and it is therefore carried out thoroughly, especially at the beginning. It is during this period that timely judgement, monitoring, and intervention can help to prevent and combat the post-transplant complications. These can be divided into regimen-related toxicity, infections, GVHD, relapse, and rejection/graft failure.
1.2.8 Toxicity

The chemotherapeutic agents used in conditioning protocols are not malignancy-specific and the intensity of the regimens is limited by the toxicity for vital organs. Injury of vascular endothelium gives rise to a group of syndromes, where two main constellations can be identified. Veno-occlusive disease (VOD) or sinusoidal-obstruction syndrome of the liver has been associated both with Bu and TBI. It usually develops within a month of transplantation, with an incidence of 5% and is characterized by hepatomegaly, ascites, jaundice, and abdominal pain (136). In a randomised study, ursodiol was found to decrease liver toxicity and improve survival after allo-HSCT (137). Ursodiol prophylaxis is now used routinely in most centers. Anticoagulant defibrotide and supportive/intensive care measures are used to treat VOD (138) and this has recently been shown to have a preventive effect in a high-risk pediatric population (139). Another complication, transplant-associated microangiopathy (TAM), is characterized by anemia, the presence of schistocytes, thrombocytopenia, elevated lactate dehydrogenase, fever, and renal insufficiency. Discontinuation of immunosuppressants may resolve the syndrome. Cardiac toxicity is associated with use of Cy in doses over 120 mg/kg or in combination with TBI (140). Hemorrhagic cystitis represents a complication where conditioning toxicity in the early onset-disease (141-143), and viruses as well as GVHD in the late-onset disease have been implicated. Currently, the causative factors and pathogenesis are still unclear and treatment is based on hyperhydration.

1.2.9 Infections

Damage to the epithelial/endothelial barriers and hematopoiesis from conditioning regimens and an immature immune system in the context of immunosuppressive treatment and, not uncommonly, HLA mismatch pave the way for infectious complications. They are a major cause of morbidity and mortality after allo-HSCT (144). During different periods of the transplant procedure, different groups of pathogens are more commonly occurring; this correlates to the status of immune reconstitution in the patient.

In the pre-engraftment period, during the neutropenic phase, gram-positive bacteria from the skin, mouth, and gastrointestinal tract are the most common agents in bacteremia (144, 145). Gram-negative infections that may encroach from the gastrointestinal tract can be successfully controlled with prophylaxis and early administration of broad-spectrum intravenous antibiotics if fever arises (146). Herpes simplex viruses are the agents that become reactivated most often during this phase, and antiviral prophylaxis is used for seropositive patients with high pre-transplant titers (147). Regarding fungal infections, oro-esophageal candida is most common, but invasive candidiasis or aspergillosis may occur (148-151). Prophylaxis with oral nystatine and systemic fluconazole is used at our center.

During the post-engraftment period (until day 100 post-transplant), cellular immunity is recovering. However, recovery is sequential and T-cells recover last, as reflected by the magnitude of viral complications in this phase. Cytomegalovirus (CMV) reactivation is most prevalent during this time and is often associated with GVHD (152-154). PCR
monitoring of viral load and pre-emptive treatment have reduced the risk of fatal outcomes (155-157). EBV infection/reactivation may cause clinical infection syndrome or post-transplant lymphoproliferative disease (PTLD) (156, 158, 159). In this post-transplant period, the risk of fungal infections is reduced; but late aspergillosis is associated with high mortality (160). Trimetoprim-sulfametoxazol prophylaxis is administered to prevent opportunistic infections (e.g. toxoplasmosis and Pneumocystis jiroveci).

During the late period, the numerical reconstitution of cells has been achieved, but the humoral and cellular responses are not fully recovered. Reactivation of VZV, CMV, and HSV, and infections with encapsulated bacteria are common, especially in patients with cGVHD (159). Due to loss of specific immunity, active immunization is recommended in all transplant populations (161).

1.2.10 Immune reconstitution post-transplant

Immune cells have differential reconstitution patterns after allo-HSCT. Innate effectors reconstitute faster and play important roles in the early post-transplant period. Neutrophils are the first leukocytes to appear after transplantation; their appearance and persistence in peripheral blood samples is regarded as a marker of engraftment. Most patients have normal levels of neutrophils within one month post-transplant; their chemotaxis is impaired but the phagocytic function is normal (162). NK-cells are detected from 10–20 days after transplantation and reach normal numbers within a month. Up to 6 months after transplantation, there is a NK subset imbalance, with increased CD56\textsuperscript{high} CD16\textsuperscript{low} (IFN\textgamma-producing) subset over the CD56\textsuperscript{low}, CD16\textsuperscript{high} (cytotoxic) subset. Recovery of NK-cells appears to be faster in CMV-seropositive individuals than in CMV-seronegative individuals (163). DC precursors are low in the blood up to 3 months after transplantation, and later on, myeloid DC counts normalize while plasmacytoid DC counts remain low at one year after transplantation. The clinical significance of this difference is unknown. Attempts to predict GVHD using DC counts have shown conflicting results (163).

Recovery of cells of the adaptive immune system is delayed, and the cell content of the graft plays a significant role in reconstitution. As for T-cells, CD4 T-cell numbers are very low after transplantation, rise slowly from 3 months, and are not normalized at 2 years in most adults. CD8-positive cell numbers are low during the first 3 months and have usually expanded rapidly by 1-year post-transplant, even reaching supranormal levels. This unbalance accounts for the quite characteristic inverted CD4:CD8 ratio. Early CD4s and CD8s are mostly of memory/effector phenotype with limited TCR diversity; T-cell pool is further diversified with the appearance of naïve T-cells later on. Production of T-cells from grafted HSCs in adults starts only after 3 months, so the early recovery is driven by graft content and homeostatic proliferation. De novo generation of CD8+ T-cells appears to occur faster. The proliferative response to mitogenic and allogeneic stimuli is restored by 6 months after transplantation, while cytotoxic activity is restored at three months in patients without GVHD. Thymic involution is recognized as an important factor influencing delayed T-cell recovery and accounting for differences in T-cell reconstitution between pediatric and adult allo-HSCT patients (163-165).
B-cells recover faster, probably due to their independence from the thymus during development. Still, normal counts are reached at approximately 6–9 months post-transplantation. B-cells do not have the ability of T-cells to expand peripherally; transferred and residual B-cells are considered to constitute the early pool, and B-cells that are generated de novo appear at 4–6 months after allo-HSCT. The B-cells that arise are mostly naïve, the percentage of B-cells bearing somatically mutated VDJ genes is abnormally low up to 1 year post-transplant, and IgM production predominates in vitro. Plasma cells appear to be more resistant to ablation and antibodies of donor origin are detected initially after transplant (163).

Figure 3. Acute GVHD
Reprinted with kind permission from Dr. Patrik Hentschke

1.2.11 Graft-versus-host and graft-versus-tumor reactions

Allogeneic reactions after HSCT are responsible for major transplant events: GVHD, GVT effect, and rejection of the graft. Interactions occur between effectors of both immune systems and the recipient tissue. Graft-versus-host disease is a major complication after allo-HSCT. However, despite the fact that it has received great attention in both clinical and laboratory research, in the current clinical reality graft-versus-tumor reactivity is still inevitably associated with GVHD (166).

GVHD can occur in as many as 85% of allo-HSCT recipients, depending on the type of donor and the degree of matching (118, 167-169). Two forms of GVHD have been defined. Acute GVHD most often occurs during the first 100 days after allo-HSCT or
donor leukocyte infusion (DLI). However, patients who have received RIC may develop aGVHD beyond three months. Clinically, it mainly affects the skin, the gastrointestinal tract, and the liver. The severity of the reactions is graded on a scale of 1 to 4, by clinical evaluation by the treating physician (170). Histological examination of the target tissue, most often of the gastrointestinal mucosa and in some cases of the skin, may aid in establishing the diagnosis. Severe acute GVHD has a profound effect on survival after allo-HSCT (171).

Today, a three-step model of aGVHD pathophysiology has been accepted. Firstly, tissue damage occurs from underlying disease and its previous treatment, conditioning therapy, and infections. The damaged tissue will upregulate adhesion molecules, secrete cytokines, and thus initiate an immune response - including activation of the DCs. In the second step, recipient DCs activate donor T-cells in the lymph nodes and induce their differentiation into effector T-cells. Lastly, inflammation and cell-mediated killing and cytokine release cause tissue damage, which supports continuation of the vicious circle of “inflammation-tissue injury-inflammation” (124, 172). T-cells are generally believed to be the main effectors in this reaction. Matching of MHC antigens reduces the severity of GVHD (173), but it still develops in a substantial number of patients due to protein polymorphisms and differences in minor histocompatibility (mHi) antigens (174, 175).

The current standard therapy for development of acute GVHD is high-dose steroids. Unfortunately, the results of steroid therapy are far from satisfactory: steroid-refractory aGVHD develops in 10-20% of cases (176). Various other approaches have been tried, such as ATG, CsA, MTX, Tacrolimus, Sirolimus (mainly for GVHD prophylaxis), psoralen, and ultraviolet light (PUVA) therapy, and regimens of monoclonal antibodies (daclizumab [anti-CD25], infliximab [anti-TNFα]) and other agents (167, 177, 178). Still, these regimens have not been effective enough to control the steroid-refractory disease. Currently it is still difficult to achieve the combination of low-grade GVH and preserved GVT effect, especially when the reaction has reached severe grade (178). Use of mesenchymal stem cells for GVHD therapy has been studied at our center (179). The true clinical efficacy of MSC in GVHD and other indications are still unproven and further studies are warranted. MSC is now being evaluated in a randomized trial by the EBMT and many other studies are ongoing.

Chronic GVHD is generally considered to occur beyond three months, and it may be a continuation of aGVHD (180). Clinically, aGVHD and cGVHD are considered to be distinct, presenting with different manifestations. Chronic GVHD may reappear after a previously resolved aGVHD, or present “de novo”, without previous aGVHD. Chronic GVHD affects 30 - 50% of long-term survivors, which is a considerable number (180-183). Its manifestations resemble those of autoimmune conditions such as dermatitis, keratoconjunctivitis, oral mucositis, and hepatic dysfunction. It is graded as limited or extensive (184). In a study performed at our center, significant risk factors for cGVHD were found to be advanced recipient age, aGVHD of grades I–IV, chronic myeloid leukemia, and alloimmunized female donor to male recipient (183).

Treatment of cGVHD mainly consists of steroids, CsA, thalidomide, PUVA, total lymphoid irradiation, rituximab (177, 178, 185). The reason for multiple treatments is
that for cGVHD, which does not respond to steroids and calcineurin inhibitors, there are no effective treatment alternatives. There is also a lack of prospective randomized studies in the field. Prevention of acute GVHD reduces the risk of developing cGVHD, since 40-100% of aGVHD patients develop cGVHD in contrast to the ones who did not develop aGVHD (15-20%) (181).

1.2.12 GVT effect and relapse of malignancy after allo-HSCT

Several lines of evidence stand in support of the graft-versus-tumor (GVT, also called graft-versus-leukemia [GVL], graft-versus-malignancy effect) after allo-HSCT. First, increased relapse rates in recipients of syngeneic grafts were noted when compared to recipients of allogeneic grafts in a mouse model (186). This led the authors to speculate that cells with immune reactivity are present in allogeneic grafts. In humans, lower relapse rates were observed in patients who had developed GVHD (187). Upon development of T-cell depletion regimens, lower GVHD was shown to be associated with increased relapse rates (128, 188, 189). This gives an indication that T-cells are responsible for a fraction of GVH/GVT reactivity. The ultimate proof for a GVT effect and involvement of cellular effectors in the reaction comes from administration of donor lymphocyte infusion (DLI) after transplantation, where eradication of relapsing malignancy could be achieved, with most success in chronic myeloid leukemia (CML) (190-193). Unfortunately, DLI bears the risk of inducing GVHD in up to 50% of patients. Since GVT effects have also been noted in patients who have not developed GVHD, this may show a potential involvement of different effector pathways of the two responses (194, 195). This has led to extension of allo-HSCT to the patients with solid tumors and a GVT effect has been reported for breast cancer, renal carcinoma, colon carcinoma, ovarian carcinoma and others (196).

Another effector of importance for a GVL effect is the NK-cell. Its function is regulated by signalling through activation and inhibition receptors: “missing self” (self being HLA Class I molecules) induces NK-mediated killing (197). Although the molecular basis of NK-cell activation is still a subject of investigation, there is an increasing amount of data on NK-mediated effects in the allo-HSCT setting, especially after haplo-HSCT, with promising results for acute myeloid leukemia (AML) (198).

Segregation of GVHD from GVL has been portrayed as a search for the “holy grail” in allo-HSCT research. Some of the immunotherapeutic approaches with focus on adoptive cellular strategies are reviewed in section on immunotherapy.

1.2.13 Rejection and graft failure

Stable and complete engraftment of donor HSCs is a prerequisite for successful allo-HSCT. Rejection of the graft is mediated by immunocompetent effectors of recipient origin. Rejection may also be caused by allo-antibodies (199). The frequency of this complication varies depending on the diagnosis and conditioning regimen. Rejection is detected by chimerism analysis, showing only the presence of recipient cells (defined as < 5% donor cells at our center), and it is a major cause of graft failure. Other causes of graft failure are viral infections (e.g. parvovirus, HHV-6, CMV), drug toxicity, GVHD and septicemia. Being a rare complication in myeloablated patients in the
beginning (200, 201), rejection has increased with the introduction of RIC (202), T-cell
depletion (128), haplo-HSCT (203), and UCBT (204). From more universal transplant
variables, HLA mismatch (205) and low cell dose (84) are known predictors of
rejection. Sensitization to HLA because of multiple transfusions, pregnancy, or
previous failed grafts also increases the risk of rejection (201).

Transfusion-related sensitization can be avoided by leukodepletion (206) and
irradiation of transfused products (207). Cell dose limitations are addressed with the
possibility of PBSC collection. DLI has been administered with success in cases of
increasing recipient T-cell chimerism (208). When a fulminant rejection is present, re-
transplantation is necessary; however, the results in leukemic patients after
myeloablative conditioning and rejection are very poor with a 5-year overall survival
(OS) below 20% (209).

1.2.14 Monitoring for engraftment and relapse after allo-HSCT

In the allogeneic setting, the advantages of genetic disparity between the donor and
recipient are used successfully for monitoring of the outcome. Both chimerism
(Chimera in Greek mythology, as described by Homer, was portrayed as a creature
bearing different parts of the body from different animals) and minimal residual disease
(MRD) can be surveyed. In allo-HSCT, chimerism indicated “% donor” refers to the
number of donor-derived hematopoietic cells post-transplant, analyzed in separate
lineages (B-cells, T-cells, myeloid cells, and HSCs) from blood and marrow (210).
Modern chimerism analyses are PCR-based and take advantage of the occurrence of
tandemly repeated DNA sequences in the genome. The number of those Mendelian
concomitant inherited repeats varies between individuals. Variation of chimerism is
analyzed over time and deviations may indicate rejection or relapse (211). MRD is
identified as the presence of small numbers of cells expressing molecular markers of
disease detected at a threshold far below what could be detected by standard clinical
pathology examinations. PCR amplification of disease-specific and patient-specific
markers is carried out (212). Thus, potential relapses can be identified. Data from our
center has shown that intervention with DLI in patients with acute leukemia and MDS
with molecular relapse significantly improves outcome (213).

1.2.15 Umbilical cord blood transplantation

1.2.15.1 Historical developments

Both organ and hematopoietic stem cell transplantation are in a constant struggle with
the shortage of a suitable graft. This pressure has led to a number of clinical and
laboratory research efforts in order to increase accessibility to the transplantation
procedure. In allo-HSCT, such an example is the development of umbilical cord blood
transplantations (UCBT), where both related and unrelated umbilical cord blood (UCB)
grafts are used.

Historically, UCB was used as a source of blood for regular blood transfusions (214,
215). These early papers contain description of the in utero collection technique and
report transfusions of whole CB to patients with different conditions and age groups from infant to adult. The first attempt to perform allo-HSCT from cord blood was reported in 1972 (216). Authors reported a change in MN blood group of a patient with acute leukemia and claimed it as a result of temporary engraftment of a transfused CB unit. However, the paper was disbelieved. Further studies were needed for the establishment of cord blood as a stem cell source. Abundant progenitor cells were demonstrated to be present in CB and they were capable of producing hematopoietic colonies when cultured in vitro (217). Subsequent studies have further characterized the stem cells and showed their viability after cryopreservation (218, 219).

First successful cord blood transplant was performed by Eliane Gluckman in 1989, when a patient with Fanconi anemia was reconstituted with UCB from his sister (220). This has encouraged further extension of the approach to the transplantation from unrelated cord blood in children and adults (221-224). Cord blood as a stem cell source differs from BM or PBSC both in the properties of hematopoietic stem cells and in the immunological status of the accompanying hematopoietic cells in the graft. This shapes the clinical course and results of the UCBT. With peculiarities of the logistics taken into account, several features of stem cell transplantation from this cell source can be defined, being both advantageous and not for the successful outcome of the transplant procedure.

1.2.15.2 Cell dose

As for other SC sources, a threshold in cell dose per kilogram of recipient weight must be reached in order to achieve consistent engraftment and minimize transplant-related events. Cell dose directly correlates with rates of neutrophil and platelet recovery (223, 225, 226). Low cell dose has been associated with delayed engraftment and increase in TRM (226), and increasing importance of cell dose with increase in HLA mismatch has been described (227). The low cell dose in a UCB unit results from a limited volume of cord blood which can be collected, on average around 120 ml (228). This is one of the greatest limitations of the UCBT and therefore the initial clinical developments were made in paediatric transplantations, where the recipient weight is lower. Current recommendation is to aim for $\geq 3 \times 10^7$ TNC/kg or $\geq 2 \times 10^5$ CD34+ cells/kg, when well-matched units are considered (e.g., 5/6 or 6/6 match). In cases of 4/6 match, transplants where GVH reactions are not favoured or where the rejection risk is high, a dose of $\geq 3.5 \times 10^7$ TNC/kg (229, 230) or, by another study, even a dose of $\geq 5 \times 10^7$ TNC/kg is desirable (231). It is important to have in mind that it is the pre-cryopreservation cell dose which is reported and considered in transplant outcome analysis in absolute majority of the studies. Up to 20% of cellular content may be lost during the thawing and release for infusion at the laboratory of transplanting institution. The TNC is the most standardized marker of CB unit cellular content, while CD34+ cell enumeration and CFU assays have encountered standardization problems between different labs. However, different techniques of CB unit processing at the CB bank may influence the correlation between TNC and progenitor amount in the unit (228). Taken all the factors together, the current goal is “the bigger-the better”, which is mostly problematic in adult recipients.

Still, patients do engraft with reliable clinical results from these SC doses, which are 1-2 log fewer than doses required for successful engraftment in BMT. This is attributed to...
the unexpectedly high proliferative capacity of progenitor cells in UCB, which was observed already in the early days of the CB transplants (219, 232). For overcoming the dose limitation in adult patients, several approaches have been developed. Most successfully, infusion of two separate UCB units, partially matched to the patient and between each other, has been employed (233, 234). Other strategies for overcoming the cell dose limitation include in vitro expansion of UCB SC, with several different protocols (235-238). Also, a so called “dual transplant” strategy has been tried, where UCB unit has been co-transplanted with CD34+ enriched graft from a third-party donor, being haploidentical or bearing shared haplotypes with the patient (239). Mesenchymal stem cells have been used in hope to support engraftment (240) and intraosseous injection of UCB units has been performed with same intention (241, 242). However, these strategies have met limited success and are in need of further optimization.

1.2.15.3 HLA matching

Clinical results from unrelated UCB transplants have confirmed the less stringent requirements for HLA matching. Results from UCBT with 4/6 antigen match have been shown to compare to those of mismatched and matched unrelated BM, and more recently, even to matched sibling donors (119, 130, 243). This feature has facilitated use of UCB where no suitable unrelated donor could be found. There is a debate ongoing whether it is SC dose or degree of HLA mismatch, which determines the transplant outcome. As described earlier, current consensus advocates for importance of SC dose, finding HLA disparities to be of minor importance for survival (244, 245). However, several studies have demonstrated importance of HLA matching earlier (226, 246) and this has been confirmed in a recent analysis of outcomes for 1061 patients receiving UCB grafts from New York Cord Blood Program (NCBP). In this study, 4/6 match associated with higher TRM if cell dose of ≥5x10^7 TNC/kg was not reached, while a higher dose than ≥2.5x10^7 TNC/kg did not influence the TRM in 5/6 match situations. This suggests that when searching for UCB units, priority should be given to better HLA-match even if the unit is smaller in size, as long as the minimum requirements are met (231).

HLA matching for UCB units is conventionally defined as low resolution for HLA-A and -B and high resolution for HLA-DR. This is different from a minimum requirement in unrelated donor matching - 8/8 high resolution HLA typing. Importantly, a recent analysis of Cord Blood Transplantation Study (COBLT) carried out by NIH, patients have included an interesting approach to retrospectively type donor-recipient pairs for HLA-A and B at high resolution. The typing available at the time of transplant was low resolution. Approximately one third of pairs were found to be more disparate than originally determined, with a substantial increase in 3/6 (2.6% to 19.6%) and 2/6 (0% to 6.1%) match groups. The statistical power seemed to be insufficient to elucidate the effects of these findings on outcome variables (247). These observations pose several issues to consider: firstly, the low resolution HLA-match data has to be regarded with caution when drawing conclusions on HLA impact from the large retrospective studies. A high-resolution 6/6 typing should be advocated for, however, this is met by the technical obstacles and cost issues. At the same time, a generous HLA-permissiveness of the UCB graft content is once more confirmed by these observations.
Permissiveness for HLA-mismatches, when transplanting from UCB, increases the possibility of finding a suitable donor for patients with uncommon tissue types. The chance of finding a 4/6 match is 99% and for a 5/6 or 6/6 match the chance is 70%, as postulated by an empirical analysis of New York CB Bank possessing (248). This is especially valuable when conducting a donor search for ethnic minorities. Another analysis from Memorial Sloan-Kettering Cancer center showed that use of UCB for transplant can extend transplant access to patients from all ethnicities, but especially for Southern Europeans and Non-Europeans (249). In context of increasing globalization, these aspects are important to consider.

In summary, permissive HLA-matching is a feature, which favours use of UCB as a stem cell source, especially when it seems to be possible to compensate for negative effects of higher mismatch grade with increment of cell dose.

1.2.15.4 Graft availability

UCB units are available “off the shelf” i.e. almost immediately, in contrast to unrelated marrow donors, where a handful of logistic events consume time. All UCB units are readily HLA-typed for HLA A, B and DRβ1, TNC count is known, inclusion of CD34+ cell count into the search database is being discussed (250). Maternal sample is tested for HIV, Hepatitis B and C, HTLV 1, 2, Syphilis, Malaria and Chagas disease and CMV serological status. All this information is not readily available in case of marrow donors. In case of UCBT, there is no risk for donor attrition. These factors facilitate faster identification of an appropriate unit, which is particularly important in cases of a rapidly progressing disease. One study reported a median of 13.5 days to identify a UCB unit in contrast to 49 days for bone marrow (251). However, as exemplified by another analysis, the time from diagnosis to transplant was found to be 21.7 months for CB compared to 8.2 months for marrow (252). This indicates the fact that UCB has historically been considered as a last choice.

Most CB units are CMV and EBV negative due to the placental barrier. However, the bidirectional match for CMV and EBV status is important, since poorer outcomes have been reported in CMV positive recipients of CB grafts and EBV mismatch is a risk factor for PTLD development (158, 253).

1.2.15.5 Summary of current clinical results

Several important multicenter retrospective studies were carried out under the last decade and presented data which forms current understanding of the clinical outcomes after UCBT. A study from Center for International Blood and Marrow Transplant Research (CIBMTR)/Eurocord study has reported comparison of BM (n=2052) and UCB (n=113) transplants from related siblings in children under years 1990-97. Reported 3-year survival did not differ between the groups (55% and 46%) and UCBT associated with delayed neutrophil and platelet engraftment as well as with reduction in risk for acute (24% and 14%) and chronic (15% and 6%) GVHD (254). Transplant indications included both malignant diseases and non-malignant conditions.
Another study from Eurocord has more specifically looked at children with acute leukemia, receiving either unrelated unmanipulated bone marrow (mostly HLA 6/6, n=262), T-cell depleted marrow with a higher degree of HLA mismatch (n=180), or mismatched UCBT (n=99), during years 1994-98 (204). The UCBT group, compared to BMT and depleted-BMT had delayed engraftment but reduced risk for both aGVHD (35%, 58% and 20%) and cGVHD (25%, 46% and 12%) compared to BMT. Early mortality (up to day+100) was increased in the UCBT group (39%, compared to 19% and 14% in BMT and depleted-BMT) and resulted mainly from infections (40%), relapse (12%) and aGVHD (14%). However, 2-year EFS (31%, 43% and 37%), OS (35%, 49% and 41%) and incidence of relapse (38%, 39% and 47%) were comparable between the three groups.

CIBMTR and NCBP have also presented analysis of their data in paediatric patients with acute leukemia (119). They compared 503 children receiving unrelated UCBT (35 were HLA matched 6/6, n=201 - 1 antigen mismatch and 269 - 2 antigen mismatch) to 282 patients receiving unrelated BM (116 were HLA-matched 8/8), between years 1995 and 2003. Cumulative probabilities for neutrophil and platelet recovery were not significantly lower in matched UCBT compared to matched or mismatched marrow (85%, 97% and 97%) and confirmed that that was the case in mismatched UCBT (80-59% depending on mismatch and cell dose). The risk for developing acute and chronic GVHD was similar between UCBT and BMT groups. Early TRM was higher for UCBT with 2 antigen mismatch or 1 antigen mismatch and low cell dose, but comparable for UCBT with 1 antigen mismatch and high cell dose. Interestingly, relapse rate was lower in the 2 antigen mismatched UCBT group compared to matched BMT. Survival analysis demonstrated an interesting finding for HLA-matched UCBT which seemed to display the highest probability (60%) of 5-year LFS, otherwise probabilities were similar for all other groups (38% matched BM, 37% mismatched BM, 36% 1-mismatch CB and low cell dose, 45% 1-mismatch CB and high cell dose, 33% 2-mismatch CB), see Figure 4.

A study on transplantation outcomes in infant (up to 18 months of age) leukemia has been published on behalf of CIBMTR (255). Patients were grafted either from matched siblings (n=101), unrelated BM donors (n=85) or unrelated CB (n=81) between years 1990-2001. There was no difference in 3-year LFS (49% and 54%) and OS (54% and 62%) when related transplants were compared with unrelated ones, i.e. unrelated BM and CB were pooled together in the second group. UCBT was associated with significantly higher TRM (31%) in comparison to unrelated BM (15%) and sibling transplants (6%).

In the COBLT study, the clinical outcomes for 193 children with hematological malignancies transplanted from unrelated UCB were analyzed, although the study was not comparative. (247). Most of the patients suffered mostly from ALL and were at high risk; all received common TBI/Cy/ATG conditioning. Incidence of grade II-IV aGVHD was 42%, 2-year OS was 50%.
Similar analyses have also been carried out for adult transplants. Two studies published in the same issue of The New England Journal of Medicine in 2004. Rocha et al in an EBMT-Eurocord-Netcord study has analyzed outcomes of patients with acute leukemia, receiving unrelated UCB (n=98, 94% HLA mismatched) and 6/6 HLA-matched unrelated BM (n=584) between 1998-2002. (243). They found risk for aGVHD to be decreased (26% and 39%) and risk for cGVHD to be similar (30% and 46%) between the groups, and no significant differences in TRM (44% and 38%), risk for relapse (23% and 23%), 2-year OS (36% and 42%) and LFS could be demonstrated. Deaths related to toxicity were more common in the CB group (35% vs 6%) whereas GVHD related deaths were more common in the BMT group (12% vs 31%). Laughlin et al in an IBMTR-NCPB study has compared recipients of matched BM (n=367), 1 antigen mismatched BM (n=83) and mismatched CB (n=150) during years 1996-2001 (130). This study was more heterogeneous in terms of disease. The authors demonstrated that rate of grade II-IV aGVHD was similar between UCB and matched BM groups and that cGVHD was more prevalent in the UCB versus the BM group, although the proportion of extensive cGVHD cases was lowest in the UCB group. UCB and mismatched BM had comparable 3-year LFS (23% and 19%) and OS (26% and 20%), but significantly lower than for matched BMT patients (LFS 33% OS 35%). Relapse rate was similar between all 3 groups but again, the early TRM resulting primarily from infectious complications was higher in the UCB group.
Two Japanese comparative studies have reported single-center experience with cord blood transplants in adults. Conditioning and supportive care was similar among the groups in both studies. In the first one, 113 patients with hematological malignancies received either UCB (n=68) or UBM (n=45). For UCB group, grade III-IV aGVHD was reduced (6% vs. 27%) as well as TRM (9% vs. 29%) and 2-year LFS was advantageous (74% vs 44%) (256). In a follow-up analysis, 100 patients underwent unrelated UCBT and 71 received related BM/PBSC. No differences in TRM (9% vs. 13%), relapse (17% vs. 26%) and 3-year LFS (70% vs. 60%) could be found (257).

Recently, UCB has been introduced in transplantations for non-malignant disorders. The rationale for this development lies on several ideas. Firstly, these diseases are inherited, which reduces the chance of finding a HLA-matched healthy sibling. Secondly, GVHD is only a complication in this transplant situation and therefore the optimistic reports on lower incidence of GVHD after UCBT fell well in place. Thirdly, mostly true for metabolic storage diseases, the speed of graft availability is crucial for halting the neurological disease progression.

For hemoglobinopathies, optimistic results have been reported from related UCB transplants with 2-year OS of 100%, EFS 79-90% depending on diagnoses and low GVHD incidence (258). However, more disappointing results from other centers followed with EFS of only 43% mostly due to graft failure, of note this study reported outcomes of unrelated transplants (259). Current studies had low patient volume and were mostly single center. In case of Fanconi anaemia, as with hemoglobinopathies, reasonable outcomes after UCBT have been reported (253, 260), but there are still no studies comparing outcomes between different graft sources. As for metabolic storage disorders, a comparison between different graft types has showed an advantage in achieving complete donor chimerism for UCB group with no difference in graft failure (261), while others have reported lower engraftment rates with UCB than expected from unrelated BM (262).

Reduced incidence of GVHD is named as a feature of UCBT, but the positive reports have been confronted from several instances and CB grafts are able to mediate severe GVHD. However, regarding evaluation of all outcomes, one has to bear in mind that no prospective randomized multicenter studies have been conducted yet, CB transplant was historically a last choice treatment and that HLA-matching has improved during the last decade. Moreover, different studies are heterogeneous in terms of diagnoses, conditioning, supportive care and methods of data analysis.

To summarize, regarding the OS and LFS data, it seems that UCBT can be chosen for paediatric patients with acute leukemia, if no HLA-id donor is available. Still one has to bear in mind that engraftment is delayed and early TRM is increased. As for adults, UCBT is justified as a good alternative when no matched unrelated donor is available and time is short. Therefore, UCB unit search should be started early. For adult patients cell dose is an important factor, and therefore double CB transplants should be performed in adults, according to “the bigger-the better” strategy, with as good as possible HLA-match.
1.2.15.6 Infectious complications after UCBT

Ability of UCB immune cells to counter infections has been at question since the beginning of its clinical use, mainly due to the immunological naivety attributed to the effector cells both in UCB grafts and after engraftment. Several retrospective comparative studies have reported increased rate of deaths due to infection in UCBT patients when compared to patients transplanted from URD (119, 204, 254). Severe infectious complications, which contribute to mortality, occurred during the first 100 days post transplant; prolonged neutropenia and low cell dose are additional risk factors (263, 264). Bacteremias occur in around 1/3 of adult patients (265). Two larger studies reported incidences of CMV infection and CMV disease, 22% and 6% (266) and 47% and 11% (267), respectively. Risk factors were reported to be T-cell depletion and severe GVHD. As for EBV, in a myeloablative setting no increased risk for PTLD could be demonstrated (268). Still, another analysis showed that in a nonmyeloablative setting, when ATG was used, incidence of PTLD was markedly increased to 21% (269). In summary, infectious events contribute to increased morbidity and mortality of UCBT patients. It is important to recognize this limitation and address it with prophylactic and preemptive treatment strategies, where available. Increased cell dose (and by this - dose of passenger immune effectors) and measures to enhance engraftment could also contribute to the reduction of infectious complications rate and TRM after UCBT.

1.2.16 Allo-HSCT for solid tumors

Hematological malignancies are successfully treated with allo-HSCT, and recognition of the GVT effect, as well as data from adoptive immunotherapy trials in autologous setting, have inspired the attempts to allograft patients with solid tumors. Solid tumors express immunogenic antigens which drive T-cell responses in autologous setting (270, 271) and spontaneous regress of the tumor has been reported. However, the tumor can evade or inhibit the actions of the immune system (196). Allo-HSCT may overcome these limitations.

When allografting patients with solid tumors, it is important to consider the conditioning regimen. Several solid tumors, for example renal cell carcinoma (RCC), are not sensitive to chemotherapy (272). Myeloablative regimens in allo-HSCT for hematological malignancies are associated with toxicity and higher rate of TRM. Solid tumor patients are usually aged and of poor performance status, therefore, development and usage of non-myeloablative condition regimens favours these patients (111, 273). More than 1000 patients have received allo-HSCT for different solid tumors from 1995 to 2006 in several European countries (274). When referred to “response”, even if studies have followed RECIST guidelines (275), there are almost always additional aspects detailed in the Materials & Methods of the respective study. Generally, a complete response (CR) is defined as complete disappearance of the tumor, partial response (PR) and progressive disease (PD) definitions are usually subject to a more detailed specification by the authors and stable disease (SD) is a status, which falls in between PR and PD. Overall response (OR), or just “response”, combines CR and PR.
1.2.16.1 Renal cell carcinoma

Allo-HSCT for treatment of solid tumors is most extensively studied in renal cell carcinoma, a work pioneered by Childs et al at NIH in a nonmyeloablative setting (276, 277). A 53% overall response to treatment was reported in initial series. Subsequently, data on 74 enrolled patients were reported, with only 8% of TRM and a response in 39% of cases, CR in 9% of cases (278, 279). Later reports from other centers mostly included less than 10 transplanted patients and the response rates varied between 8-57% (274, 278). Other series reported negative results with none of the treated patients responding (280, 281). However, as evident from NIH studies and highlighted by discussions in the field, GVT in RCC tends to occur late after allo-HSCT, at a median of 5 months post-transplant, and often after aggressive strategy applied in order to promote it (immunosuppression reduction and/or DLI). Therefore, patients with good performance status need to be selected for transplant, in order to be able to withstand the procedure and to survive until the GVT effect can take place. The tumor growth kinetics is also affecting outcome and patients with fast tumor kinetics should be avoided. These patients show a rapid tumor progression after HSCT and, hence do not benefit from allo-HSCT since the GVT effect is delayed (196). Importance of aggressive strategy for GVT propagation was not considered in the studies reported by Rini et al and Pedrazzoli et al, since only a few of the patients received DLI, and patients with poor performance status were included in these studies (280, 281). The European study (124 RCC patients) reported TRM of 16%, confirmed late occurrence of responses (at a median of 150 days) and highlighted importance of low tumor burden (<3 metastatic sites), good performance status (Karnofsky score >70%) and importance of active GVT induction. The 2-year survival of 70% was demonstrated in patients who received DLI and developed cGVHD, in comparison to 3-year overall survival of 30% for the whole group of patients (282). It is important to note that studies of GVT effect in RCC patients have contributed a very important finding, which stands out as a mechanistic proof of the GVT effect in allografted solid tumor patients. Takahashi et al have been able to expand a donor CD8+ T-cell clone from a RCC patient after allo-HSCT and to identify the target antigen of these T-cells (279). The antigen was associated with human endogenous retrovirus type E and it seems that expression of the genes derived from this virus were active only in developed RCC. These results prove specific reactivity of allogeneic T-cells against the cancer tissue and thereby support further clinical development of allo-transplant programs for patients with metastatic solid tumors.

1.2.16.2 Other tumors

Experience with allo-HSCT in other solid tumors is less extensive. In allo-HSCT for breast cancer, response rates between 16 -37% were reported from different studies (274, 283). Again, most studies were of small patient volume and the reported TRM incidences were up to 20%. The EBMT study (Ueno et al) on 66 women with metastatic breast cancer demonstrated that use of RIC contributed to decrease in TRM, however, 2-year progression-free survival (PFS) was just 5% for the myeloablative regimen group and no patients receiving RIC survived. For breast cancer patients, a “tandem transplant” concept was suggested, where first the cytoreduction (or “debulking”) is performed by chemotherapy and autologous HSC transplant, and allo-
HSCT comes in after (284). An OR of 24% was reported in this study. In allografting for colorectal cancer, a study from the EBMT registry reported OR of 46% in 39 patients (285). EBMT experience in allo-HSCT for ovarian cancer was summarized in a recent report on 30 patients, OR was reported in 50% of cases, 1-year TRM was 20% and median overall survival was 10 months. Still, improved responses were seen in patients with cGVHD (286).

During the latest period, activity in the field of allo-HSCT for solid tumors has been steadily decreasing. The main reasons for this are the poor reported survival rates and high TRM rates. Most importantly, allo-HSCT for solid tumors has failed to demonstrate a survival benefit for the patients in comparison to the other regimens of oncological treatment. Still, the observed complete responses after allo-HSCT need to be considered, as well as the repetitive clinical observations of GVT effect in the existing reports. Multicenter studies aiming at patients with good performance status and low tumor burden, combined with implementation of active pro-GVT strategies, are warranted.

### 1.3 IMMUNOTHERAPY AND ALLO-HSCT

#### 1.3.1 Immunotherapy

Immunotherapy can be viewed as a constellation of deliberate manipulations of immune responses, carried out in order to modulate: enhance or suppress functions of the immune system and by this to prevent or to treat a disease. Such manipulations are applied not only in oncology/hematology, but also in other areas of medicine. Vaccination for prevention of infectious diseases is the first obvious example (287). Further on, allergology is another area where allergen-specific immunotherapy represents an intriguing and successful treatment strategy (288). Treatment of autoinflammatory syndromes by recombinant IL-1 receptor antagonist (anakinra) represents another recently developed effective immunotherapeutical tool (289). TNF-inhibitors are another group of novel disease-modifying anti-rheumatic drugs, which have greatly improved outcomes of rheumatic diseases (290). An elegant example of a vaccination to prevent cancer is immunization against the human papilloma virus (291). In the fields of rheumatology, oncology and allo-HSCT a number of pharmacological compounds influencing function of immune effector cells are used. These few examples illustrate extensive development and progress which is continuously ongoing in the field. The scope of this thesis lies on adoptive immunotherapy, primarily for infections and malignancy, after allo-HSCT. Therefore only a brief overview on these aspects in the autologous setting is provided.

Immunotherapeutic approaches for treatment of malignancies are traditionally (with reference to vaccination as the first example of immunotherapy) grouped into passive and active. In passive immunotherapy, the effector component is provided, whereas in active immunotherapy the immune system itself is triggered to respond and to establish memory. Both groups can be further divided into specific or non-specific, depending on which antigen recognition pathway is targeted. Example of an active specific approach for malignancy is cancer vaccines, where host immune system is challenged with e.g.
peptides, proteins, DNA or irradiated tumor cells with an aim to initiate an immune response. Active non-specific immunotherapy is represented by administration of immunostimulatory cytokines like IL-2 and IFNα. Usage of the Bacillus Calmette-Guerin instillations for patients with urinary bladder carcinoma is an example of passive non-specific immunotherapeutical approach (292). The largest group, passive specific approach, employs therapies with monoclonal antibodies, receptor antagonists and adoptively transferred immune cells, where antigen-specific recognition mechanisms are exploited by the transferred effectors.

1.3.2 Cellular Immunotherapy for malignancy in autologous setting

Lymphocytes can be isolated from cancer patients, activated and/or expanded in vitro and subsequently infused back to the patient. Cells can be isolated from peripheral blood, lymph nodes, malignant effusions and tumors. In the early studies, PBMCs activated with IL-2 (lymphocyte-activated-killer [LAK] cells) were used, with a very limited response (293). Later on it was noticed that tumor-infiltrating lymphocyte (TIL) populations have a high frequency of specific T-cells, but TILs were more difficult to obtain (294). Most of the early studies employing cellular therapy were carried out in patients with malignant melanoma, with the Rosenberg group as pioneers (295). The objective response to therapy was seen in around 30% of patients (296). Further on, an interesting modification of the procedure was to induce lymphodepletion with Cy+Flu before the cell transfer, which has favoured expansion of the transferred T-cells, most likely due to enhanced homeostatic expansion (297). Another modification of the approach was use of antigen-specific CD8+ clones for the infusion (298). However, the same study reported the loss of the targeted tumor antigen, thus demonstrating development of tumor escape. Later studies attempted to modify autologous T-cells and induce them to express transgenic TCRs recognizing tumor antigens (299). This study reported cancer regression in 2 out of 15 patients. An interesting approach focusing on T-cells derived from sentinel lymph nodes has been developed at our Institute. In these studies, cells were restimulated in vitro with tumor lysate, expanded and infused back to the patients with colon or bladder cancer. In pilot studies, responses were observed in 4/9 patients (300). A summary of the latest developments in the field of cancer vaccines for treatment of prostate cancer is provided in a separate section of this thesis.

1.3.3 Immunotherapy after allo-HSCT

1.3.3.1 Donor lymphocyte infusion

The effectiveness of allo-HSCT is a proof by itself that an adoptive immunotherapy can be successful in practice. In the early animal studies, it was demonstrated that mice grafted from non-identical marrow could develop signs of immune reactions and clear leukemia (186, 301). Later on, the allogeneic immunotherapeutical effect was identified when allo-HSCT advanced in humans (302). It was noticed that development of GVHD was associated with lower risk for relapse (187), and that transplants from syngeneic donors were at higher risk for relapse, when compared to matched sibling donors (194, 303). Also, if T-cells were depleted from the graft with an aim to prevent GVHD, the
risk for relapse was increased (128). These findings advocated for the importance of allogeneic GVT effect and of T-cells as the main effectors in this response.

Donor lymphocyte infusions (DLI) were used as a mean of adoptive immunotherapy for treatment of relapse of the underlying malignancy after allo-HSCT. Already from the start, results were most encouraging for patients with relapsing CML (304), and less successful response was seen in acute leukemias and other malignancies (190, 191, 304-308). Immunological features of CML cells, such as antigen presentation and 

bcr/abl expression, could be responsible for this effect (309-311). Also, the more aggressive nature of acute leukemias probably plays a significant role. It was observed that effect of a DLI requires time, as responses are detected 2-3 months post infusion. Therefore, close monitoring of the minimal residual disease and early DLI give better results in acute leukemia patients, as suggested by data from our group and from other centers (213, 312).

After RIC transplantation, DLI can be administered as a therapeutic measure for incomplete donor chimerism (192, 313-315). This can aid to achieve complete chimerism in about 1/3 of the cases. DLs have also been infused in a prophylactic (preemptive) manner in patients transplanted with a T-cell depleted graft. This approach seems to be most feasible in high-risk malignancy patients (316-318).

Administration of DLI evokes acute GVHD in 40% to 60% of the patients. As anticipated, GVT response is associated with development of GVHD (319), but severe GVHD increases mortality (314). DLI can be administered as a bulk infusion, but with an idea of controlling the severity of GVHD, an escalating-dose regimen has been developed. In patients with CML, this strategy limits GVHD (320), but at the same time the GVT effect is delayed (6-12 months), and therefore is not practical in case of relapse of rapidly progressing disease. The studies available have not demonstrated a dose-response relationship for diseases other than CML (193, 321). Still, the dose escalation approach seems to be effective also in patients with acute leukemia when treatment is initiated at molecular signs of the disease (213)

1.3.3.2 Adoptive T-cell immunotherapy to prevent and treat infection

1.3.3.2.1 CTLs for EBV and PTLD

Adoptive T-cell immunotherapy for post-transplant lymphoproliferative disease (PTLD) is one of the most successful examples of T-cell therapy used in the clinical practice. PBMCs can be infected with EBV in vitro and by this an immortalized B-cell cell line, lymphoblastoid cell line (LCL), can be established. LCLs were demonstrated to express proteins of the latency III program of the EBV replication cycle, the same protein expression pattern was detected in PTLDs (322). Therefore, autologous LCLs could successfully be used as stimulators for raising clonal populations of EBV-specific T-cells in vitro. In the initial clinical applications, polyclonal T-cell cultures were raised from peripheral blood of the donor and were administered to patients with or at risk for PTLD. The therapy demonstrated a sustainable effect and promoted tumor regression
(323, 324). These results were further confirmed, and preemptive infusions were shown to be effective (325, 326).

The main drawback associated with this procedure is the extended time needed for isolation and expansion of the specific T-cells (6-8 weeks at a minimum). Since the course of PTLD is usually rapid, administration is often too late. As a remedy for this, Crawford et al have proposed to produce and bank EBV specific CTLs, as a source of third-party therapy product. These off-the-shelf available allogeneic, partially HLA-matched T-cells were successfully infused to patients in a multicenter trial and demonstrated responses in 52% of patients at 6 months (327). However, with the rise of more rigorous regulatory constraints (good manufacturing practice – GMP), generation of cultures by LCL re-stimulation and banking of such CTLs becomes practically impossible in university/public health care system setting, mainly due to the very high cost of the end-product.

Additional modifications of the stimulation technique were suggested, where transfection of autologous LCLs or DCs with an adenovirus vector, containing an immunodominant CMV protein, was shown to be successful. By this method tri-virus (EBV, adenovirus, CMV) specific CTLs could be derived either from peripheral blood (328, 329) or even cord blood (330). Still, this approach doesn’t eliminate the time factor and high associated costs of the cell culture under GMP conditions. Therefore, novel approaches are required in order to be able to provide a treatment possibility to allo-HSCT patients, suffering from a therapy-resistant PTLD. It is also important to be able to deliver the therapy in time, since PTLD is often aggressive. In this thesis an example of such an approach is presented (paper III). Recently, Kolb and co-workers have taken the advantage of the IFNγ-capture technology, which is available at the GMP-grade. They stimulated donor lymphocytes with a mix of immunodominant EBV peptides overnight, and T-Cells, which responded to the stimulation by IFNγ production, were beaded out and infused. A positive response to the infusion was demonstrated in 3 out of 6 patients (331). This study represents another approach, which also provides the advantage of speed and relatively low cost in production of EBV-specific T-cells.

Since EBV is implicated in development of other tumors, the success of EBV T-cell transfers in PTLD patients has inspired similar efforts in other EBV-associated malignancies. However, the EBV protein expression and immunogenicity pattern might be different and more challenging to exploit in other types of tumors. Transgenic DCs expressing LMP2 have been used to raise T-cell clones for EBV-positive-Hodgkin’s lymphoma patients in the autologous setting. A tumor regression could be seen in some patients (332). Also, strategies of raising EBV-specific T-cells in patients with EBV-positive nasopharyngeal carcinoma are being explored (333). In summary, adoptive immunotherapy approaches for EBV PTLD have been successful and have a life-saving potential, when administered in time for allo-HSCT patients. It is important to develop the approach further in order to be able to increase accessibility to this treatment in the daily clinical practice.

1.3.3.2.2 CTLs for CMV infection and disease
Even if pharmacological options are available as a successful primary treatment option for CMV infection, therapy-resistant mutants may develop or patients may suffer substantially from drug toxicity (334). In these situations secondary treatment options are needed. The feasibility of adoptive CTL therapy for CMV infection was for the first time demonstrated by the Riddell group, where CD8+ T-cell clones were transferred into 14 patients (335). Later on, polyclonal CMV-specific CD4+ and CD8+ T-cell preparations were infused into therapy-resistant patients, demonstrating impressive responses despite low cell numbers (10^7 cells/m^2) infused (336). Similarly to the situation in EBV, production of CMV specific CTLs by restimulation is associated with high GMP costs and is a time consuming procedure. These problems have facilitated further development of the techniques, such as approaches employing tetramers (337) or IFN-γ-capture assays (338) for quick isolation of memory precursor cells.

1.3.3.3 Adoptive T-cell immunotherapy for malignancy

In contrast to successful use of CTLs to treat infections, the attempts to adoptively transfer malignancy-specific T-cells after allo-HSCT was met with less success. In this area, more promising results were achieved in autologous setting. One of the main reasons for such differences is the type of malignancy which allo-HSCT is mostly performed for - malignancy of hematopoietic origin. The main difficulty lies in the identification of antigens, which could be targeted. Two classes of immunogenic epitopes have been proposed in hematological malignancies. Allo responses have been demonstrated against either minor histocompatibility antigens (miHA) or tumor-associated antigens (TAA). Allelic polymorphisms generate differences in amino acid sequence of normal cellular proteins, and these polymorphisms can result in cellular processing of immunogenic peptides. Such peptides are displayed on HLA molecules and trigger alloreactions in HLA-identical setting. Since they are peptides, and not the antigen-presenting molecules, the term miHA is somewhat misleading in relation to the terms MHC and HLA, however such a definition has been suggested first and is commonly accepted. The miHAs HA1 and HA2 have been implicated as possible targets for CTL therapy, when their selective expression in hematopoietic cells was demonstrated (339). However, no clinical evidence is present to date in definite support of this concept. One of the reasons might be that HA1 disparities were also shown to correlate with GVHD in HLA-identical related donor setting, indicating that specific GVT is directed towards other epitopes (340, 341). The efforts to transfer miHA-specific T-cell clone results in poor survival of the transferred cells (342, 343). There are reports that BM environment might be “hijacked” by the malignancy, turning it into a protective niche for leukemic cells (344, 345). Such effects could impair access of the adoptively transferred T-cells to the tumor, and this could eventually compromise their survival upon transfer.

Other group of antigens, which induce specific alloresponses, can be TAA.s. In recent reports, responses towards a TAA Wilms’ tumor antigen 1 (WT1) were associated with a superior clinical outcome in ALL patients. The observed effect seems to be independent of GVHD influence (346). This might represent a potent pathway for adoptive intervention (347). Most recent developments in this field are represented by transgenic approaches, where chimeric antigen receptors specific for tumor antigens are
introduced into lymphocytes. For example, in a conceptually interesting in vitro study PBLs were transfected with a receptor for CD19 and later on stimulated with adeno-CMV-transfected APCs and LCLs. Resulting T-cells were empowered with antitumor and antiviral specificities (330).

In summary, lack of identified target antigens seems to compromise the adoptive therapy for malignancy after allo-HSCT. One of the potential approaches could be engagement of co-stimulatory pathways what has been explored, with some success in the autologous setting (see separate section on prostate cancer and immunotherapy).

1.4 EBV AND POST-TRANSPLANT LYMPHOPROLIFERATIVE DISEASE

1.4.1 Overview of EBV biology

Epstein-Barr virus is a double-stranded DNA \( \gamma \)-herpesvirus which infects more than 90% of the world’s population. Primary infection occurs most often in childhood by salivary transmission and is usually asymptomatic. If primary infection is delayed to adolescence, in approximately 25% of cases it manifests as infectious mononucleosis (IM). Upon salivary transmission, EBV replicates in the oropharynx and virus shedding into the throat occurs. EBV can infect both epithelial cells and B-cells, but latency most likely is achieved only in B-cells. Occasional reactivation of the virus leads to viral spread and infection of new hosts.

As in all herpesviruses, the lytic replication of EBV involves three sequential phases characterized by differential expression of: I) immediate-early (IE) proteins; II) early (E) proteins; III) late (L) proteins. IE proteins are transactivators of viral early gene expression, E proteins include components of viral DNA replication machinery and L proteins are mainly structural ones.

EBV infection is mainly controlled by T-cells. A feature of T-cell response against EBV is that responses are directed against epitopes, which are termed immunodominant. This means that in different individuals, and despite different HLA backgrounds, responses are mounted against antigens, which are derived from the same subset of lytic or latent EBV proteins. In this way, hierarchies of immunodominance among these proteins were established. As determined in IM patients, in the lytic cycle, two IE proteins, BZLF1 and BRLF1, and a set of E proteins, namely BMLF1, BMRF1, BALF2, BALF5 and BGLF4 drive most responses of CD8+ T-cells. (322)

EBV latency is established in B-cells, which are generally not permissive for viral replication. EBV is self-sufficient in inducing B-cell transformation, whereas this function of other \( \gamma \)-herpesvirus Kaposi’s sarcoma associated herpesvirus is dependent on host factors, such as CD4 help. EBV achieves this through a set of latency proteins which are expressed during proliferation of infected cells. These are the Epstein-Barr nuclear antigens (EBNAs) -1, -2, -3 (or 3A), -4 (or 3B), -5 (or LP) and -6 (or 3C) and latent membrane proteins (LMPs) -1, -2A and -2B. EBV latency genes also encode for small nonpolyadenylated RNAs (EBERs). EBERs are nontranslated and have been implicated in oncogenesis; they are also commonly used as a diagnostic marker. During latent infection EBV is believed to sequentially activate different latency gene
expression programs that drive the cells through steps that mirror those of the natural B cell differentiation process. (348). Upon infection of naïve B-cells, the Latency III or “growth” program is activated involving all EBNA s and LMPs, which induces B-cell activation and proliferation, resembling blastogenesis in response to antigen. This amplifies the virus-containing cell pool, and induces the migration of cells into secondary follicles and the formation of germinal centers (GC). In GC the latency II program is activated and EBNA1 and LMPs are expressed. Upon leaving GC, infected B-cells activate “latency” or Latency 0 program, where all genes are shut down leaving the infected cells in stealth for T-cell recognition. During homeostatic division, Latency I program is activated, where only EBNA1 and occasionally LMP 2a are expressed (348, 349).

From the latent proteins, EBNA-3A, -3B and -3C are the most immunodominant proteins eliciting CD8+ T-cell responses. Subdominant responses are directed against LMP2, and less often against EBNA1, EBNA2, EBNA-LP and LMP1.

EBV was the first human virus implicated in oncogenesis and is associated with a heterogeneous group of malignant diseases, such as Burkitts lymphoma/Non-Hodgkin lymphoma, nasopharyngeal carcinoma, Hodgkin disease, hemophagocytic lymphohistiocytosis and lymphoproliferative diseases in immunocompromised individuals (recipients of transplants or HIV infected patients) (350). The current understanding implies that different latency programs are active in transformed cells in EBV-associated malignancies, correlating with the presumed phenotype of the originally transformed B cells. Latency III is activated in immunoblast-like cells from PTLD lesions, Latency II is found in EBV positive Hodgkins lymphomas which are believed to be of germinal center origin and Latency I is found in EBV positive Burkitt’s lymphomas that are phenotypically similar to memory B-cells and probably stem from a late germinal center reaction (349). As in PTLD lesions, Latency III proteins and EBERs are expressed in lymphoblastoid cell lines (LCLs) which are generated upon EBV infection in vitro (351).

1.4.2 EBV infection and PTLD development

After organ or SC transplant, especially in the early post-transplant period, patient T-cell function is deliberately compromised by immunosuppressive treatment regimens, which are used to counter prevent and treat rejection and GVHD. Moreover, this immunosuppression is often intensified when rejection/GVHD arise. This can lead to suppression of EBV-specific T-cell function and allow B-cells harbouring a latent EBV infection to escape T-cell surveillance (352).

In case of serological mismatch between the donor and recipient, the EBV-negative compartment gets infected. In a seronegative recipient receiving graft from a seropositive donor, EBV infection occurs at a median time of 6 weeks posttransplant as detected by EBV shedding in the throat. B-cells of a seronegative graft also get infected (353, 354). Also, an already seropositive recipient can get infected with another strain of EBV (355). UCB units are seronegative and PTLDs of donor origin in UCBTs have
PTLD is a heterogenous group of lymphoproliferative diseases, that occur after transplantation and is in many cases associated with EBV infection. The diagnosis of EBV-PTLD requires at least two of the following criteria: I) disruption of underlying cellular architecture by a lymphoproliferative process; II) presence of monoclonal or oligoclonal cell populations as revealed by cellular and/or viral markers; III) evidence of EBV infections in many of the cells by detection of EBV DNA, RNA or protein. Detection of EBV DNA in the blood is not sufficient for PTLD diagnosis. Histologically, 3 main categories can be defined: early lesions, polymorphic PTLD and monomorphic PTLD. The most of PTLDs are of B-cell origin. EBV genome is found in more than 90% of such B-cell PTLDs. In allo-HSCT patients PTLD most often occur during the first year post-transplant, when the patients are deeply immunocompromised. Recipients of solid organ transplants are at risk for PTLD throughout the lifetime due to the continous immunosuppression, however, the risk is at it’s peak during the first year. Late PTLDs, up to 10 years post solid organ transplant, have also been reported. Up to 45% of late-onset PTLDs presents as EBV-negative lymphoma, the role of EBV in carcinogenesis of such cases is uncertain.

Pathogenesis of PTLD and mechanisms of EBV action are not completely understood. As mentioned earlier, blasts in the most of B-cell PTLDs express Latency III program. It has been identified that LMP-1, EBNA-1, -2, -3, -5 and -6 are critical for transformation of B-cells in vitro, while LMP2A, LMP2B and EBNA4 are not. The cooperative activity of proteins deranges cellular pathways controlling growth and survival of B-cells. However, expression of the viral gene was reported to be heterogeneous both within and between the tumors, suggesting that full expression of Latency III may be required only in the initiation of PTLD. Also, in SCID mice, EBV mutants defective of lytic replication fail to promote induction of PTLD, indicating a possible role for lytic replication of EBV in PTLD. Also, circulating lytic-antigen specific CD8+ T-cells have been reported in organ transplant recipients with a history of PTLD (357). A majority of PTLDs are derived from B-cells of GC and post-GC origin. Cells from about half of PTLDs fails to express functional B-cell receptor. Anti-apoptotic EBV proteins have been implicated to rescue cells with crippling mutations of Ig genes, thereby expansion of such B-cells is assured. High number of infiltrating CD4+ T-cells in PTLD lesions and requirement of CD4s to induce PTLDs in mouse model support a role for CD4+ help in outgrowth of PTLD. In addition to EBV, genomic instability due to the defects in DNA repair mechanisms and mutations in the BCL-6 gene were implicated in tumorogenesis of PTLD (349, 351).

1.4.3 Clinical aspects of PTLD

Several definitions for different manifestations of EBV infection after allo-HSCT have been suggested (159, 350), these include: I) EBV DNA-emia - detection of EBV DNA in the blood; II). Primary EBV infection - EBV detected in a previously seronegative patient; III) probable EBV disease - significant end organ disease with high EBV DNA load in the blood, in the absence of other etiologic factors or established diseases; IV)
proven EBV disease (PTLD or other end-organ disease) – EBV detected in the tissue together with symptoms from the affected organ. Clinically, EBV infection can present as enteritis, hepatitis, encephalitis and fulminant hemophagocytosis. Chronic active EBV infection has also been described, where infectious mononucleosis-like symptoms are recurrent (350). These clinical manifestations, in contrast to PTLD, rarely cause significant morbidity.

PTLD frequently arises in extra-nodal sites. After allo-HSCT, PTLD is most often of donor origin and has more aggressive course than PTLD after organ transplantation. It is a serious complication of allo-HSCT associated with mortality in up to 85% of cases (358). Pulmonary involvement is associated with even worse prognosis (359). Incidences of EBV reactivation and PTLD after allo-HSCT range from 0.45% to 29% (359, 358, 360), depending on different types of donor and transplant protocols. The known risk factors for PTLD after allo-HSCT are HLA-mismatch, serological mismatch between the donor and recipient, T-cell depletion (either manipulation of the graft in vitro or as a part of conditioning regimen in vivo), splenectomy before transplant, presence of cGVHD (158, 358) and pre- or post-transplant usage of nucleoside analogs or alemtuzumab (anti-CD52) (361, 362). Among solid organ transplant patients, seronegative recipients are reported to be at the highest risk to develop PTLD (363).

In patients, EBV viral load is quantified in the peripheral blood by EBV DNA amplification with polymerase chain reaction PCR. In leukopenic allo-HSCT recipients, quantification in serum or plasma is considered to be the method of choice. Virus quantification can also be performed in lymphocytes and there are differences in the techniques, which has resulted in the usage of several different units of measurement. A system for conversion between different units has been proposed (364). In high risk patients it is recommended to monitor EBV load weekly up to 3 months post transplant. Still, a definite threshold value, associated with the development of PTLD, has not been defined (159, 350). Therefore, initiation of therapy in relation to viral load varies between different centers.

Interventions against EBV infection in a patient after allo-HSCT can be classified as prophylactic (asymptomatic patient, no EBV DNA load), preemptive (asymptomatic patient, EBV-DNA detected by screening) and therapeutic (treatment of the EBV related disease). Prophylactic approach is uncommon, since antiviral drugs, such as ganciclovir, foscarnet and cidofovir do not have any impact on PTLD development and are not recommended. B-cell depletion before the transplant or rituximab early after transplant have been suggested for patients with high EBV load (159, 365). It is also recommended to modify the transplant protocols (e.g. to reduce ATG dose) if this is possible (366).

The threshold values of EBV viral load for initiation of the preemptive therapy varies among different authors (from 1000 copies/10^5 cells to 10 000 copies/ml) (367, 368) (369). Weekly treatment with rituximab (anti-CD20 monoclonal antibody) at 375mg/m^2 doses is the most effective measure according to the current evidence. The number of doses is usually subject to local practice and is usually governed by the response (decrease in EBV-DNA load). Using this approach PTLD is prevented in up
to 90% of cases (350). However, downregulation of CD20 following rituximab treatment has been reported (325) and this poses a question if subsequent administration of rituximab can be considered as an effective approach. The drawback of this approach is the total B cell depletion, which lasts for 6-9 months, what results in even more profound immunosuppression (159, 350). According to the observations at our center, a number of patients (18%) can cope with the emerging substantial EBV viraemia without intervention (up to >1000 copies/ml blood, the highest spontaneously-cleared value of 39 000 copies (369 and not published data). This is important to consider when making a decision for an intervention when EBV-load is relatively low, since there is a risk for overtreatment and rituximab B-cell ablative usually last for 6-8 months.

Reduction or discontinuation of immunosuppressive therapy is recommended as another preemptive measure if the clinical situation allows. This potentially restores reactivity of the whole T-cell pool and has a positive effect on EBV control. When tapering immunosuppression, risks associated with rejection/GVHD have to be considered in preemptive setting; however, in case of therapeutical setting for PTLD the argumentation is different. EBV-specific CTLs as preemptive measure were also infused (326, 370) with reported responses of up to 94% (350). However, until recently, one of the major limitations for this procedure was the laborious and time-consuming laboratory approach.

The recommended first line measures for the treatment of EBV disease are the same ones as for the preemptive approach. The reported response rates to rituximab in this setting are up to 63% (350). It has been demonstrated, that sustained control of EBV infection is dependent on maintenance/restoration of T-cell immunity (371-373). This can be achieved by adoptive transfer of EBV-specific T-cells generated from the stem cell or third party donor (324, 370), with an overall response in up to 88% of cases when used for PTLD therapy). Infusion of total DLI for PTLD has been performed with an estimated response of 41%, however, total DLI harbours the risk for inducing GVHD. As a second line therapy, chemotheraphy or hidroxyurea monotherapy has been used with a low response rate and is not recommended by the current guidelines (350).

UCB transplant situation has a peculiar immunological status of the graft: low numbers of T-cells, most of them are naïve, the grafts are EBV seronegative, the engraftment is usually delayed and immune reconstitution is impaired. Therefore use of UCB raised major concerns regarding post-transplantation EBV infection and PTLD development (374-376). Interestingly, incidences of EBV-related events after UCBT with myeloablative conditioning have been demonstrated to be comparable (2- 3%) to the incidences reported from transplantations with other cell sources (268, 269, 377). A marked increase in the incidence of PTLD was reported among UCBT patients receiving RIC, especially the ones receiving ATG (21%). Having in mind these rates it is speculated that the remaining B cells of the patient might play an important role (269, 356).

In paper III of this thesis I present a method which was successfully applied at our center and successfully treated a therapy-resistant PTLD.
1.5 PROSTATE AND PROSTATE CANCER

1.5.1 The prostate

The prostate is an exocrine gland of the male reproductive system. Anatomically, the gland is located below the urinary bladder and in front of the rectum. Urethra passes through the anterior part of it. The base of the gland is connected cranially to the bladder neck and the apex of the gland caudally or inferiorly. Middle, right and left lateral lobes can be identified. Below the apical part of the prostate the urethra is surrounded by the external sphincter, which is important for the continence. Histologically, the secretory part of the tissue has an acinar structure, follicles open into canals which eventually join to form the excretory ducts. The gland can be divided into three zones: the peripheral zone, which constitutes about 70% of the glandular part of the prostate, the central zone which constitutes about 25% of the glandular prostate and the transitional zone (5%). Main function of the gland is production and storage of serous fluid, which constitutes about 70% of the ejaculate. The function and development of the prostate is androgen-dependent and dihydrotestosterone, the active metabolite of testosterone, is the main regulator (378).

In females, paraurethral (Skene’s) glands are considered to be the homologue of male prostate. The Skene's glands were found in 70% of the females examined at autopsy. These glands are considered to be the main source of PSA in females but are reported to be not as well differentiated and functional in a female adult as the prostate in the male (379, 380).

1.5.2 Prostate specific antigen (PSA)

PSA is a tissue kallikrein-related peptidase (KLK3). It is mostly expressed in prostate, and to a much lesser extent in mammary gland, nerve tissue, bone, bone marrow, salivary gland and muscle (381). PSA is the major effector molecule in semen liquidification, other functions are not known (381). In the end of the 80’s, PSA was introduced as a marker in screening for prostate cancer (PC) in the US. The intact basal membrane layer of the prostate epithelium prevents PSA from entering the bloodstream, and only small amounts of PSA leak out, keeping serum levels below 1ng/ml.

Serum PSA rises when the number of epithelial cell in prostate increases (as in most cases of benign prostate hyperplasia, BPH, and in PC) or when the epithelial barrier suffers a damage (prostate palpation, biopsy, inflammation, PC). Approximately 65-95% of PSA molecules form complexes with protease inhibitors in circulation. Benign lesions of the prostate present with increased ratio (%fPSA) of free (fPSa) to total (tPSA) (382). Therefore, evaluation of %fPSA has been applied in differentiation of BPH from PC.

1.5.3 Adenocarcinoma of the prostate
PC is the most frequently diagnosed cancer in Swedish males (at 35% of all cancer variants), incidence is around 9000 cases/year (383). Diagnostic methods used at various stages of PC diagnostics are PSA estimation in the blood, digital rectal examination, transrectal ultrasound, biopsy sampling, scintigraphy of the skeletal system and other radiological methods (CT, MRI, CT-PET). Two principal systems are used for classification of PC. Histological grading is performed according to the Gleason system (384). A pathologist assigns the primary (most common tumor pattern) and secondary (next most common tumor pattern) grade according to the criteria. Grades range from one to five (1-5), where 1 means a well preserved glandular structure and 5 means unrecognizable gland formation. The sum is noted by the pathologist (e.g. 4+3=7). Gleason scores correlate to prognosis and the natural course of PC after radical prostatectomy or irradiation (385).

Clinical staging is performed according to the international TNM (tumor / node / metastasis) system, where it is accounted for spread of the tumor outside the boundaries of the organ. In a simplified manner, T defines tumor involvement within the primary organ. T1 means non-palpable tumor which is most often detected incidentally; T2 tumors are palpable but localized within the prostate; T3 tumors extend through the capsule and may invade the seminal vesicles; T4 tumors invade adjacent structures. N accounts for involvement of lymph nodes and M - for distant metastases (386). Also, patients with localized PC can be grouped into low, intermediate and high risk groups according to level of PSA in the blood and Gleason score (387).

Treatment for localized prostate cancer is curatively intended where radical prostatectomy, full dose irradiation or a combination can be used (388). Unfortunately, around a third of these patients relapse (389) and consequently, among other measures, receive hormonal manipulation therapy. Patients with metastatic disease have a median survival of 2-3 years and 5-year survival is 20% (389). These patients also receive hormonal manipulation therapy. Such therapy aims to deprive the tumor of androgen support and by this to slow down progression. One way to achieve this is to administer androgen receptor antagonist (e.g. bicalutamide), which acts directly on cancer cells. Another alternative is castration (ceasing of androgen production) by surgical (bilateral orchidectomy) or pharmacological means. Pharmacological (chemical) castration is achieved by continuous administration of gonadotropine-releasing-hormone (GnRH) analogue (e.g. euproreline), which interferes with the physiological pulsative manner of GnRH action in the body and causes inhibition of sex steroid synthesis. When an antiandrogen is administered in combination with castration regimen, the approach is termed total hormonal blockade (387).

Hormonal manipulation has a strong suppressive effect on the cancer. However, it is not curative and patients are reported to eventually progress into a hormone-refractory prostate cancer (HRPC) state, meaning, that PSA levels start rising (increase in >25% over baseline and increase in the absolute PSA value of ≥5 ng/ml ) despite ongoing therapy. Patients with metastatic disease eventually progress to HRPC stage (390). This is a relatively long process since survival over 60 months is reported for HRPC patients without metastases and around 40 moths for HRPC patients with metastatic disease (390). Generally, many approaches are tried, but no effective treatments are available for these patients (391). Moreover, 20-30% of patients have metastastatic disease
already at time of PC diagnosis. However, this number has been decreasing during the latest years, most likely due to establishment of PC at earlier stage. As consequence, more younger patients are diagnosed with PC (390). Altogether, a large patient group with a disease status, which has no curative options, is in need of effective treatment measures.

In a study on allo-HSCT for advanced PC which is ongoing at our center, we aim to include PC patients who are in good clinical shape, have underwent debulking by surgery or radiation therapy, progressed to HRPC and preferably does not have metastasis, in particular bone metastasis. We believe that measures undertaken early enough for these patients can at least prolong their survival with HRPC due to the GVT effect on the tumor. Hopefully, with experience gained and further development of immunotherapy, we may aim for achieving a sustained control of malignant disease in combination with other treatment options.

1.5.4 Cancer immune escape and prostate cancer (PC)

According to immune surveillance hypothesis (392), cells with abnormal properties should be detected by the immune system. However, tumors still develop in immunocompetent hosts. A modern revision of immune surveillance theory emphasizes the role of “immune selection” (“immune editing”, “immune sculpting”), as an evolutionary pressure on tumor development. It is postulated, that outgrowth of tumor variants resistant for immune recognition occurs (“immune escape”), once the detectable malignant variants have been eliminated (393). Another theory explains tumor persistence by shifting focus on to shift the focus on tumor and its microenvironment – where tumor establishes a “state of tolerance” by influencing the surrounding systems with cytokines and other active compounds (394). None of these theories are fully accepted by the scientific community, but the main idea of a disabled autologous immune system at the time of cancer diagnosis is evident.

Several immune evasion mechanisms have been described in PC and other cancers. One of the best documented ways to evade T-cell recognition is defective expression/loss of HLA Class I molecules by prostate and other cancers (395, 396). Also, tumor cells often lack costimulatory molecules (397), and this can compromise successful T-cell activation. Effective antigen presentation and activation of cellular effectors can be further disturbed by affecting APC function. APCs were found to be functionally impaired in cancer patients, also among them PC patients were also examined (398, 399). Tumor-induced production of IL-10 was implicated as a possible mechanism in these observations (398, 400). It has been postulated, that a shift towards Th2 phenotype might induce immunosuppression and favour development of cancer (401). Elevated levels of IL-4, IL-6 and IL-10 were detected in PC patients (402, 403) and increased serum IL-6 correlates with poor prognosis (404). Also, this cytokine was reported to directly modulate prostate cancer cell growth (405). T-cells isolated from ascitis of a PC patient predominantly secreted Th2 cytokines (406). Conclusively, it seem that PC possesses a series of mechanisms, which can inhibit effective generation of adaptive immune response.
Following a recent interest in properties of regulatory T-cells, infiltration of CD4+ Tregs in tumor tissue and their specificity for tumor antigens was described. Also, increased levels of Tregs were reported in blood and cancer tissue of PC patients (407, 408). IL-10 and TGFβ, cytokines that are important in both induction and function of Tregs, have been detected in PC microenvironment (409, 410). This polarization towards Treg cytokine profile indicates that neoplastic tissue might be inducing / supporting immunosuppressive effectors of the adaptive immune system.

Prostate cancer can also cause local metabolic inhibition of T-cell proliferation by inducing defective L-arginine metabolism (411). Same might be true for induction of cyclooxygenase-2 driven prostaglandin E2 production, which may inhibit both innate and adaptive effectors of the immune system (412).

1.5.5 Adoptive immunotherapy for PC

Immunotherapeutical approaches for PC have primarily been associated with different vaccination strategies. A number of T-cell epitopes from prostate tumor associated antigens (TAA), have been identified and used in development of vaccines for PC (413). Initial approaches were based on reasoning that upon delivery of the antigen, autologous DCs will take up antigen and activate autologous T-cells. Antigen was delivered in different forms: as peptides, whole proteins, or cDNA coding for tumor proteins, and several modifications of vehicle were used. Also, autologous DCs pulsed in vitro were used. However, clinical trials have mainly reported the safety of such approach and transient clinical effects, which were not durable (414). The identified TAA s are poorly immunogenic. Therefore, the need for targeting the costimulatory and cytokine pathways together with activation through antigen-specific pathway was identified (413, 415). Main costimulatory molecules targeted in prostate cancer therapies are B7.1 (CD80), ICAM-1 and LFA-3 (416), as well as CTLA-4 pathway, which has a role in negative regulation of T-cell proliferation (417). Also, cytokines have an obviously important role in T-cell activation. Therefore, GM-CSF is a frequently used adjuvant, and it was shown to induce growth, maturation and lymph node trafficking of DCs and to enchaunc T-cell responses (418). IL-2, besides it's direct immunotherapeutic effects (419) has also been used as adjuvant in vaccine therapy (420).

Different ways of delivery of signals to the immune system have been explored. Currently, several methods have prevailed and are tested in clinical trials. One approach uses allogeneic irradiated cancer cell lines (LNCaP, PC-3), transfected with GM-CSF, product name GVAX (421). Multiple antigens are targeted with this approach and production of vaccine is less costly. Phase III trials were initiated in patients with metastatic PC, but had to be terminated because of negative interim analysis in one and increased treatment-arm mortality in the other (422). Another vaccine contains 3 different irradiated cell lines (LNCaP, P4E6, OnyCap-23), product name ONY-P1. Here, phase II trials are ongoing, notably, in patients at an earlier stage of the disease (423).
From the approaches where autologous DCs are employed, GM-CSF and prostate acid phosphatase (PAP) recombinant fusion protein is used to pulse DCs \textit{ex vivo}, product name Sipuleucel-T. In a phase III randomized, double blind, placebo-controlled multicenter study, a significant survival advantage was demonstrated, 21.7 vs 25.8 months. It is the first trial in the field of autologous vaccination therapy of cancer demonstrating positive evidence and further studies are under evaluation for approval by the FDA (422).

Vaccinations of PC patients are also carried out with the use of attenuated vaccinia viruses as vectors for TAA and costimulatory molecule genes. Initially, hopes were raised that vaccinia-entry itself would boost the immune response. However, even if the immunostimulation could be detected, no effective responses against TAA's could be generated, since neutralizing antibodies formed against the vector upon vaccinations (424). Therefore, a protocol has been developed, where the patient is first primed by vaccinia virus and later on the genes are delivered with a replication-defective poxvirus. PSA and B7.1, ICAM-1, LFA-3 genes have been transferred with this method in Phase-II trials, where survival benefit was demonstrated (423).

Vaccination strategies are also combined with other treatment methods used in onco-urology, where radiation (425), hormonal blockade (426) and docetaxel (427) are implicated to have adjuvant effects. Antibody against CTLA-4, ipilimumab, has been employed in several trials in PC patients. CTLA-4 is expressed on T-cells and was shown to influence T-cell proliferation in negative fashion when crosslinked by CD80/CD86. However, the mechanism of CTLA-4 function is not completely defined, e.g. it was found to induce Tregs in some studies (428, 429). Adverse events such as adrenal insufficiency, hepatitis and autoimmune colitis were observed upon administration of the antibody, indicating importance of the targeted molecule in immune regulation. Ipilimumab has been used in monotherapy, in combination with GM-CSF (430) and GVAX vaccine (422). The studies reported observed positive changes in PSA doubling time and radiological responses; still, no major survival benefit could be demonstrated.

To summarize, the latest studies carried out in the field of autologous adoptive immunotherapy for PC demonstrate that it is important to recognize benefits delivered by costimulatory signalling in T-cell activation. Durability of the responses has still to be proven, and it is questionable if these approaches will be powerful enough to overcome the tolerization, which is present in the cancer situation. These findings may also be important in adoptive immunotherapy after allo-HSCT as complimentary strategies.
2 AIMS

The studies presented in this thesis were initiated in response to observations and questions raised from daily clinical practice. The general aim of the thesis was to facilitate the translational collaboration between the fields of laboratory immunology and clinical stem cell transplantation, and to implement the findings into the clinical practice, in order to benefit the patients.

More specifically, we aimed to:

- Compare the outcomes between two alternative graft sources, which can be used when no suitable donor can be found: the MMURD versus unrelated UCB;
- To develop a protocol for expansion of UCB T-cells, that could be used for donor lymphocyte infusion after UCBT, and to characterize their phenotype and function;
- To develop an alternative protocol for rapid acquisition of EBV CTLs for treatment of EBV-PTLD;
- To analyze the response against prostate antigens after allo-HSCT for prostate cancer.
3 RESULTS AND DISCUSSION

3.1 OUTCOMES AFTER UCBT AND MM URD TRANSPLANTS (PAPER I)

UCBT has evolved as an alternative graft source for patients where no HLA identical sibling or MUD is available. The first study in this thesis was a retrospective analysis of outcomes in 41 patients admitted to our institution between 1992 and 2006, where search for a donor could not come up with a sibling or M U donor. In our patient group, the MM URD was a historically older “second” choice, before CB became more accepted. Results were discouraging using MM URD, but it has to be taken into account that transplant procedures, management of complications, and supportive care have advanced significantly during this period. The time effect has also been reported when analyzing outcomes after cord blood transplantation, where poorer results before 1998 have been described (431). It has been speculated that improvement in the technique of HLA typing could be one of the reasons, as well as the general progress in the field (243, 432).

Fourteen patients in our study were grafted from MM URD and 27 from UCB. Both pediatric and adult patients were included, mostly the ones with acute leukemia (52% in the CB group and 50% in the MM URD group) and with non-malignant diseases (up to 30% in both groups). There was no difference between the two groups in terms of diagnosis. A pediatric patient group is more likely to receive an UCB graft because of low weight. Adults are generally more likely to have co-morbidities at the time of transplant and their outcomes after transplant are usually associated with higher risks. In the context of cord blood, they are more likely to have a weight disadvantage, which influences the choice of the graft. Indeed, 9 double UCBTs were included in our study in order to achieve a suitable cell dose of > 2 × 10^7 TNC/kg. The differences listed are also of importance when evaluating the results of our study. Regarding cell dose requirements, the recommendations have changed over time as data from larger studies have become available (see Introduction). The dose indicated was relevant according to the recommendations at the time of intervention.

URD grafts were 5/6 HLA-matched (HLA-A, -B, -DRß1) with most mismatches at Class I antigens (in 11 out of 14). All HLA typings were of high resolution. Majority of URD grafts were BM (13 out of 14) and T-cell depleted (11 out of 14). As for UCB, one patient received a matched graft, another one received a graft with 2 allelic mismatches and rest of the patients have received grafts with antigen-level mismatches. From HLA-antigen mismatched UCB grafts, 56% (14 of 25) were 1 antigen mismatched and the rest - 2 antigen mismatched (12 of 25). Additional allele mismatches were present in both 1 (8 of 14) and 2 (6 of 12) antigen mismatch groups. In the MM URD group the majority of the patients received radiation-based myeloablative conditioning, whereas RIC was used for 26% of UCBT recipients. GVHD prophylaxis was mostly CsA + prednisolone for UCB and CsA +MTX for MM URD group.
Analysis of the clinical outcomes in our study confirmed a delayed neutrophil and platelet engraftment in the UCB group, which supports earlier reports (119, 130, 243).

A significant OS advantage for UCBT group could be detected. TRM was lower for all patients in the UCB group. For patients transplanted for a malignancy, relapse incidence and relapse-free survival (RFS) was lower using UCB, although these differences were not statistically significant. There were no differences in incidence of either acute or chronic GVHD.

A large Eurocord study has compared recipients of T-cell depleted BM grafts and mismatched UCBT (204). They reported similar cumulative incidences of GVHD between the groups, although this was somewhat higher in the UCBT group concerning grade IV GVHD. In multivariate analysis with adjustment for prognostic factors, the risk of cGVHD was increased for the UCBT group. Early TRM was increased for UCBT recipients while relapse, EFS, and 2-year OS were comparable. Our results are more in favor of the UCBT group compared to those of the Eurocord study; however, the patient population was more homogenous in the latter with only pediatric patients with malignancies included. Outcomes are different in patients with malignant and non-malignant disorders, and this must be taken into account when interpreting results (433). The survival data are more favourable after UCBT for non-malignant disorders (434, 435) and this might have influenced the OS figures in our study. More detailed comparisons were difficult to achieve due to low patient numbers.

Another large single-center study from the Minnesota group has compared outcomes for ALL patients, both pediatric and adult, receiving different types of grafts (436). MM URD transplants, with the majority of grafts being T-cell depleted, showed poor survival, mainly resulting from increased TRM. In addition, LFS was also lowest in this group. UCB was superior to MUD and RD transplants regarding these parameters. Risk of aGVHD was higher both in the MM URD and UCB groups compared to MUD and RD transplants.

Initial reports of UCBT outcomes gave more optimistic data regarding cumulative incidence and risk of GVHD development (243, 254, 256, 437). This was attributed to the immunological naivety of the graft. However, several recent studies have shown GVHD risks to be similar or even elevated when UCBT is compared to matched donor transplants (119, 130, 255, 436, 438). Still, most importantly, this does not seem to compromise EFS and OS of patients receiving CB grafts and trends towards improved survival can be seen in data from centers with substantial experience of UCBT. The GVT effect appears to be maintained, with RFS being comparable to that of MUD and matched RD transplantations.

In our study, EBV PTLD has developed in 2 patients from each group. The numbers are too low to draw any conclusions, it was reported by other studies that HLA-mismatch and UCBT with use of ATG are risk factors for PTLD development (158, 439). Interestingly, 4 patients included in the MM URD in this analysis have received prophylactic EBV CTLs (326). This could indirectly indicate that MM URD patients were at higher risk for PTLD development, but again, the possible differences in transplant procedures due to the time factor has to be kept in mind. Still, if PTLD arises
and rituximab fails, no effective treatment is available. Moreover, donor lymphocytes are not available post-transplant in case of UCBT. In paper III of this study we describe a transfer of EBV-specific CTLs from a haploidentical donor to a patient transplanted from UCB. These CTLs were acquired with a use of a more rapid and less laborious approach, when compared to the conventional methods, and represent a strategy, which could be employed in the future.

We have evaluated chimerism data in our patients. Analysis of donor chimerism in CD3/CD19/CD33 lineages did not reveal statistically significant advantage for UCBT group, however, a trend towards more complete donor chimerism could be seen, especially when evaluating the CD3 lineage. The low number of patients has most probably limited the statistical power in this case. In other studies, complete donor chimerism was reported in 50 – 94% of surviving patients after UCBT (204, 440, 441).

To summarize, this retrospective analysis of outcomes for patients lacking a “gold standard” donor at our institution advocates selection of a mismatched UCB unit rather than a MM URD. This is in line with the observations of others (119, 204, 440, 441). However, it is possible that the results using MM URD could be better with improved supportive care and add-back of donor T-cells after HSCT in patient without signs of GVHD. Moreover, CBTs have several specific disadvantages that could not be addressed in our study, mainly due to the heterogeneity of the material and low number of patients. Large comparative studies have shown that in the case of malignancy, risk of relapse is considerable and relapse is one of the main causes or non-TRM related deaths. Also, rejection can be another obstacle (119, 204, 243, 254, 255). These two complications of the transplant procedure are also present in transplants from other graft sources. There, the donor is available and DLI can be performed, with varying rate of success according to the diagnosis (192, 313-315). DLI has been a challenge for UCBT, since no donor is available. We have addressed this limitation further in the next study in this thesis by establishing a clinically feasible protocol of T-cell expansion from CB graft aliquots, and we characterized the expanded T-cells.

### 3.2 EXPANSION OF UCB T-CELLS FOR USE AS DLI (PAPER II)

One of the obstacles present after UCBT is the subsequent lack of access to the donor and thereby to the possibility of collecting and administrating donor lymphocytes infusions (DLI) to the patient. Both bulk DLI and manipulated products after selection in vitro have been successful used in therapeutic and prophylactic approaches to counterfeet malignant relapse, engraftment failure and infectious complications post-transplant (see introduction for details). The possibility of access to cellular therapy after UCBT is important for the further development of this SCT strategy, since relapse occurs at comparable rates as for transplants from other graft sources and antiviral immunity seems to be impaired (130, 243, 267, 269).

In this study, we have evaluated a clinically adapted protocol of T-cell expansion from the UCB grafts. A fraction of 5% of the TNC content was allocated for expansion. After this reduction of cellular content of the graft, cell doses were still within required ranges. T-cells were positively selected from the CB samples with help of superparamagnetic polystyrene beads covalently coupled to monoclonal mouse anti-human
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Figure 5. Cord blood T-cell expansion for donor lymphocyte infusion

Thaw, quality control and infuse when needed

Cyropreserve in escalating DLI doses

Remove CD3/28 beads & bead bound cells then discard

Culture at 0.3 x 10⁶ cells/ml for 7–11 days, r IL-2 200 IU/ml

Remove magnet

Positively selected CD3+ cells

Expose suspension to magnet, remove non-selected cells

Cyropreserve CD3-negative cells

Labeling with αCD28 αCD3 Paramagnetic bead CD28 CD3

5% of volume removed for expansion at thaw

Cord blood unit

Patient

Transplantation

5% of volume removed for expansion at thaw

Labeling with CD3/28 beads

Cord blood unit

Figure 5. Cord blood T-cell expansion for donor lymphocyte infusion
antibodies against CD3 and CD28 (ClinExVivo, Invitrogen Dynal). This tool, which can be viewed as an “artificial APC”, delivers both an activating signal via the CD3/TCR complex and co-stimulation via CD28 crosslinking. See Figure 5 for an overview of the procedure. Additional co-stimulation, in comparison with CD3 stimulation only, has been shown to enhance T-cell proliferation and cytokine production (442, 443). Together with addition of recombinant human IL-2 (rhIL-2), CD3/28 stimulation efficiently induces proliferation of CD3+ cells (444, 445) and has earlier been used to expand T-cells from peripheral blood for immunotherapeutic approaches both in autologous and allogeneic setting (446-449).

T-cells expansion from UCB for DLI have recently been studied by others, either employing CD3/28 costimulation and rhIL-2 (450, 451), CD3 crosslinking by adsorbed OKT-3 and rhIL-2 (452, 453) or different cytokine combinations (454-456). The CD3/28-bead and rh-IL2 approach is the most feasible and cost-effective, since the availability of GMP-grade cytokines, which were proposed in these studies, is limited. In our setting, cells have expanded to a median of 150 - fold, yielding sufficient amounts for cryopreservation of DLI doses in duplicates for an escalating dose protocol in all cases. Other studies have reported lower expansion rates (450, 451). Judging from the protocol descriptions, we have kept cells at lower concentration (0.3x10⁶ cells/ml vs. ~1x10⁶ cells/ml) when compared to other studies and it is possible to speculate that this is one of the factors that has allowed for a more efficient expansion.

In vitro manipulation is associated with risks of skewing the phenotypic and functional status of the cells. Throughout our evaluation of the expanded UCB T-cells, we have compared them to peripheral blood mononuclear cells (PBMCs) from healthy adult donors and ex vivo acquired CB mononuclear cells (CBMCs). PBMCs represent the current clinical modality of DLI. The ideal control to represent the unmanipulated UCB in our experiments would have been an aliquot from the same UCB graft, which was used to initiate the expansion. We could not obtain such aliquots since grafts were used for clinical transplantations. Therefore, CB acquired ex vivo from caesarean deliveries was our second best option.

We could not detect expansion of NK-cells or B-cells in our experiments and T-cells were almost exclusively TCRαβ. The CD4/CD8 ratio was slightly skewed in favour of CD8 cells, which is consistent with data of Mazur et al, who have attributed the skewing to more CD8+ cells being in active phases of cell cycle and increased apoptosis in the CD4+ compartment (450). However, others have reported maintained CD4/CD8 ratio post-expansion both in cases of CB (451) and PB expansions (449, 457). Interestingly, the number of CD4+CD8+ double positive cells increased significantly post-expansion in our cultures. Elevated levels of such cells have earlier been reported in autoimmune conditions such as myasthenia gravis, systemic sclerosis, as well as in breast cancer effusions (458-460). These T-cells were reported to be of effector memory phenotype and producers of IL-5 and IL-13. However, it seems that more data is needed for establishment of a more definite role for such cells.

The expanded CB T-cells were in a more activated status compared to controls, as indicated by increased expression of CD69 and CD25, which are markers of
lymphocyte activation (461, 462). When co-staining for CD25 and FoxP3, increase in the frequency of FoxP3+ cells of expanded CB cells was not significant, although more evident in CD8+ T-cells. It has to be acknowledged, that CD3/28 stimulation in presence of IL-2 has been efficiently employed to expand T-regulatory cells from UCB (463), however the difference is that the starting population in that case is purified to be CD4+CD25+. Earlier, CD8+FoxP3+ cells have been described in human tissues and attributed suppressive properties, however the significance of these findings is still unclear (464, 465). Moreover, T-cells can transiently up regulate FoxP3 when activated (466, 467) and only further commitment, such as the demethylation state of the FoxP3 promoter seems to be required for stable suppressive function (468). We cannot exclude the possibility of expanding regulatory T-cells as a part of the final product, since we have not assessed cytokine production or functional properties of just these cells, but a more extensive evaluation is currently ongoing.

Peripheral CD8+ T-cells can be grouped on basis of expression of CD45 splice variants CD45RA and CD45RO and adhesion molecules CD62L (L-selectin) and/or CCR7. The expression pattern of these molecules associate with differences in the functional status of T-cells (19, 469-471). T-cells were defined as naïve (Tn, CCR7+CD45RO-), central memory (Tcm, CCR7+CD45RO+) effector memory (Tem, CCR7-CD45RO+) and terminally differentiated effectors (Ttde, CCR7-CD45RO-). In the expanded CB T-cell pool, fractions of Tcm and Tem increased significantly. Expansion of memory subsets was also reported by others (451), however, in those studies a substantial fraction of naïve and terminally differentiated CD8+ T-cells was still present after expansion. While the definition of the subsets differed between the studies this could explain the differences.

CB grafts are generally considered to be “naïve” or “immature”. In the clinical community this naivety is often directly associated with reports on high content of CD45RA+ cells, lower production of proinflammatory cytokines upon stimulation and a shift towards a Th2 cytokine profile (472-474). Indeed, we found CB ex vivo to produce low amounts of IL-2, TNFα and IFNγ. Interestingly, this could be reversed by the expansion procedure. Our data is confirmed by earlier reports where CB T-cells were demonstrated to respond with increased IFNγ production to CD3/28 activation in presence of IL-12, whereas presence of IL-4 shifted CB-cells towards a Th2 phenotype (475).

CB T-cells acquired ex vivo were reported to proliferate in vitro upon stimulation with allergens, infectious agents and self-antigens (476). Also, proliferative responses to alloantigens were reported at a level comparable to that of PB cells (474, 477), suggesting that an alloreactive capacity is present in CB T-cell pool, despite their naivety, as demonstrated by CD45RA expression. Our results support these findings, since we have detected a considerable response in MLCs with T-cells from ex vivo acquired CB. The expansion procedure seems not to have activated cells much more in this sense. On the other hand, Mazur et al have reported a poor cytotoxic response and deficient cytolytic machinery in expanded CB T-cells, which could be a compromising ability for such cells to be able to act in vivo (450). Still, recent reports on generation of functional T-cells from naïve CB, which were specific for viral antigens, stand in support of the functional capacities present in CB T-cell repertoires (478). As
demonstrated by spectratyping data from our study and that of (451), clonality is preserved after the expansion procedure. Altogether, it’s tempting to speculate that expanded cord blood T-cells can be expected to be able to demonstrate antiviral and allogeneic responses upon adoptive transfer.

Considering the low number of T-cells present in CB graft and delayed engraftment period, it is possible to advocate for prophylactic infusions of such expanded cells for high-risk patients, to treat complicated viral infections or malignant relapse. The lymphopenic environment could contribute to homeostatic expansion of the transferred cells. Still, it must be admitted, that the relatively lymphodepleted state after CB transplantation may contribute to reduced GVHD, since priming of effectors occurs at a lesser rate. Corroboration of this state may only be justifiable for high-risk patients. So far, one case of use of expanded CB DLI in patients has been reported, but the expansion procedure differed considerably (479). We have administered expanded CB T-cells for 3 high-risk patients without any side effects. In 1 patient we have observed a positive change in chimeric pattern. Further studies are ongoing at our institution to confirm clinical effects of adoptively transferred expanded CB T-cells.

To conclude, our results confirm feasibility of T-cell expansion from CB grafts used for patient transplantation and suggest that the functional capacity of expanded T-cells is preserved. Expanded CB T-cells exhibited comparable responses to PB and CB \textit{ex vivo} controls in functional assays. However, the Tem pool increased substantially after expansion, which may mean that such cells will have shorter persistence when transferred \textit{in vivo}. Approaches to modify cell culture conditions in order to counter this problem are on the way at our center.

3.3 ACQUISITION OF EBV-SPECIFIC T-CELLS FOR PTLD (PAPER III)

Recipients of allo-HSCT are at risk of infectious complications following transplant. Reactivation of latent viral infections is common early after transplantation due to the profound immunosuppression, especially of T-cell functions. If a rise in the EBV DNA load in the peripheral blood is detected or PTLD is diagnosed, reduction of immunosuppression and administration of rituximab are the first-line measures. However, when these approaches fail, no effective second-line treatment exists. In UCBT, the risk of EBV-PTLD is markedly increased when ATG is used in the conditioning regimens (269). In the third paper of this thesis, we describe a novel approach that was developed because of the need for a remedy for a patient who developed EBV-PTLD after UCBT.

EBV-specific T-cells are of utmost importance in controlling EBV infection, preventing PTLD development, and in mediating clearance of transformed cells (371-373). Administration of EBV-specific CTLs to allo-HSCT patients, for both preemptive and therapeutic purposes, has proven to be successful (323, 326, 366). However, assured availability of EBV-specific T-cells for adoptive therapy has been limited until recently, since the generation process is time consuming (at least 6-8 weeks) and therefore not feasible in patients with fulminant PTLD. Moreover, in the
UCBT setting the donor is not available. We circumvented these limitations by adopting a rapid HLA-multimer based EBV-specific cell isolation method from a haplo-identical donor.

The patient described in paper II was classified as a high-risk individual at admission for transplantation, due to her previous medical history. She had AML in complete remission (CR) 4, was heavily pretreated, and had received an autologous HSCT earlier. No matched URD could be found and she was grafted from a single UCB unit. She was EBV-seropositive before transplantation and an EBV load of < 500 DNA copies/ml was first detected on day +50 post-transplant. The engraftment was delayed up to 1 month post-transplant, she was lymphopenic for a prolonged period, and received G-CSF several times. On day 83, she was admitted due to fever and an EBV DNA load of 2000 copies/ml was detected. Lesions in the lungs, liver, adrenal gland, and kidneys were verified by computed tomography (CT). EBV-positive PTLD was verified in lung and liver biopsies. Despite three doses of rituximab, progression was noted radiologically. EBV PTLD with lung involvement is associated with mortality rate of > 90% (359). The patient has rapidly deteriorated during a 3-week period and was in a poor status at the time of adoptive transfer, requiring oxygen administration and suffering from hectic fever.

Since the patient was grafted from CB, no access to the donor was available. Thus, we considered her mother, who was HLA-A2 positive and mismatched on 1 HLA-B, 1 HLA-C, and 1 HLA-DPB1 antigen against recipient and on 2 HLA-B and 1 HLA-DRB1 against CB graft, as a possible donor for EBV CTL therapy. We found that up to 1.9% of all CD8+ T-cells in peripheral blood of the mother were specific for HLA-A2 pentamer, assembled with the GLC peptide from EBV (derived from E protein BMLF1). We used a pentamer assembled with a peptide from a protein expressed in the lytic cycle of EBV for practical reasons, when the aggressive clinical course warranted sudden action.

Transformed cells from PTLD lesions are commonly thought to express mostly latency III program gene products (480). Expression of lytic cycle antigens has been difficult to detect in tumor tissue due to the lack of specific antibodies, and therefore the role of lytic antigens in PTLD was historically considered to be of less importance (481-483). However, there have been reports indicating the opposite. The presence of CTLs specific for RAK peptide from lytic BZLF1 EBV protein in organ transplant recipients with PTLD and their expansion in peripheral blood after reduction of immunosuppression has been demonstrated (357). Expression of lytic EBV protein BZLF1 has recently been documented from PTLD tissue in a patient after allo-HSCT (484) and mRNA of another lytic protein BXLF-1 was detected in 8 out of 8 PTLDs (480). Also, a heterogeneous state of PTLD tumors (meaning that more than one malignancy was present at the same anatomical site of PTLD) has been reported, which may indicate that different states of replication can be present at the same time (485). These reports indicated the possible importance of lytic cycle antigens in PTLD and thus supported our approach of using GLC-pentamer for our patient. Recently, data were published from a study where EBV CTLs specific for multiple latent and lytic epitopes were adoptively transferred and subsequently recovered from patients for
Figure 6. Selection of EBV-specific T-cells

- Leukapheresis product
- PBMC separation by gradient centrifugation
- Labeling with HLA-pentamer containing peptide from EBV
- Antibody against fluorochrome
- Paramagnetic bead
- Coupling pentamer-T-cell complexes to paramagnetic beads

- Add cells to separation column, expose to magnet
- Non-selected lymphocytes discarded
- Positively selected cells
- Remove magnet
- Quality control
  - Wash
  - Analyze
  - Count

- Infuse to the patient
- Donor
functional evaluation. T-cell responses to lytic cycle antigens were also confirmed in that study (331).

The EBV-specific CTLs were selected from PBMCs, adopting a method reported by Cobbold et al. (337). For an overview, see Figure 6. For the first adoptive transfer, T-cells were selected by GLC-HLA-A2 tetramers. When selecting the cells for the second time, besides GLC-pentamer, we could include a pentamer assembled with CLG peptide from a latent protein (LMP2). Multimers were conjugated to allophycocyanin (APC). PBMCs were first incubated with multimers; later on, paramagnetic microbeads coupled to anti-APC were added and finally magnetic selection was performed. The preparations were checked for purity, sampled for microbiology tests, and infused into the patient. Multimer-positive cells constituted > 60% of the infused cells on both occasions. We did not analyze the non-multimer fraction, mainly due to the very low cell numbers. From other reports, it is evident that phagocytes and antigen presenting cells can be present in this fraction (331).

Three doses of rituximab were administered to the patient, the last one 2 weeks before CTL transfer. From the literature currently available, the effect of Rituximab is expected to be apparent within one week (331). In our patient, we could observe a steep increase in EBV load, worsening of the condition, and radiological progression of the lesions despite rituximab treatment. However, a late effect of rituximab could still have contributed to the observed treatment effect. Still, several studies have demonstrated that recovery of T-cell function is the most important component in clearance of PTLD and maintenance of disease-free status (371-373). The immunosuppression was discontinued 2 days before the CTL infusion, and this may have potentiated the effect of the CTLs, which could have been present in the patient before transfer and were not detected by HLA-A2 tetramer. Still, at the time of the second CTL infusion, the patient had been off immunosuppressive treatment for several months and no rituximab was administered prior to transfer of CTLs. We again observed a clinical response and clearance of EBV load together with persistence of the third-party donor T-cells. This strongly suggests that adoptively transferred third-party CTLs mediated the response to EBV. The patient is alive and well 16 months after transplantation, and has returned to school. At 14 months post transplant, she developed a transient increase in EBV load over a period of 2 weeks, from 2,500 copies/ml to 10,000 copies/ml, with very limited cervical adenopathy. She developed fever and was admitted to the ward with abdominal pain. CT scans of head, neck, thorax, and abdomen were negative; however, blood cultures revealed gram-positive bacteria. The patient improved quickly when given intravenous antibiotics, and was discharged. Regarding increased EBV load, no action was taken and the viral load had decreased spontaneously to 2,600 copies/ml, as of late March 2010. This transient viremia and spontaneous decrease in viral load could indicate an effect of persisting adoptively transferred T-cells. Her PBMCs, sampled during the period described, will be analyzed for immune reconstitution.

The main advantage of this EBV-CTL acquisition procedure is that it is a rapid and relatively low-cost T-cell selection method. This is an important improvement in comparison to approaches used in earlier studies. In the very first trials, total unmanipulated DLI was administered for the treatment of PTLD; however, the risk of GVHD was substantial (486). Later on, polyclonal and clonal T-cell cultures, derived in
vitro by restimulation with EBV-LCLs, were sucessfully administered both in therapeutic and prophylactic settings (323-326, 487). However, at least 6 to 8 weeks are needed to obtain the cultures by restimulation. In a rapidly progressing PTLD such an amount of time is not usually available; also, costs are high in the GMP setting. However, multimeric selection procedures also have drawbacks. The greatest one is the limited availability of identified stable peptide:multimer complexes over different HLA types. It is also important to consider that infusion of clonal populations might promote tumor escape, as hinted by previous reports. (488). Thus, several multimers should be considered for selection at the same time when available, in order to mimic transfer of oligoclonal/polyclonal cultures. Another way of addressing such limitations of HLA and epitope restriction was recently presented by Moosmann et al., where 23 different immunodominant peptides spanning several HLAs were used in a rapid isolation protocol employing IFN-γ-capture (331).

The use of multimers for routine CTL isolation might be problematic due to the high binding avidity between the multimer and the T-cell. This high avidity facilitates effective binding and selection, but some reports have demonstrated that such crosslinking of the TCR activates T-cells and can drive them into activation-induced cell death (51, 56). Such effects would not be beneficial in the case of isolation of specific T-cells. A possible solution to the problem has arisen in the form of a novel multimer assembly approach, the AviTag multimer, which might reduce the rate of cell death. Increased physical distance between crosslinked TCRs and subsequently reduced T-cell activation were implicated to be responsible for the effect on apoptosis (58). Even so, this approach still has to be confirmed and valuated clinically.

Due to the lack of material, we could not perform follow-up studies on immune reconstitution after adoptive cell transfer to the patient. Such studies are currently being performed in a cohort of patients who have received CTLs selected using the same protocol. However, we could perform chimerism analysis, which provides an important proof of persistence and expansion of haploidentical CTLs.

Despite the relatively high degree of HLA mismatch between the third-party donor and cord blood graft (the patient has always been 100% donor chimera since day 28), the infused cells were not rejected. From the available reports of adoptive immunotherapy, it is clear that persistence of transferred T-cells differs considerably between tumor-specific T-cells (489, 490) and virus-specific T-cells (335, 337, 487), favoring the latter. There could be several explanations; one addresses the fact that virus-specific CTLs are more often transferred into a lymphopenic environment. It might be speculated that in a lymphopenic state, there is less competition for “survival” cytokines IL-15 and IL-7, the lymphoid niche is not fully populated, and Tregs may be absent. The significance of lymphodepletion for the survival of adoptively transferred T-cells is evident from studies by Rosenberg et al. with melanoma patients in an autologous setting, (491, 492) where induced lymphodepletion was found to be associated with an increase in IL-7 and IL-15 levels. Another explanation for persistence of third-party haploidentical cells in our patient may be suggested by reports on promotion of tolerance by maternal microchimerism (493, 494). However, we have not evaluated such a possibility.
One of the most important findings in our study was the demonstration of engraftment, persistence, and expansion of the third-party CTLs. We could demonstrate this due to the fact that the CTL donor was third-party and disparities could be identified using short tandem repeat typing. In other studies, where specific T-cells were derived from the original HSC donor, T-cells were isolated from the patients after transfer and functional studies were performed, which demonstrated expansion and reactivity attributable to specific T-cells that had been transferred (331, 337).

In summary, our results support the introduction of a novel approach for rapidly selecting EBV-specific T-cells for adoptive transfer into patients with EBV-PTLD. This approach could be extended to patients suffering from adenovirus and herpes virus infections, if multimeric complexes with high-avidity antigens derived from the proteins of these pathogens were available. Another interesting possibility is to introduce such a selection method subsequent to the expansion of CB T-cells, as described in paper II. The presence of T-cells with appropriate specificities in ex vivo cord blood has been demonstrated by studies where “tri-virus specific” CTLs could be raised from CB upon in vitro restimulation (478). CD3/28 T-cell expansion does not appear to alter the T-cell repertoire, as indicated by paper II and (451). Thus, we will explore such a possibility in subsequent studies at our center.

3.4 PSA-SPECIFIC T-CELLS AFTER ALLO-HSCT FOR PROSTATE CANCER (PAPER IV)

Accumulation of proofs on the GVL effect against hematological malignancies and identification of T-cells, as main effectors for this alloreaction, led to the further extension of indications for allo-HSCT. Intentional allo-HSCT studies for solid tumor patients started in the late 90’s. Developments were further facilitated by development of RIC regimens, which reduced TRM for this patient population. (495-497).

Prostate cancer, the most common cancer in males, is obviously underrepresented among the recipients of allo-HSCT for solid tumors (274)(Bregni BMT 2006). Still, it is the most common cancer in males of the Western world and a considerable fraction of young patients progress into more advanced disease (498). An allo-HSCT program for prostate cancer patients has been initiated at our center. With experiences from earlier trials, great amount of attention has been paid on identifying the patients with good performance status and with as low tumor burden as possible.

The 52-year old patient which has been analyzed in this study presented with an inoperable advanced stage (T4, Gleason score 9) tumor and retroperitoneal metastasis. He responded to initially administrated total androgen deprivation therapy with androgen receptor antagonist bicalutamide and GnRH analogue leuprotein; the latter was continued during the post-transplant period.

He was later grafted from his HLA-identical sister in March 2003 and, due to mixed chimerism, received two doses of DLI, to which he responded with conversion into full donor chimera and grade II GVHD. At 1 year post-transplant he was diagnosed with extensive cGVHD.
In autumn 2006, at 3.5 years after transplant, his serum PSA levels started to increase, although dynamics were occurring under the normal threshold level. In June 2007 prostate cancer was revealed in one of four biopsies, Gleason 3+3. In order to increase the GVT effect, the patient received five doses of donor T-cells collected from the patient’s peripheral blood and injected directly into the prostate. This seemed to have a transient effect and the patient showed stable disease until the end of 2009 when the prostate cancer started to progress with abdominal metastasis. He is now receiving palliative therapy.

We have set out to analyze the possible specific reactivity of CD8+ T-cells against prostate tissue in the patient. First, we identified potential HLA-A2 restricted peptides derived from prostate antigens PSA, PSMA, PAP. The peptides have either been previously described in the literature, or determined by epitope prediction algorithms. Further, we tested the ability of these peptides to stabilize HLA-A2 molecules on TAP-deficient T2 cells. Four candidate peptides could stabilize HLA-A2 to sufficient levels and were further incorporated into multimers with HLA-A2.

Relatively high frequencies of CD8+ T-cells, which reacted with pentamers containing peptides P9 and P10 (both from overlapping region of PSA), could be detected in peripheral blood of the patient over a prolonged period of time. Further, such cells could be detected in the donor and a set of healthy male and female controls. Both P9- and P10-T-cells were more frequent in females than males, and this difference was significant for P9-T-cells. P9 has also showed best results earlier in stabilizing HLA-A2 expression.

Further on, we determined the functional status of PBMCs from the patient, donor and healthy controls. We found that cells from the patient, but not from the donor or controls, exhibited peptide-specific killing. In the PBMCs of the patient, killing did not decrease at the time point, when an increase in PSA was detected. Also, expansion of P9- and P10-specific T-cells were more efficient after 3-week in vitro stimulations of PBMCs from the patient than compared to cells from the donor.

Prostate cancer offers interesting possibilities for achieving GVT in context of allo-HSCT. Firstly, prostate is an organ that does not possess vital function and can be offered for elimination, if needed. In context of T-cell immunotherapy this means possibility to elicit responses against tissue, not only tumor, specific antigens. Secondly, prostate is a functional male sex organ and it is tempting to speculate that female T-cells should be less tolerized to prostate tissue antigens. However, it is important to take into account that a prostate homologue in females is known (paraurethral glands or Skene’s glands) and expresses PSA and other prostate antigens. Still, antigen expression level seems to be low and these glands are less mature structurally and functionally than their homologue in males (379, 380). In addition, low expression of PSA mRNA is detected in other tissues (see introduction).

We have observed a difference in functional status of PSA-specific T-cells between the transplanted patient and his female donor. We did not have the possibility to investigate if these P9 and P10 cells could directly lyse patients’ cancer cells, therefore all the
speculations on their involvement in the anticancer activity are still indirect. PSA specific T-cells were also detected at high levels in healthy females and at lower levels in healthy males, suggesting that additional tolerization mechanisms in males may be present.

A number of groups have reported T-cell responses against prostate-derived antigens in prostate cancer patients (499-504). In a recent report, responses in healthy females were also investigated and no differences in specific T-cell frequencies (when assessing IFN-γ response) between females and males could be demonstrated (502). However, to our knowledge, we’re the first ones to report on responses of female T-cells transferred into a male recipient, a situation which is different from responses in autologous setting. Evidence of contribution to prostate antigen expression in thymus during T-cell development was lacking until recently. In a murine system, Aire-regulated thymic expression of prostate autoantigen SVS2 was demonstrated and associated with development of chronic prostatitis (505). In the same report, observations were extended to evaluation of antibody levels against semenogelin, a protein found in human seminal plasma, in patients with chronic prostatitis. All the patients who had biopsy-proven inflammation in the prostate possessed autoantibodies to semenogelin. This report provides evidence for thymic establishment of tolerance against prostate-related antigens and implicates that an inflammatory process might break tolerance (505). Unfortunately, the authors did not investigate the impact of different sexes for establishment and maintenance of tolerance against prostate autoantigens. It is known that PSA is expressed in females to a lower extent (see introduction) but it is unclear if this could have differential impact on tolerance establishment.

It is tempting to hypothesize, that the tolerogenic state of PSA-specific T-cells detected in our study could be broken upon adoptive transfer in the prostate cancer patient. Apart from the T-cells in the stem cell graft, this patient has received unmanipulated DLI on two occasions and GVHD was induced. GVHD was associated with GVT in solid tumor patients (196). It is possible, that proinflammatory milieu which is perpetuating the vicious cycle of GVHD, has contributed to enhancement of antigen presentation and costimulation. This in turn could have outweighed the tolerization. A report was published on generation of CEA-specific CD8+ T-cells (detected with pentamers) in allografted colon-cancer patients who developed GVHD. Such T-cells were not detected in colon cancer patients without GVHD development or in patients transplanted for other diagnoses than colon cancer (506). This supports our speculation on the role of GVHD in breaking the tolerance. In another study, PSA-specific T-cells isolated from prostate cancer patients, displayed defective killing (507) which was not in agreement with our observations. This indirectly stands in support of our hypothesis that adoptive transfer in context of allo-HSCT could have allowed the PSA-specific T-cells to become active.

Another interesting aspect of adoptive immunotherapy in patients with prostate cancer is the effect of GnRH agonists on T-cell generation in the thymus. Experiments in mice have indicated that thymic output was increased upon sex steroid blockade (508-510). These effects were confirmed in auto- and allo-HSCT recipients, where increased thymic output, diversification of the peripheral TCR repertoire and enhancement of T-cell function were noted (511). In relation to GVT effect after allo-HSCT for solid
It is possible to speculate that enhancement of thymic function is beneficial for reducing TRM events; however, theoretically, in the long term this could also speed up tolerization of the adopted immune system and result in GVT impairment. Interestingly, effects on enhancement of T-cell function (proliferation and cytokine production in vitro) were noted in the early post-transplant period (<6 months). It is of interest, if there are any direct effects of sex-steroid blockade on the peripheral T-cell pool. GnRH receptor is expressed on PBMCs (512), as are the receptors for androgens (513). Authors comment that they could not detect isolated effects on peripheral T-cells in their preliminary studies (511). The patient in our study has received GnRH analogue treatment before and after transplantation.

Figure 7. PSA curve of a PC patient after allo-HCT. It is the third patient in the allo-HSCT study for PC at our institution. Arrows indicate DLI administration.

In our report, the GVT effect comes from the allo-HSCT procedure and 2 unmanipulated DLIs, i.e. no specific immunotherapy was administered. Still, it provides an interesting platform to explore the possible T-cell specificities and action mechanisms directed against prostate tissue. The clinical course of the third patient in the allo-HSCT study for PC at our center can serve as a support to our observation of a GVT effect in the patient described in paper IV. The third patient is currently at 1.5 years after transplant, and has signs of progressive disease. Unfortunately, he had bone metastases at the time of transplant. Still, his clinical course suggests a clear GVT response (Figure 7). Here, at the time points of GVHD development (arrows), an increase in PSA with subsequent decline was noted. We believe that these increments signal a possible T-cell mediated attack on the tumor. P9 and P10 T-cells were also detected in this patient and further analysis of the response is ongoing.

The patient described in paper IV has progressed in his clinical disease and we made several attempts to infuse the cells locally into the prostate of the patient. The rationale here was to induce local injury and, hopefully, by this deliver danger signal, which
could enhance the immune response. However, our approaches were generally unsuccessful and the cancer has progressed. Still, we have noted an eventual transient effect, resulting in increasing, but still low PSA levels within normal range. This transient effect lasted more than two years. In biopsies taken during the late period, minimal infiltration of T-cells could be shown; however, no T-cells were seen in vicinity of the tumor. That was in contrast to the localization of T-cells in the biopsies taken earlier after transplantation. This would suggest that local immunosuppressive factors were in place in the later biopsies, most probably compromising T-cell trafficking and function at the tumor site, as reported by others (411, 514, 515). Further on, we plan to visualize trafficking of such infused cells in order to gain better understanding on the requirements for immunotherapeutic approaches in this setting.

Also, we have been presented with a possibility to challenge our hypothesis by identifying three patients, who were transplanted due to hematological malignancies but all had prostate cancer diagnosed before HSCT. These patients were all transplanted with male donors, it will be interesting to further investigate the possible contribution of gender influences on responses to prostate tissue after allo-HSCT in patients with prostate cancer.

In summary, we have detected PSA-specific T-cells in an allo-HSCT patient and these T-cells were functional in vitro, in contrast to such T-cells detected in the stem cell donor and healthy controls. A number of factors might have accounted for the activation of PSA-specific T-cells, and possible contribution of these factors to improvement of allo-HSCT outcomes need to be addressed in further studies.
4 CONCLUSIONS

- Allogeneic HSCT, when using UCB as a stem cell source, is associated with a higher survival probability and is superior to allo-HSCT, when using MM URD;

- T-cells from UCB grafts can be successfully expanded to sufficient levels for administration as DLI after UCBT, expansion procedure does not skew the TCR repertoire;

- Expanded CB T-cells display an activated central-effector memory phenotype and are functional in vitro;

- EBV-specific T-cells can be rapidly isolated from peripheral blood of adult donors by selection with HLA-multimeric complexes and paramagnetic beads;

- HLA-multimer-selected third-party EBV specific T-cells can be transferred into the patient without toxicity, engraft and persist in the patient and most likely mediate regression of EBV associated PTLD;

- T-cells specific for PSA-specific T-cells can be detected after allo-HSCT for prostate cancer and are functional in vitro, in contrast to such T-cells detected in healthy controls.
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My Lithuanian friends – Aurelija and Valentinas - if it were not you, we would have not be here. You are the ones who have helped me and my wife countless times and we really appreciate that. We wish you all the best in Ludvika, can’t wait to get to see Lukas! Tomas and Agnė - among other pleasant things, you are my teachers in alpine skiing, thank you! To teach me fishing is the next task, I can hang along when Bernardas grows bigger. Jurga, Evaldas, Mėgė and Sofija – thank you for your hospitality and your interest in dance, tango is waiting...

Gedas - I hope you’re doing OK in Singapore, we miss you here! Thank you for our “Talks of the town” when you were still here at MTC. Laura and Audas, finally you both are here! I appreciate that we share attitudes and sense of humor with you. I think we’re going to have fun. Rolandas – we met recently, but it seems that we have known each other for a long time. Thank you for your company and nice discussions and I look forward to the following years!

Back in Lithuania, I wold like to acknowledge my mentors and colleagues. Prof. Algimantas Sinkus, you have introduced critical thinking into my mind and I’ll never forget that. Prof. Apolinaras Zaborskis, thank you for your guidance through the world of epidemiology! My special acknowledgement to the first scientific co-authors ever, Jurgita Petronytė and Tautvydas Vaišvilas, it was fun! A big acknowledgement goes to Auksė Mickienė, first, for teaching me infectious diseases, and recently - for the great time and all the discussions at the courses here at KI! Laimonas Griškevičius, Igoris Trociukas, Valdas Pečeliūnas and other colleagues from Vilnius University Hospital – for developing stem cell transplantation in Lithuania and for all the nice discussions during EBMT meetings.

Finally, I want to express my gratitude to my family in Lithuania.

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Mėly wife Vilija, thank you for your LOVE. And a little miracle that we are still waiting for.


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