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ANTITUMOR EFFECT IN ALLOGENEIC HEMATOPOIETIC STEM CELL TRANSPLANTATION IN PATIENTS WITH SOLID CANCER

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To Jan
To Ellen
To my parents
and
The memory of my grandparents

ABSTRACT

Allogeneic hematopoietic stem cell transplantation (HSCT) has been presented as a promising immunotherapy not only against hematological malignancies but also against solid tumors. Different solid tumors such as renal cell, breast, colon, prostate and advanced primary liver cancer have been treated with HSCT at our center. Treatment with HSCT gives rise to an anti-tumor response so called graft-versus-tumor (GvT) effect that has been demonstrated as tumor regression of metastasis.

Before transplantation, patients receive non-myeloablative conditioning also known as low intensity conditioning (LIC) or reduced intensity conditioning (RIC) that eradicates the bone marrow to a lesser extent, allowing place for the new hematopoietic stem cells from the donor. The presence of remaining tumor cells or mixed chimerism threatens the patient with disease relapse after the LIC/RIC regimen. Therefore, the GvT effect after LIC/RIC regimen relies on donor lymphocyte infusions (DLI), i.e. adoptive immunotherapy that can contribute to full donor chimerism.

A common complication after HSCT is graft-versus-host disease (GvHD), i.e. the attack of the transplanted cells against the patient's epithelial cells in the skin, bowel and liver. It seems that donor T lymphocytes mediate both GvHD and the GvT effect, since the risk for tumor recurrence is higher when donor T lymphocytes are depleted from the transplant. Therefore, a patient having mild acute or chronic GvHD, has a predictable high chance of the GvT effect.

In paper I, we studied inflammatory and anti-inflammatory cytokines in serum using ELISA in four patients with metastatic renal cell and two with colon cancer. We found dominating TNF-α and IFN-γ levels in serum, which correlated with tumor regression.

In paper II, we retrospectively determined the risk factors for complications in 48 patients with solid tumors receiving LIC as compared to RIC regimen. We reported that engraftment and development of donor B cell chimerism occurred earlier in patients receiving LIC than in patients receiving RIC. The best GvT effect was demonstrated in patients with advanced primary liver cancer who had previously undergone liver transplantation. The most favorable GvT effect was found in patients who received DLI and developed chronic GvHD. A tendency for prolonged survival was found in patients receiving RIC compared to the LIC group.

In paper III, we measured cytokine secretion using ELISpot during DLI given to four patients with solid tumors and four with hematological malignancies. Increased expression of TNF-α, IFN-γ, IL-10 and IL-12 in mononuclear cells (MNC) was found in patients with favorable outcome of disease response after DLI.

In paper IV, we detected and identified tumor-reactive T lymphocytes from an HLA-identical sibling against tumor cells from a patient with pancreatic cancer using flow-cytometric assay of specific cell-mediated immune responses in activated whole blood (FASCIA). Using CDR3 size spectratyping tumor-reactive T lymphocytes could be distinguished from T lymphocytes activated against peripheral blood MNC from the patient.

In conclusion, these findings might give a better treatment aiming at a more effective GvT effect. Monitoring cytokines before DLI could predict those patients who will gain from immunotherapy. Combining FASCIA and CDR3 size spectratyping might be a way to identify, isolate, *in vitro* expand and infuse tumor-specific T lymphocytes from donors in order to intensify the GvT effect.

LIST OF PUBLICATIONS

I. Inflammatory cytokines predominate in cases of tumor regression after hematopoietic stem cell transplantation for solid cancer
Réka Conrad, Mats Remberger, Kerstin Cederlund, Patrik Hentschke, Berit Sundberg, Lisbeth Barkholt
Biology of Blood and Marrow Transplantation 2006. 12:346-354

II. A comparison between low intensity and reduced intensity conditioning in allogeneic hematopoietic stem cell transplantation for solid tumors
Réka Conrad, Mats Remberger, Kerstin Cederlund, Olle Ringdén, Lisbeth Barkholt
Haematologica 2008. 93(2):265-272

III. Cytokine expression in patients with solid tumors and hematological malignancies treated with donor lymphocyte infusions after allogeneic hematopoietic stem cell transplantation
Réka Conrad, Mats Remberger, Kerstin Cederlund, Lisbeth Barkholt
Submitted to Immunotherapy.

IV. Novel methodology for detection of tumor-reactive cell-mediated immune response against pancreatic cancer cells in HLA-identical setting
Réka Conrad, Brigitta Omazic, Mats Alheim, Sam Ghazi, Mehmet Uzunel, Dan Hauzenberger, Lisbeth Barkholt
Manuscript.

CONTENTS

LIST OF ABBREVIATIONS

6-FAM	6-fluorescein phosphor amidite
AML	Acute myeloid leukemia
APC	Antigen presenting cells
Apo-nec	Apoptotic/necrotic
BMT	Bone marrow transplantation
Bp	Base pair
Bu	Busulphan
C	Constant
CDR	Complementarity-determining regions
CEA	Carcinoembryonic antigen
CML	Chronic myeloid leukemia
CMV	Cytomegalovirus
CsA	Cyclosporine-A
CT	Computer tomography
CTL	Cytotoxic T lymphocytes
Cy	Cyclophosphamide
DC	Dendritic cells
DLI	Donor lymphocyte infusions
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-linked immunosorbent assay
ELISpot	Enzyme-linked immunospot assay
FASCIA	Flow-cytometric assay of specific cell-mediated immune response in activated whole blood
Flu	Fludarabine
G-CSF	Granulocyte colony stimulating factor
GM-CSF	Granulocyte/macrophage colony stimulating factor
GvHD	Graft-versus-host disease
GvL	Graft-versus-leukemia
GvT	Graft-versus-tumor
Gy	Gray
HLA	Human leukocyte antigen
HSCT	Hematopoietic stem cell transplantation
IFN	Interferon
Ig	Immunoglobulins
IL	Interleukin
KIR	Killer cell immunoglobulin-like receptors
LAK	Lymphokine-activated killer
mHag	Minor histocompatibility antigens
MHC	Major histocompatibility antigens
MMF	Mycophenolate mofetil
MNC	Mononuclear cells
MTX	Methotrexate
MUD	Matched unrelated donor
NK	Natural killer

NKT	Natural killer T
PCR	Polymerase chain reaction
PUVA	Psoralen and ultraviolet light A
RAG	Recombination-activating genes
RECIST	Response evaluation criteria in solid tumors
RIC	Reduced intensity conditioning
RSS	Recombination signal sequence
RT-PCR	Reverse transcription PCR
SFC	Spot forming cells
TAA	Tumor-associated antigens
TBI	Total body irradiation
TCR	T cell receptor
TGF	Transforming growth factor
Th	T helper
TIL	Tumor infiltrating lymphocytes
TNF	Tumor necrosis factor
Treg	Regulatory T lymphocytes
V	Variable
VCZ	Varicella zoster virus
VEGF	Vascular endothelial growth factor

“Ami végtére is megtart az életben,
az nem más, mint képesség,
hogy valaminek örüljünk,
valamit kedvvel tegyünk,
hogy valamit szeressünk.”

(F. Riemann)

1 SUMMARY

Allogeneic hematopoietic stem cell transplantation (HSCT) has been presented as a promising immunotherapy not only against hematological malignancies but also against solid tumors. Different solid tumors such as renal cell, breast, colon, prostate and advanced primary liver cancer have been treated with HSCT at our center. Treatment with HSCT gives rise to an anti-tumor response so called graft-versus-tumor (GvT) effect that has been demonstrated as tumor regression of metastasis.

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2 INTRODUCTION

2.1 IMMUNE SYSTEM

The era of immunology started in 1796 when Edward Jenner inoculated cowpox, or vaccinia, to induce protection against human smallpox; thereby the term vaccination was introduced. But it took one century until Robert Koch discovered that microorganisms cause infectious diseases. Today, we know about four categories of foreign microorganisms: viruses, bacteria, pathogenic fungi and parasites. The first success of vaccination was achieved by Louis Pasteur in 1880, when he, with a rabies vaccine, cured a boy bitten by a rabid dog. In 1884 Ilja Metjnikov discovered that phagocytic cells, which he called macrophages, can engulf microorganisms. One decade later, Emil von Behring and Shibasaburo Kitasato discovered that the serum of animals immune to diphtheria or tetanus contained antitoxic compounds, later called antibodies. Today we know that the immune system is an extraordinarily complex and well-organized system, which consists of the innate and the adaptive immunity.[1]

The innate immunity, also called natural or native immunity is the first line of defense against microbial infectious agents and reacts always in a similar fashion to the microbes. This immunity is non-specific and recognizes different microbial structures such as pathogen-associated molecular patterns, e.g. gram-negative bacterial lipopolysaccharide, bacterial flagellin and peptidoglycan. However, the innate system is able to discriminate between self and non-self, which is an important determination before an immune response takes place. Activation of the innate immune system happens immediately and does not develop an immunological memory. The components of the innate immunity are epithelia in the skin, gastrointestinal tract and respiratory tract, phagocytes such as neutrophils and macrophages, dendritic cells (DC), natural killer (NK) cells, cytokines, and plasma proteins, including the proteins of the complement system.

The adaptive immunity, also called acquired immunity is initiated by specific antigens, which are recognized by antigen receptors on lymphocytes. This immunity creates a memory after exposure of an antigen and reacts more rapidly after repeated exposure to the same antigen. The components of the adaptive immunity are antigen presenting cells (APC), DC, B and T lymphocytes, which all interact with cytokines, chemokines and immunoglobulins (Ig) to create a specific immune response against foreign antigens.

The innate immune system interacts with the adaptive immune system; after engulfment and phagocytosis of microbes and foreign antigens by macrophages or DC the adaptive immunity becomes activated by expression of the co-stimulatory molecules B7.1 (CD80) and B7.2 (CD82) on DC. The activated DC migrates to peripheral lymphoid organs, the lymph nodes and become mature APC. In the lymph nodes, protein antigens from the microbe are displayed for recognition by T lymphocytes. The recognition engages antigen processing on APC together with major histocompatibility complex (MHC) on T lymphocytes.

Proteins degraded in the cytosol of any nucleated cell are processed in the cytoplasm, displayed by class I MHC molecules and presented to CD8+ T lymphocytes, also called cytotoxic T lymphocytes (CTL). Extracellular proteins degraded in the endocytic vesicles of APC are displayed by class II MHC molecules and presented to CD4+ T lymphocytes, also called T helper (Th) lymphocytes. A naive T lymphocyte is activated when it receives two signals. The first signal is provided when the T cell receptor (TCR) on T lymphocytes binds to the peptide:MHC complex. The second signal or co-stimulatory signal is provided when CD80 or CD86 on APC binds to CD28 on the T lymphocyte. These two signals are required for full lymphocyte activation; whereafter intracellular signals give raise to proliferation and differentiation of T lymphocytes.[2]

In addition, the immune system plays an important role in defense against malignant diseases, by recognizing and eliminating the transformed cells.[3]

2.1.1 Components of the immune system

Macrophages are phagocytic cells located in different tissues in the body. After maturation, they leave the circulation and migrate into tissues, ready to recognize, ingest and destroy foreign microbes. Macrophages are found in connective tissue, in the submucosal layer of the gastrointestinal tract, in the lung, along certain blood vessels in the liver (Kupffer cells) and in the spleen. Immature DC in peripheral tissue are also capable for phagocytosis. When they engulf a microbe or a foreign antigen they mature into APC and migrate to the lymph nodes where the antigen recognition and processing take place.[4] Neutrophils are short-lived cells in the blood but they are not present in healthy tissues. They also have the ability to phagocyte foreign microbes entering the blood stream.

Natural-killer cells are capable of killing certain tumor cells without the need for prior immunization or activation.[5, 6] NK cells are activated in response to interferon (IFN)-β, IFN-γ and interleukin (IL)-12 and they can serve to contain virus infections until the adaptive immune response is generated and specific CTL are produced in order to clear the infection.[7, 8] NK cell killing is mediated when infected cells or tumor cells lack the class I MHC molecule on their surface; thereby no signal is processed by the inhibitory killer cell immunoglobulin-like receptors (KIR) on the NK cell. This “missing” signal results in the killing of the infected/tumor cell by apoptosis via perforin/granzyme B and Fas/Fas ligand pathways.[9, 10]

T lymphocytes are involved in the recognition of antigens and belong to the adaptive immune system. The TCR on T lymphocytes is a membrane-bound specific antigen recognition receptor. There are two main subsets of T lymphocytes, CD4+ T or Th lymphocytes and CD8+ T lymphocytes or CTL. Upon activation of antigens, naive CD4+ Th lymphocytes can differentiate to different subsets of effector cells, type 1 Th (Th1) or type 2 Th lymphocytes (Th2) depending on the cytokines they produce.

Th1 lymphocytes stimulate phagocyte-mediated ingestion, killing of microbes and induce cell-mediated immunity. Th1 development is induced by IL-12 produced by activated DC and macrophages, and by IFN-γ produced by NK cells or by the responding T lymphocytes themselves (paracrine activation). Th1 lymphocytes produce tumor necrosis factor (TNF)-α, IFN-γ, IL-2 and also to a lesser extent IL-10. Th1 stimulus is maintained especially by the paracrine activity of IL-2.

Th2 lymphocytes stimulate phagocyte-independent, mast cell/eosinophil-dependent allergic inflammation and induce a humoral immunity. Th2 development is induced by IL-4 produced by mast cells or by the T lymphocytes themselves in the absence of IL-12. Th2 lymphocytes produce IL-4, IL-5, IL-10 and IL-13.

Another CD4+ T lymphocyte subpopulation is regulatory T (Treg) lymphocytes, sometimes also called type 3 Th (Th3) lymphocytes, characterized by the phenotypic markers CD25[11] and FoxP3.[12, 13] They produce IL-10 and transforming growth factor (TGF)-β, and are important in suppression of the immune response. Development of Treg lymphocytes is generated when CD4+ lymphocytes recognize self-antigens in the thymus or peripheral tissues; thereby they play an important role in the maintenance of central and peripheral tolerance.[14] Treg has been found at a higher frequency in the peripheral blood and in the tumor microenvironment of patients with pancreas, breast and ovarian cancer, and may induce peripheral ignorance of tumor cells, helping metastatic spread of the disease.[15, 16]

More recently, type 17 Th (Th17) lymphocytes have been described which promote inflammation in a number of immunological reactions. Th17 development is induced by IL-1, IL-6 and IL-23 produced by activated DC and macrophages. Th17 lymphocytes produce IL-17 and IL-22. In tumor immunology, these cells have been found in the tumor microenvironment in patients with ovarian cancer[17] as well as in other solid tumors, such as hepatocellular, renal cell, prostate, pancreatic and colon cancer.[18] These cells have also been reported in the development of graft-versus-host disease (GvHD) after hematopoietic stem cell transplantation (HSCT) (both HSCT and GvHD are discussed later).[19]

CD8+ T lymphocytes have cytotoxic function and upon activation of antigen and co-stimulatory molecules they differentiate to CTL that are able to kill infected cells expressing the antigen. CTL kill infected cells by delivery of granule proteins, such as granzymes and perforin, into the infected cell. CTL can also kill by Fas/Fas ligand pathway, thereby inducing target cell apoptosis.

B lymphocytes are also involved in the adaptive immunity. The B cell receptor exists both as membrane-bound antigen receptor or Ig and as secreted Ig. Upon activation B lymphocytes mature to Ig (antibody) producing plasma or memory cells. Secreted antibodies are the effector molecules of the humoral immunity.

2.1.2 T cell receptor genes

T cell receptors and B cell receptors (Ig) on lymphocytes are responsible for antigen recognition, as mentioned above. Lymphocytes in the body have numerous copies of a single antigen receptor with a unique antigen-binding site, which determines the antigens that the lymphocyte can bind. To generate lymphocytes with a wide range of antigen specificities, a complex genetic mechanism for generating highly variable (V) regions of the TCR and Ig has evolved. These highly variable parts are encoded in several gene segments, a mechanism known as gene rearrangement. The basic mechanism of rearrangement for the TCR and Ig genes is common but in this thesis rearrangement of TCR is highlighted.

The TCR consists of one α chain (TCRα) and one β chain (TCRβ), and each chain has a V region and a constant (C) region. The TCRα locus contains V and J gene segments (V_α and J_α), whereas the TCRβ locus contains V, D and J gene segments (V_β, D_β and J_β). The TCR gene rearrangement takes place in the thymus.
The TCR rearrangement mechanism is guided by conserved noncoding DNA sequences that flank the gene segments encoding the V region. These sequences are called recombination signal sequence (RSS) and consist of a conserved block of seven nucleotides (heptamer), which is always contiguous with the coding sequence followed by a nonconserved region (spacer) of 12 or 23 nucleotides, which is followed by a second conserved block of nine nucleotides (nonamer). A gene segment flanked by an RSS with a 12-base pair (bp) spacer can only be joined by a RSS with a 23-bp spacer. This is known as the 12/23 rule and makes sure that the gene segments are joined in the correct order.[20] The recombination process is completed when recombination-activating genes (RAG)-1 and RAG-2 together with several DNA-modifying proteins are assembled with the 12-bp spaced and 23-bp spaced RSS.[21]
The antigen-binding site of the V region of the TCR is formed by the complementarity-determining regions (CDR) 1, 2 and 3. All three CDR are expressed on both TCRα and TCRβ. CDR1 and CDR2 loops are less variable and contact the MHC component of the ligand, whereas CDR3 is a hypervariable loop which contact the unique peptide component.[22] The TCR rearrangement of TCRα and TCRβ chains is demonstrated in Figure 1.

2.2 CYTOKINES

Cytokines are polypeptide growth factors that regulate the growth, differentiation and activation of various cell types. They can be either secreted or membrane-bound. On the target cells, cytokines bind to its receptors, and activate downstream signaling events that result in the required biological response.[23] Cytokines are involved in many aspects of immunity and inflammation, including innate immunity, adaptive immunity, e.g. antigen presentation, bone marrow differentiation, cellular recruitment and activation, and adhesion molecule expression. Which cytokines are produced due to an immune response, depends on whether the response is cytotoxic, humoral, cell-mediated or allergic.[24] The cytokines examined in paper I and III are highlighted, as follows: TNF-α, IFN-γ, IL-4, IL-10, IL-12, IL-13 and TGF-β.

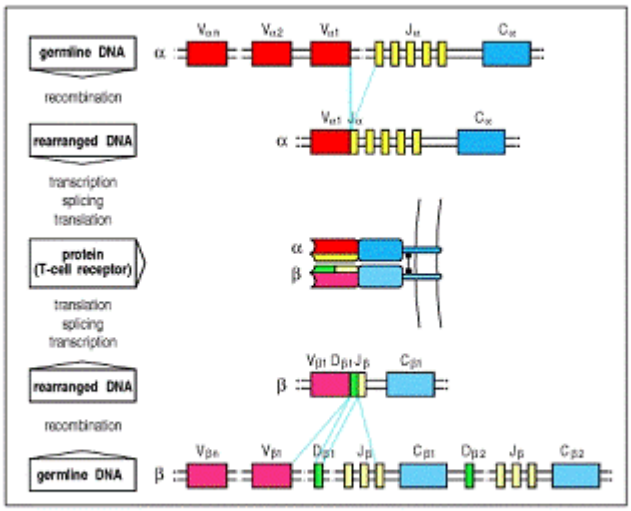

Figure 1. T cell receptor rearrangement of α and β TCR chains. Reprinted with permission from Janeway et al editors 2005.

TNF-α is involved in systemic inflammation and is a member of a group of cytokines that stimulate the acute phase reaction. TNF-α is produced by activated lymphocytes, NK cells, macrophages, neutrophils, endothelial cells and mast cells. As mentioned above, in adaptive immunity, TNF-α is produced by Th1 lymphocytes. The primary role of TNF-α is regulation of different immune cells. TNF-α is also able to induce anti-tumor immunity by either applying cytotoxicity directly on tumor cells or by stimulating anti-tumor response via apoptosis.

Even though TNF-α is a potent activator of immune cells, interest for potential immunotherapeutic value of this cytokine to treat cancer has been moderated by its severe side effects. TNF-α is responsible for the severe cachexia that occurs in cancer and chronic infections.[25] Furthermore, TNF-α induces vascular leakage and is the primary endogenous mediator of toxic shock and sepsis.[26] These severe inflammatory reactions lead to fever and tissue destruction with endothelial damage. Acute treatment with neutralizing anti-TNF-α antibodies has reversed toxic shock and late organ failure in patients surviving 28 days after septic shock.[27] Treatment with anti-TNF-α antibodies has been evoked as an effective therapy for autoimmune diseases such as rheumatoid arthritis, multiple sclerosis and inflammatory bowel disease.[28-30]
In the context of allogeneic HSCT, TNF-α has been recognized as an important inflammatory cytokine of the 'cytokine storm', which is a relevant mediator in the pathogenesis of GvHD.[31]

In murine studies, blockade of TNF-α with anti-TNF-α antibodies or by using TNF-α receptor-deficient recipients has resulted in diminished GvHD and graft-versus-leukemia effect (GvL) (discussed later).[32-35] Furthermore, murine donor T cell-derived TNF-α has been shown to contribute to GvHD and GvL effect.[36]

In human studies, patients with acute GvHD have been reported with elevated TNF-α levels in serum[37-40] and clinical trials have shown recovery of GvHD during treatment with anti-TNF-α antibodies.[41, 42]

IFN-γ plays an important role in the innate and adaptive immunity against viral and intracellular bacterial infections. IFN-γ is produced predominantly by T lymphocytes, NK cells and natural killer T (NKT) cells and to a lesser extent by DC and macrophages. As mentioned above, in adaptive immunity, IFN-γ is produced by Th1 lymphocytes. The importance of IFN-γ in the immune system derives from its immunostimulatory and immunomodulatory effects. It stimulates NK cells for phagocytosis as well as mediates increased expression of class I and II MHC molecules. IFN-γ is also able to induce anti-tumor immunity by applying cytotoxicity directly on tumor cells.
In murine studies, double-knockout mice lacking T and B lymphocytes and a transcription factor required for IFN-γ signaling, spontaneously developed adenocarcinomas of the colon, breast and lung.[43] Mice deficient in cytokines IL-12 an IL-23, cytokines that stimulate IFN-γ production, were more sensitive to carcinogens and have shown enhanced tumor development as compared with normal mice.[44]
In human studies, enhanced production of IFN-γ has been found in T lymphocyte clones isolated from patients with GvHD.[45] In the study by Remberger et al, higher levels of IFN-γ in serum was found in patients who developed acute GvHD grade II-IV than in those with no or mild GvHD.[39] IFN-γ has reported to contribute to the graft-versus-tumor effect (GvT) (discussed later), where it was produced in CD8+ T lymphocytes in patients with renal cell cancer.[46]

IL-4 plays an important role in humoral immunity, specifically in the development of allergic immunity. IL-4 is produced by Th2 lymphocytes, mast cells, eosinophils and basophils. As mentioned above, IL-4 induces differentiation of naive Th lymphocytes to Th2 lymphocytes. Upon activation by IL-4, Th2 lymphocytes produce additional IL-4. This cytokine induces B lymphocyte class switching from IgM to IgE. Increased production of IL-4 has been associated with allergies.[47]
In murine models, T lymphocytes that had been experimentally manipulated to produce IL-4 and IL-10 were shown to inhibit development of acute GvHD.[48, 49]
In human studies, one study has reported increased IL-4 producing cells in patients with acute GvHD as compared to patients without GvHD[50], whereas another study showed no difference[51], and a third study showed decreased IL-4 producing cells in patients with acute GvHD.[52]

IL-10 inhibits activation and effector function of T lymphocytes, monocytes and macrophages. IL-10 is produced by both Th1 and Th2 lymphocytes, CTL, B lymphocytes and mast cells. The principal function of IL-10 is to limit and terminate inflammatory responses. This is the case, when IL-10 inhibits cytokines production, such as IFN-γ, IL-2 and TNF-α, and proliferation of Th1 lymphocytes by down-regulating class II MHC molecules and the co-stimulatory molecule B7.
In contrast, IL-10 has stimulatory effects on CD8+ T lymphocytes and induces their recruitment, cytotoxic activity and proliferation.[53-56]

In murine tumor cell lines, IL-10 suppressed T lymphocyte-mediated immunity by down-regulating class I MHC on tumor cells.[57] In mice models of GvHD, administration of IL-10 after transplantation resulted in lethal outcome with increased mortality.[58-60]
In human studies, IL-10 was present in large amounts in tumor biopsies from patients with ovarian cancer.[61] Furthermore, increased IL-10 levels prior HSCT were correlated with lower incidence of GvHD and improved survival.[62-64] In other studies, high levels of IL-10 after HSCT indicated a higher incidence of GvHD[39, 65] and a poor prognosis for survival.[66]

IL-12 is important in the induction of Th1 responses, in activation of NK cells and IFN-γ production. IL-12 is produced by DC, macrophages and monocytes. As mentioned above, it is involved in the differentiation of naive Th lymphocytes into Th1 lymphocytes. IL-12 mediates enhancement of the cytotoxic activity of NK cells and CTL. It is known as a factor, which can stimulate the growth and function of T lymphocytes. IL-12 stimulates the production of Th1 cytokines, including IFN-γ and TNF-α and suppresses the Th2 cytokines, such as IL-4 and IL-13.
In murine studies, it was shown that IL-12 restores the ability of CD4+ CD25- T lymphocytes to proliferate and express activation markers during co-culture with Treg.[67] IL-12 has been reported to play an important role in acute GvHD[68, 69] and it also preserves the GvL effect.[70, 71]
Also in human studies, high levels of IL-12 in plasma have been associated with the development of acute GvHD grade II-IV[72] and with the GvL effect without increasing the risk for GvHD.[73]

IL-13 has very similar features to IL-4, thus it also has important role in humoral immunity. As IL-4, IL-13 is produced by Th2 lymphocytes, mast cells, eosinophils and basophils but can also be produced by DC and NK cells.
In murine studies, IL-13 has been reported to down-regulate anti-tumor responses to allow tumor growth. This anti-tumor response appeared when NKT cells were induced by tumors to secrete IL-13, thereby suppressing CTL responses against the tumor.[74]
In human studies, development of acute GvHD grade III was associated with high levels of IL-13 produced by donor T lymphocytes prior to HSCT.[75]

TGF-β is an immunosuppressive cytokine and inhibits effector function of both innate and adaptive immune cells. TGF-β is produced by monocytes, platelets, some T lymphocytes, chondrocytes, osteocytes, fibroblasts and tumor cells. In tumor immunology, TGF-β has been reported to promote cancer metastasis by enhancing tumor cell invasion and by inhibiting function of effector immune cells.[76, 77] These findings have encouraged scientists to target TGF-β and its signalling pathway as immunotherapy against different cancer diseases.
In murine studies, combination of a TGF-β monoclonal antibody and a vaccine resulted in the inhibition of tumor growth that was mediated by increased number and activity of CD8+ T lymphocytes.[78-80]
In human studies, elevated levels of TGF-β in serum were associated with disease progression in patients with colorectal cancer[81, 82] and with reduced amount of circulating DC.[83] Furthermore, elevated TGF-β in plasma was reported in patients with breast[84], hepatocellular[85, 86], and lung cancer.[87]

2.3 ALLOGENEIC HEMATOPOIETIC STEM CELL TRANSPLANTATION

Allogeneic HSCT is transplantation of adult hematopoietic stem cells from the bone marrow or peripheral blood to a patient from a healthy donor. The first clinical studies on allogeneic bone marrow transplantation (BMT) in humans were performed in 1957 by Edward Donnal Thomas and colleagues.[88] For the first time it was shown that relatively large amounts of bone marrow could be infused to patients when bone fragments and fat were removed. However, the results from these early studies were poor. The era of modern allogeneic BMT started after the discovery of the MHC and the human leukocyte antigen (HLA).[89, 90] In 1972, it had become evident that allogeneic BMT could cure leukemia, severe aplastic anemia and severe combined immunodeficiency.[91, 92] In 1975, Thomas et al reported results on BMT with HLA-identical sibling donors.[93, 94] In Sweden, the first allogeneic BMT was performed at our hospital in 1977.[95] In 1979, it was reported that patients with leukemia who developed GvHD had a decreased risk of relapse compared to patients without GvHD; thereby the GvL effect was discovered.[96] In 1980, the first allogeneic BMT was performed with a matched unrelated donor (MUD).[97] Today, the majority of patients who lack an HLA-identical sibling donor will find a suitable MUD among more than fourteen million donors and four hundred thousand cord blood units (Bone Marrow Donors Worldwide 2010).

2.4 ALLOGENEIC HSCT FOR SOLID TUMORS

The anti-tumor potential of the GvL effect in patients with hematological malignancies provided the basis to select solid tumor patients that could benefit by a GvT effect following allogeneic HSCT. The existence of a GvT effect has been shown in mice with lymphosarcomas[98] and mammary adenocarcinoma[99], where development of spontaneous tumors could be protected by HSCT using either MHC antigen matched[98] or mismatched donor.[99] In 1996, the first evidence of a possible allogeneic GvT effect in patients with metastatic breast carcinoma undergoing allogeneic HSCT with myeloablative conditioning was reported.[100, 101] In one of these reports, the GvT effect was suggested due to regression of a metastatic breast carcinoma lesion in a patient receiving HSCT for relapsed acute myeloid leukemia (AML).[100] In the other report, the GvT effect was supported when regression of liver metastasis was demonstrated together with severe acute GvHD in a patient transplanted for metastatic breast carcinoma.[101] These reports were followed by a report from a single institution of 10 patients with metastatic breast carcinoma treated with myeloablative conditioning followed by allogeneic HSCT.[102] The first clinical evidence of donor immune-mediated antitumor effect was demonstrated in this study, where one patient had complete regression and five patients experienced partial responses of metastases. However, enthusiasm for this immunotherapy decreased by fatal toxicities associated with the transplantation procedure.

The outlook on using the GvT effect against solid tumors improved again after the introduction of non-myeloablative conditioning (discussed later). Non-myeloablative conditioning was introduced first in patients with hematological malignancies in order to reduce transplantation-related mortality and to enable transplantation in older patients or those with organ impairment, who could not tolerate myeloablative therapy.[103-107] The first argument for non-myeloablative conditioning for solid tumors was that this conditioning could also be safer for elderly patients, as it has been the case for the older patients with hematological malignancies. Another argument for non-myeloablative conditioning was that chemotherapy already had failed in some solid tumors and conditioning therefore only was needed to prevent rejection of stem cells from the donor.

Already in 1928 spontaneous regression of metastasis was reported in a patient with metastatic renal cell carcinoma, thereby realizing that the immune system could control tumor growth.[108] The first solid tumor to be treated with non-myeloablative conditioning was metastatic renal cell carcinoma motivated by previous studies on renal cancer cell's susceptibility to immune response *in vitro* and *in vivo*.[109-111] In 1999, Childs et al reported the first allogeneic GvT effect with complete regression of metastases after non-myeloablative conditioning in a patient with metastatic renal cell carcinoma.[112] One year later, this group reported a series of 19 patients with metastatic renal cell carcinoma who underwent allogeneic HSCT.[113] Three patients had complete response and remained in remission 27, 25 and 16 months after HSCT. In addition, seven patients had partial response. In Sweden at our center and hospital, the first allogeneic HSCT with non-myeloablative conditioning was performed in August 1999 in a patient with metastatic colon carcinoma. Since the first report by Childs et al, several studies have been performed on allogeneic HSCT in patients with renal cell[114-126], breast[115, 117, 118, 120, 127-130], colon[117, 131-134], ovarian[120, 135-137], prostate[120], advanced primary liver[138] and pancreatic cancer.[139-142] The outcome and results on tumor response of these studies mentioned above will be highlighted in more detail in the section describing the GvT effect.

During the recent years, the use of allogeneic HSCT for solid tumors has been decreasing. This fact can be explained by a remaining high transplantation-related mortality and poor survival rates due to high tumor burden before transplantation, different conditioning types and immunosuppression strategies. Furthermore, development and approval of new therapeutic drugs against tyrosine protein kinases and kinase enzymes, such as sorafenib, sunitinib, temsirolimus and everolimus have also contributed to reduction in allogeneic HSCT application. Still, worldwide pilot studies on allogeneic HSCT with clinical observations of GvT effect found prolonged survival compared to conventional anticancer therapy.

2.4.1 Conditioning

Before transplantation, the patients receive a preparative regimen, so called conditioning that abolishes the bone marrow from malignant cells, allowing place for the new immune system from the donor, thereby providing immunosuppression to

prevent graft rejection. In patients with malignant diseases, it is important that the conditioning is as intensive as possible. Therefore, myeloablative conditioning is applied in these patients, where the conditioning consists of high doses of chemotherapy with or without irradiation. This conditioning eradicates as many as possible of the malignant cells, diminishes the tumor burden and prevents relapse of the disease. One commonly used myeloablative conditioning for patients with hematological malignancies is cyclophosphamide (Cy) followed by total body irradiation (TBI), 10 Gray (Gy) given at one session.[94, 143] To decrease the toxicity of TBI and to allow a higher total dose of irradiation, fractionated irradiation is given today during a longer time period with a total dose of 11 to 15 Gy.[144] An alternative to irradiation is the use of busulphan (Bu), followed Cy in patients with AML and children.[145-149]

Non-myeloablative conditioning also known as low intensity conditioning or reduced intensity conditioning (RIC) has been developed in order to reduce transplantation-related mortality for patients who could not tolerate a myeloablative regimen due to high age or organ dysfunction.[104] Thus, the purpose of RIC is not to eradicate all malignant cells but to create immunosuppression in order to avoid graft rejection. The RIC regimen does eradicate the patient's hematopoiesis but at the time of neutrophil engraftment both donor and patient lymphoid and myeloid cells are detectable, so called mixed chimerism.[106] Thus, after RIC regimen followed by allogeneic HSCT, the presence of remaining leukemic or tumor cells or mixed chimerism threatens the patient with disease relapse. Therefore, the GvT effect after RIC relies on donor lymphocyte infusions (DLI), i.e. adoptive immunotherapy that can contribute to full donor chimerism as follows myeloablative conditioning. The low intensity conditioning consists of 2 Gy TBI together with fludarabine (Flu).[104, 150] There are several RIC regimens in use consisting of Flu together with Cy, Bu and melphalan for patients with hematological malignancies and solid tumors.[104, 151]
At our center, Center for Allogeneic Stem Cell Transplantation at Karolinska University Hospital, Huddinge, we used low intensity conditioning between 1999 and until May 2001, thereafter until today RIC was used with Flu and Cy for patients with solid tumors.

2.4.2 Stem cell source

Initially, hematopoietic stem cells from the bone marrow were used for allogeneic transplantation. Hematopoietic stem cells are characterized by the expression of CD34 on their surface. Their abundance is 1-2 % in the bone marrow and 0.2% in the peripheral blood.[152] Being pluripotent cells, they can expand and mature into different types of blood cells. Hematopoietic stem cells from bone marrow are obtained from the donor by aspiration from the posterior iliac crest under general or spinal anesthesia.[94]

Since the late 1970s, it is possible to mobilize CD34+ hematopoietic stem cells to the peripheral blood by administration of granulocyte colony stimulating factor (G-CSF) or granulocyte/macrophage colony stimulating factor (GM-CSF) to the donor.

Administration of G-CSF results in a graft of three or four times more CD34+ cells and one-log higher T and NK cell dose after leukapheresis. Fear of increased risk for acute GvHD due to increased number of T lymphocytes compared to the bone marrow graft, delayed the use of peripheral blood as source of stem cells for allogeneic HSCT until the 1990s. However, later studies have shown that G-CSF-mobilized peripheral stem cells result in a faster engraftment of neutrophils and platelets[153, 154] than those from marrow without increasing the risk for acute GvHD.[155, 156] The use of peripheral stem cells compared to bone marrow for allogeneic HSCT, is similar regarding the incidence of acute GvHD, relapse, transplantation-related mortality and survival. However, increased occurrence of chronic GvHD has been reported in association with the use of peripheral stem cells.[157, 158]
At our center, all solid tumor patients (except two with advanced primary liver cancer) were transplanted with peripheral blood stem cells.

The third stem cell source is the use of hematopoietic stem cells from umbilical cord blood. Umbilical cord blood contains a higher amount of hematopoietic stem cells and progenitor cells but the total number of CD34+ cells is 1-2 log fewer compared with bone marrow and peripheral stem cells.[159] Unfortunately, the limited volume of cord blood is associated with a slower engraftment and an increased risk for graft rejection.[160, 161] However, stem cells from cord blood are more tolerant to one or two HLA-antigen mismatches and to overcome the cell dose limitation, transplantation of two cord blood units for adult patients was introduced recently.[162, 163]

2.4.3 Immunosuppression

Immunosuppressive treatment is given after allogeneic HSCT to prevent graft rejection and severe acute GvHD. The treatment consists of different drugs, and the length and the intensity of the treatment depends on the underlying disease, the choice of donor, the conditioning type and the severity of acute GvHD. Today, the most common protocol in use is combination of cyclosporine-A (CsA) and methotrexate (MTX).[164, 165] Other drugs used in combinations are mycophenolate mofetil (MMF)[166], tacrolimus[167, 168] and sirolimus.[167, 169] Another approach to decrease the incidence of GvHD, is depletion of T lymphocytes from the graft or *in vivo* with anti-T lymphocyte antibodies, like OKT-3 and ATG. The regimens reduce the occurrence of acute GvHD but increase the incidence of graft rejection and relapse.[170-172]

At our center, CsA in combination with MMF were used during the time of low intensity conditioning for patients with solid tumors, whereas CsA together with MTX is used today for patients who receive RIC. Patients with advanced primary liver cancer who underwent combined orthotopic liver transplantation and allogeneic HSCT, at our center, continued to receive the same immunosuppression for HSCT as for the liver transplantation, in order to protect against rejection of the liver graft.[138] This immunosuppression consists of either CsA or tacrolimus in combination with steroids. After allogeneic HSCT, CsA or tacrolimus is combined with MMF or MTX.

2.4.4 Graft rejection

Graft rejection or graft failure occurs when immunocompetent cells remain in the patient despite the conditioning given and induce rejection of the transplanted cells. With myeloablative conditioning, the incidence of graft rejection is around 2% if an HLA-identical sibling donor is used, and less than 5% with a MUD.[173, 174] The use of RIC protocols and T cell depletion increases the incidence of graft rejection (relative risk 9.29).[172]

2.4.5 Graft-versus-host disease

A common complication after allogeneic HSCT is GvHD, i.e. the attack of the transplanted cells from the donor against the patient's epithelial cells in the skin, bowel, liver and lung. Clinical manifestations of GvHD depend on the degree of donor/patient histocompatibility and graft alloreactivity to major host antigens. The incidence of GvHD can be as high as 85% in patients receiving allogeneic HSCT, depending on the type of donor and the degree of HLA-matching.[165, 175-177] The severity of GvHD can be reduced by well matched HLA-antigens between patient and donor, thereby also accelerate engraftment.[178] Despite full match of HLA between patient and donor, GvHD may still develop due to differences in minor histocompatibility antigens (mHag).[179] Studying the pathogenesis of GvHD, it was shown that infiltrating donor T lymphocytes play an important role in this mechanism.[180] Thus, donor T lymphocytes mediate both GvHD and the GvT effect, therefore the main goal in this field is to design methods where cells responsible for GvHD can be distinguished from those cells that exert the GvT effect.

GvHD exists in two forms, as acute and as chronic. Acute GvHD often occurs during the first 100 days after allogeneic HSCT or after administration of DLI. However, patients who have received RIC may develop acute GvHD later than three months. Acute GvHD involves tissue injury in the skin, liver and intestinal mucosa.[181] The severity of acute GvHD is graded between 0 and IV.[182] Grade 0 means absence of acute GvHD, grade I includes skin rash of less than 50% of the body surface, grade II involves skin rash on more than 50% of the body surface and/or mild liver involvement and/or mild diarrhea. Acute GvHD grade III affects the liver and/or gut more severe and grade IV includes erythroderma with bullous formation or severe liver disease with ascites and high serum levels of bilirubin or bowel inflammation with massive diarrhea with or without hemorrhages and ileus.[183, 184] The pathophysiology of acute GvHD is divided in three steps.

The first phase of acute GvHD occurs during the conditioning treatment before the donor cells are infused. Tissue damage occurs caused by the conditioning regimen, the underlying disease and infections. The damaged tissue initiates an immune response by secreting cytokines, upregulating adhesion molecules and activating DC. During this phase, the most commonly inflammatory cytokines are TNF-α and IL-1 secreted by activated DC.[185] These inflammatory cytokines increases the expression of adhesion molecules and co-stimulatory molecules.

In the second phase, after infusion of the graft, presentation of host antigens to donor T lymphocytes occurs in the lymph nodes, thereby activation of donor T lymphocytes are induced. Subsequently, the activation of donor T lymphocytes stimulates first proliferation and finally differentiation into effector T lymphocytes. The inflammatory cytokines involved in this phase are IL-2 and IFN-γ.[186] IL-2 induces clonal expansion of activated T lymphocytes and their differentiation into CTL. IFN-γ, together with IL-2, induces further T lymphocyte expansion and induces CTL and NK cell responses.

In the third phase, inflammation and more tissue damage are caused by secretion of inflammatory cytokines and effector T lymphocytes. Cell-mediated killing is induced by the perforin/granzyme B and Fas/Fas ligand cytolytic pathways, NK cells and the release of nitric oxide.[187] This process continues with more inflammation and tissue damage, thereby triggering new activated donor T lymphocytes at the inflammation site and keeps the GvHD ongoing.

The mechanism of chronic GvHD is less well studied and understood than acute GvHD. However, it has been shown that chronic GvHD develops due to the presence of alloreactive donor T lymphocytes.[188] Chronic GvHD occurs beyond three months and it can reappear after previously determined acute GvHD but also without. The clinical manifestation of chronic GvHD is different from that of acute GvHD. Its symptoms resemble those of autoimmune conditions such as keratoconjuctivitis, dermatitis, liver dysfunction and immunodeficiency.[189] Chronic GvHD is graded as limited or extensive.[190]

Treatment of acute and chronic GvHD consists of high doses of steroids, CsA, or anti-T lymphocyte antibodies, like OKT-3 and ATG, IL-2 receptor antibodies, oral psoralen combined with extracorporal ultraviolet light A (PUVA).[191, 192] Other treatments have also been reported with 1 Gy of total lymphoid irradiation and anti-B lymphocyte antibodies.[193, 194]

2.4.6 Infections

Infections are common after allogeneic HSCT due to immature immune system in the recipient followed by conditioning regimen and additional immunosuppressive treatments after transplantation in order to prevent GvHD. The abundance of different pathogens depends on the phase of the transplantation process.

During the pre-engraftment phase or neutropenic period from day 0-30 after HSCT, gram-positive bacteria of the skin and mouth are responsible for Bacteremia.[195] Gram-negative infections of the gastrointestinal tract are not as common today as they were earlier due to successful prophylaxis and administration of broad-spectrum antibiotics.[196] Regarding viral infections, reactivation of herpes simplex virus is most common during this phase and for seropositive patients antiviral prophylaxis is given.[197, 198] Among fungal infections oro-esophageal candida is most common but aspergillus and candida can also cause invasive infections.[199]

The post-engraftment phase lasts until day 100 after transplantation and during this period cellular immunity is slowly recovering. Cytomegalovirus (CMV) reactivation is most prevalent during this phase and is often correlated to GvHD.[200]
The risk of fatal outcomes from CMV disease has been reduced in the past due to the use of CMV prophylaxis and pre-emptive treatment based on sensitive qualitative/quantitative polymerase chain reaction (PCR)-based detection assays in order to monitor the viral load.[201-204] Prophylactic treatment is administered to prevent opportunistic infections.[205]

The late period starts beyond 100 days after transplantation and during this period the cellular and humoral immunity is not fully recovered. Reactivation of CMV, herpes simplex virus and varicella zoster virus (VZV) are common, especially in patients with chronic GvHD[206], whereas bacterial infections are less common.

2.4.7 Donor lymphocyte infusion

DLI has been developed as an adoptive immunotherapy against relapse of the underlying disease for patients with hematological malignancies who received myeloablative conditioning followed by allogeneic HSCT. The allogeneic immunotherapeutic effect in humans was already noticed in 1979, when the incidence of GvHD was found to be associated with lower risk of relapse.[96] Later it was demonstrated that patients receiving graft from a twin sibling were at higher risk of relapse when compared to HLA-matched sibling donors.[207] The observation that donor T lymphocytes mediate both GvHD and the GvT effect was confirmed when depletion of T lymphocytes from the graft with an aim to prevent GvHD, increased the risk of relapse.[172] These earlier findings confirmed the notion that donor T lymphocytes are the main effector cells on exerting the allogeneic GvT effect.

The evidence that DLI has the potential to eradicate leukemic cells was already reported from the start suggesting a potent GvL effect in patients with chronic myeloid leukemia (CML).[208] However, the GvL effect was less successful in patients with acute leukemias and other malignancies.[209-213] The mechanism behind the promising immunological response to DLI in CML compared to acute leukemias is unknown. One suggestion is the slower progress of CML compared to the aggressive tumor development of acute leukemias. Another is the required time of DLI response (2-3 months after administration) that allows the development of chronic GvHD in patients with CML. However, early administration of DLI and monitoring of minimal residual disease in patients with acute leukemias have given better GvL effect.[214]

Although administration of DLI improves the GvL/GvT effect, it may also induce fatal GvHD. The severity of GvHD can be reduced if DLI is administered in escalating doses. This was reported in patients with CML, where the GvL effect was delayed with a time span of 6-12 months.[215] However, the approach of administration of DLI in escalating doses is used in patients with acute leukemias and solid tumors.

After RIC regimen, DLI has been used in another approach, namely to convert mixed chimerism to full donor chimerism in patients with hematological malignancies[216-219] and solid tumors.[113, 114, 116, 117, 128, 129, 134, 140, 141] In studies performed on patients with solid tumors, the indication for DLI was often tumor progression with mixed chimerism or without.

Tumor response or prolonged survival after administration of DLI was demonstrated in patients with metastatic renal cell[126, 220], colon[220] and ovarian cancer.[136] In the first study of Barkholt et al, ^{111}-Indium labeled donor lymphocytes were infused into the hepatic artery of patients with renal cell or colon cancer. The donor lymphocytes were observed to home to metastases in the liver supporting the clinical response when one patient with renal cell and one with colon cancer showed stable size and number of metastases for 5 and 21 months, respectively.[220] In the second study by Barkholt et al, 124 patients with renal cell cancer from 21 European centers who had received DLI and developed chronic GvHD had the best overall survival (70% at 2-years).[126] DLI seemed to promote GvHD and control disease progression in one out of two patients with ovarian cancer.[136]

2.4.8 Graft-versus-tumor effect

Early clinical observations on tumor response suggesting a possible GvT effect resulted in immunotherapies with IL-2 and IFN-α, and adoptive immunotherapy with lymphokine-activated killer cells (LAK) or tumor-infiltrating lymphocytes (TIL) in autologous settings.[221-224] Furthermore, it was shown that TIL recognized melanoma-associated antigens and these lymphocytes could infiltrate the tumors and could grow *in vitro* in medium containing IL-2.[225, 226] Later, *ex-vivo* selected and expanded autologous lymphocytes were infused in patients with metastatic melanoma after lymphodepleting therapy demonstrating a response rate between 51% and 72%.[227, 228] However, patients with metastatic melanoma did not benefit from the GvT effect in the allogeneic setting.[229]

In the allogeneic setting, one approach to identify the GvT effect is the isolation of donor CTL that have the ability to kill leukemic or tumor cells from the patient with or without GvHD. Thus, one target peptide epitopes for CTL are mHag expressed on hematopoietic, epithelial or malignant hematopoietic cells. CTL specific for the mHag called HA-1 and HA-2 on hematopoietic cells induced remission in patients with CML and multiple myeloma.[230] Furthermore, CTL specific for HA-1, HA-3 and HA-8 were isolated from patients with renal cell cancer, who showed partial tumor responses or stable diseases.[123] However, it seems that infusion of mHag-specific T lymphocytes results in poor survival of the infused cells[231], which may imply that these lymphocytes never reach the tumor environment.

Another target peptide epitopes for CTL are tumor-associated antigens (TAA) expressed on tumor cells. CTL specific for Wilms' tumor antigen 1 were detectable in patients with acute lymphoblastic leukemia with favorable tumor responses.[232]

In patients with colorectal cancer, carcinoembryonic antigen (CEA)-specific CTL were detectable with the onset of GvHD.[133] In one patient CEA-specific CTL were associated with decreased CEA levels in serum and partial response of the tumor. From our group prostate-specific CTL were reported in a patient with prostate cancer associated with clinical remission.[233] Recently, Takahashi et al detected donor-derived CTL from a patient with renal cell cancer after allogeneic HSCT and identified the target antigen of renal cell cancer-specific CTL.[234] The target antigen was derived from human endogenous retrovirus type E and was selectively expressed in renal cell cancer cell lines and fresh renal cell cancer tissue but not in normal kidney or other tissues. Furthermore, one *in vitro* study showed superior renal cell cancer-reactive CTL responses of HLA-matched allogeneic T lymphocytes compared to autologous T cells.[235] These results support the specific reactivity of allogeneic T lymphocytes against renal cell cancer. However, the lack of identified TAA restricts the use of adoptive T lymphocyte immunotherapy against tumor antigens after allogeneic HSCT.

Another cell that plays an important role in the GvT effect is the NK cell. Due to the 'missing self 'hypothesis they are inhibited to act directly against class I MHC molecules on target cells.[236] In murine studies, it was shown that NK cells had the capacity to migrate to the tumor, infiltrate and selectively kill tumor cells without killing normal cells. In a mHag mismatched mouse allotransplant model, immunization against leukemia or fibrosarcoma resulted in anti-tumor responses without the development of GvHD.[237] In a recent study, NK cell-mediated GvHD reduction was demonstrated, where donor NK cells inhibited and lysed donor T lymphocytes activated during the initiation of GvHD.[238]

In human studies, after allogeneic HSCT, donor NK cells may target malignant cells if the patients lack KIR that recognizes class I HLA allele groups that are present in the donor.[239] NK cell alloreactivity could induce the GvL effect in patients with AML in both haploidentical[240-242] and HLA-matched[243-245] allogeneic settings with T cell depleted HSCT. The alloreactivity of the NK cells was demonstrated when the patients lacked the HLA-ligand for the donor-inhibitory KIR. However, this was not found in patients with acute lymphoid leukemia and CML.[246] Furthermore, the NK cell alloreactivity did not work either in allogeneic HSCT without T cell depletion. This may be explained by two studies showing that T lymphocytes in the graft alter KIR expression on NK cells[247] and Treg suppress cytotoxicity of the NK cells.[248]
One *in vitro* study showed NK alloreactivity against melanoma and renal cell cancer cell lines by lysing the tumor cells in human KIR-ligand mismatch setting.[249]
Recently, our group reported a safety analysis of donor-derived long-term *ex vivo*-expanded human NK and NKT cells given as DLI to four patients with different solid tumors.[250] Infusion of these cells did not cause acute GvHD or other severe side effects. One patient with hepatocellular cancer had decreased α-fetoprotein levels in serum following NK/NKT cell infusions suggesting a possible GvT effect.

Clinical results of studies of the allogeneic GvT effect in patients with different solid tumors (such as renal cell, breast, colon, ovarian, pancreatic and advanced primary liver cancer) achieved until today will be highlighted below.

The first allogeneic GvT effect on metastatic renal cell cancer was reported by Childs et al[112], observing a tumor response rate of 53% in 19 patients studied.[113] Regression of metastases was delayed occurring at a median of 129 days after transplantation. Since the first report by Childs et al, several studies were performed with different conditioning regimens and immunosuppression treatments against GvHD.[114-124] In all but two of these studies, GvT effect was reported with variable tumor response rates between 8% and 57%.[114, 115, 117-123] In the two studies by Pedrazzoli et al and Rini et al, the absence of tumor response was due to the lack of adoptive immunotherapy (DLI) following HSCT[124] and that patients with low performance status were enrolled in the study.[116]
The largest series of allogeneic HSCT in patients with renal cell cancer was reported in a European multicenter trial including 124 patients from 21 centers.[126] The tumor response rate was 32% and regression of metastases occurred at a median of 150 days after HSCT. Best overall survival of 70% at 2-years was seen in patients who had less than three metastatic sites, received DLI and developed chronic GvHD.

Allogeneic HSCT with non-myeloablative conditioning in metastatic breast cancer exhibited tumor response rates between 16% and 37%.[115, 118, 120, 128, 129] In the study by Bishop et al, the GvT effect was achieved by depletion of allogeneic T lymphocytes from the graft.[128] After allogeneic HSCT these lymphocytes were administered at escalating doses to the patients. The tumor response rate was 33% and tumor regression occurred concomitantly with the development of GvHD. Carella et al used another strategy to enhance the GvT effect and decrease the non-relapse mortality, namely to administer high-dose chemotherapy with autologous graft before allogeneic HSCT.[129] The tumor response rate was 24% and no non-relapse mortality was noted during the first 100 days.
The largest series of allogeneic HSCT in patients with breast cancer was reported from 15 centers including 66 patients.[130] Myeloablative conditioning regimen was used in 59% of patients, whereas 41% received RIC. In the RIC group, more patients had poor pretransplant performance status (63% vs 26%). The tumor response rate was 31% in the myeloablative group and 29% in the RIC group. Patients who developed acute GvHD after RIC regimen had lower risk of relapse or progression than those who did not. Progression-free survival at 1-year was 23% with myeloablative conditioning and 8% with RIC.

Allogeneic HSCT in metastatic colon cancer has also demonstrated the existence of a GvT effect. Hentschke et al reported six patients transplanted with advanced disease, where regression of metastases was seen in one patient and another one had mixed response.[117] The patient with mixed response had regression of lung metastases but progression of metastases in the liver. In the study by Kojima et al, four patients were treated and one patient achieved partial response and the others stable disease.[132] Carnevale-Schianca et al reported 15 patients, where one patient had partial response and three had stable disease.[133]
The largest series of allogeneic HSCT in patients with colorectal cancer was reported from nine European centers including 39 patients.[134] The tumor response rate was 46% and regression of metastases occurred at a median of 90 days after HSCT. Tumor regression occurred concomitantly with the development of GvHD and was achieved in 59% of patients who experienced either acute or chronic GvHD.

The GvT effect was also demonstrated in patients with ovarian cancer.[120, 136, 137] Bay et al presented five patients who had undergone allogeneic HSCT and four patients had tumor regression associated with the development of GvHD.[136] In the study by Blaise et al, five patients were treated and three patients had a tumor response.[120]
The largest series of allogeneic HSCT in patients with ovarian cancer was reported from six European centers including 30 patients.[137] The tumor response was 50% and three patients had tumor regression in correlation to the development of acute GvHD. The median overall survival was 10.4 months. Patients who developed chronic GvHD had a better overall survival compared to those who did not (17.6 months vs 6.5 months).

Patients with unresectable metastatic pancreatic cancer have also undergone allogeneic HSCT.[140-142] Takahashi et al reported five patients transplanted and two patients had tumor regression, whereas another two had decreased levels of the tumor markers CEA and CA19-9 in the serum.[140] In the study by Kanda et al, seven patients were treated and tumor response was observed in two patients and another had decreased levels of CA19-9 in the serum.[141]
The largest series of allogeneic HSCT in patients with unresectable pancreatic cancer was reported from three Japanese centers including 22 patients.[142] The tumor response rate was 23% and the median survival was 139 days. Patients who developed chronic GvHD tended to survive longer than those who did not.
At our center, two patients with resectable pancreatic cancer without metastasis underwent allogeneic HSCT with HLA-identical sibling donors in 2007. These two patients are alive, and with no sign of disease recurrence, three years after HSCT compared with the five control patients who all have died from their disease (unpublished data).

Combined orthotopic liver transplantation and allogeneic HSCT for advanced primary liver cancer is only performed at our center in Sweden. Five patients with non-resectable primary liver tumor have undergone orthotopic liver transplantation and were treated with allogeneic HSCT with non-myeloablative (low intensity conditioning) regimen.[138] In two patients, no recurrence of the disease was observed at a follow-up of 10 and 26 months. In two patients, no engraftment of donor stem cells was seen, whereas one rejected the graft 2 months after HSCT. In two of the patients, a stable mixed donor chimerism was established.

In conclusion, the existence a GvT effect against renal cell, breast, colon, ovarian, pancreatic and advanced primary liver cancer is suggested by the presented results. However, the use of allogeneic HSCT for solid tumors has been decreasing during the past years. One reason is the introduction of tyrosine protein kinases and kinase enzymes based medical therapies. Another reason is that the transplantation-related mortality is still high due to the patients´ physical condition to manage the transplantation. The allogeneic HSCT is therefore most successful in patients with low tumor load and good physical condition as reported by Barkholt et al.[126]

Inhibition or blockade of tyrosine kinases has increased survival in patients with poor prognosis[251] or doubled the progression-free survival in pretreated patients with renal cell cancer.[252] In patients with colon cancer treatment with antibodies against vascular endothelial growth factor (VEGF) or epidermal growth factor receptor (EGFR) have improved prognosis and have increased the median survival to 2 years.[253, 254] Inhibition of VEGF or VEGF-signaling pathway by bevacizumab, or sunitinib or sorafenib has also produced anti-tumor responses in patients with renal cell cancer.[252, 255, 256]

Finally, allogeneic HSCT would be improved even more in the future, if adoptive immunotherapies combined with new therapeutic drugs could be applied often in a stepwise space-rocket type of approach in order to reduce the tumor load. More specific adoptive immunotherapies that include genetic manipulation of donor lymphocytes as retroviral transfer of suicide genes and transfer of tumor-specific CTL against TAA, mHag or viral peptides, may also be promising strategies.

3 AIMS

The general aim of this thesis was to investigate the anti-tumor or GVT effect after allogeneic HSCT in patients with different type of solid tumors.

Specific aims were as follows:

- To study inflammatory and anti-inflammatory cytokine response in patients after HSCT and to find a correlation between cytokine levels and anti-tumor response (paper I)

- To determine the risk factors for complications in patients undergoing HSCT with low intensity as compared to reduced intensity conditioning regimen (paper II)

- To explore if measurement of cytokine secretion during DLI given to patients after HSCT correlates with anti-tumor response (paper III)

- To develop new methods for *in vitro* activation, detection and identification of tumor-reactive T lymphocytes from a patient with pancreatic squamous cell carcinoma as well as from an HLA-identical sibling (paper IV)

4 MATERIALS AND METHODS

Patients included in the first three studies for this thesis were transplanted at Karolinska University Hospital Huddinge between 1999 and 2006. Patient characteristics are summarized in Table 1.

4.1 BLOOD SAMPLES AND PREPARATION OF MONONUCLEAR CELLS

In paper I, III and IV in this thesis, peripheral blood samples were used as starting material. In paper I, blood samples were collected within 1 month before and 1, 3, 6, 9 and 12 months after SCT. At these time points, the patients were at the hospital for clinical check-ups and collecting blood samples at the same time was suitable. Blood sample collection is a non painful procedure for the patients and it is an easy and fast way to isolate mononuclear cells (MNC) or prepare serum.
In paper III, blood samples were collected on one occasion from controls, and just before DLI as well as 1 and 3 weeks after DLI, from patients. The blood samples were centrifuged and MNC were isolated by Lymphoprep (Axis-Shield PoC As, Oslo, Norway) gradient centrifugation.
In paper IV, blood samples were collected from a patient with pancreatic squamous cell carcinoma and an HLA-identical sibling.

4.1.1 ELISA and ELISpot analyses

For extracellular cytokine detection we used two different methods: enzyme-linked immunosorbent assay (ELISA) and enzyme-linked immunospot (ELISpot) techniques.

The ELISA technique measures secreted protein (cytokine) in body fluids or supernatants from different cell cultures. The disadvantage of the assay is the intervention of soluble cytokine receptors and inhibitors and the degradation of proteins. Cytokines are mainly produced locally, for that reason the detection of cytokines in serum may sometimes be a rough method. However, when systemic levels are identified, it reflects a strong reaction and some interesting correlations have been revealed.

In paper I, we used commercially available assays, i.e. automated chemiluminescence immunoassay (Immulite, DPC, Los Angeles, CA) for analyzing TNF-α and IL-10 and Quantikine ELISA kits (Quantikine R and D Systems, Minneapolis, Minn) for IFN-γ and TGF-β1 detection.

The highly sensitive ELISpot assay detects cells that actively secrete cytokines reflecting *in vivo* immune responses at single cell level. The disadvantage of the assay is that one cannot determine the phenotype of the cells since the cells are washed away during the procedure. However, this problem can be overcome if the studied cell populations are purified.

Paper	I	II	III
Time period of study	2000-2002	1999-2004	2001-2006
Number of patients	6	48	8
Males/Females	4/2	33/15	7/1
Recipient age, range	47-66	28-77	35-67
Diagnosis			
AML	0	0	3
MPS	0	0	1
Renal cell carcinoma	4	17	1
Colorectal carcinoma	2	15	2
Primary liver carcinoma	0	11	0
Prostate carcinoma	0	2	1
Other solid tumors	0	3	0
Donors			
HLA-identical sibling	3	25	1
MUD	3	23	7
Conditioning			
Bu+Cy	0	0	1
Flu+Bu	0	0	2
Flu+Melphalan	0	0	1
Flu+TBI	5	23	0
Flu+Cy	1	25	4
Graft source			
PBMC	6	46	8
BM	0	2	0
GvHD prophylaxis			
CsA+MMF	5	19	0
CsA+MTX	1	21	8
Tacrolimus+MMF	0	3	0
Tacrolimus+MTX	0	5	0
GvHD			
acute 0	2	12	1
acute I-II	2	29	5
acute III-IV	2	7	2
chronic 0	3	37	6
chronic limited	3	11	2
DLI			
Number of patients	5	31	8
Number of DLI, range	1-5	1-11*	1-5

* including infusions of *ex vivo* expanded mixed donor NK/NKT cells

Table 1. Patient characteristics.

This assay has been shown to have both a sensitivity and specificity of up to 200 times greater than conventional ELISA by enumerating dynamically the number of cytokine secreting cells both at basal level and after specific antigen stimulation.[257]

In paper III, PHA-stimulated MNC were analyzed by ELISpot assay for the cytokines TNF-α, IFN-γ, IL-4, IL-10, IL-12 and IL-13 (Mabtech AB, Stockholm, Sweden). All samples were tested in duplicates. One advantage of the analysis is that the cytokine secretion was evaluated by a single person. The ELISpot software counts the number of spots or spot forming cells (SFC) where each spot represents a single cytokine producing cell.

4.1.2 Flow-cytometric Assay of Specific Cell-mediated Immune response in Activated whole blood

Flow-cytometric assay of specific cell-mediated immune response in activated whole blood (FASCIA) analysis has been developed for the detection of specific immunity against several antigens.[258, 259] For example, VZV specific immunity in children has been studied with FASCIA.[260] In the study by Svahn et al, diluted whole blood was incubated with VZV-antigen and activated immune cells (T lymphocytes) against VZV were visualized by the identification of lymphoblasts displayed as large granular lymphocytes by their scatter profile using flow cytometry. So far, FASCIA has only been used to detect immunity against infectious agents, and the methodology has been based on peptide and/or protein antigens.

The advantage of using FASCIA assay as compared to other methods of T lymphocyte activation is that the FASCIA assay requires only small amounts of blood and little time and labours and can thus be readily carried out on single samples as well as for large-scale studies.[260] The use of whole blood samples without specific preparation of dendritic cells or cell separation saves a lot of time. Another advantage is the easy access to small volumes of blood where the cells experience an environment similar to that *in vivo*. The FASCIA technique also gives the possibility to a more sensitive read-out than mixed lymphocyte reaction, and can be considered as an enhanced mixed lymphocyte reaction. In paper IV, we have used the FASCIA technology with patient-derived tumor cells as targets and activated T lymphocytes as read-out.

Diluted whole blood samples and the patient's apoptotic/necrotic (apo-nec) tumor cells (discussed later) or irradiated MNC were examined by FASCIA for CD3+, CD4+, CD8+ cells and for the activation marker CD25 (Figure 2). Following FASCIA, CD4+CD25+ and CD8+CD25+ cells from the patient and his HLA-identical sibling were sorted with FACSAria (BD Biosciences, San José, CA) and analysed using FACSDiva software (BD Biosciences).

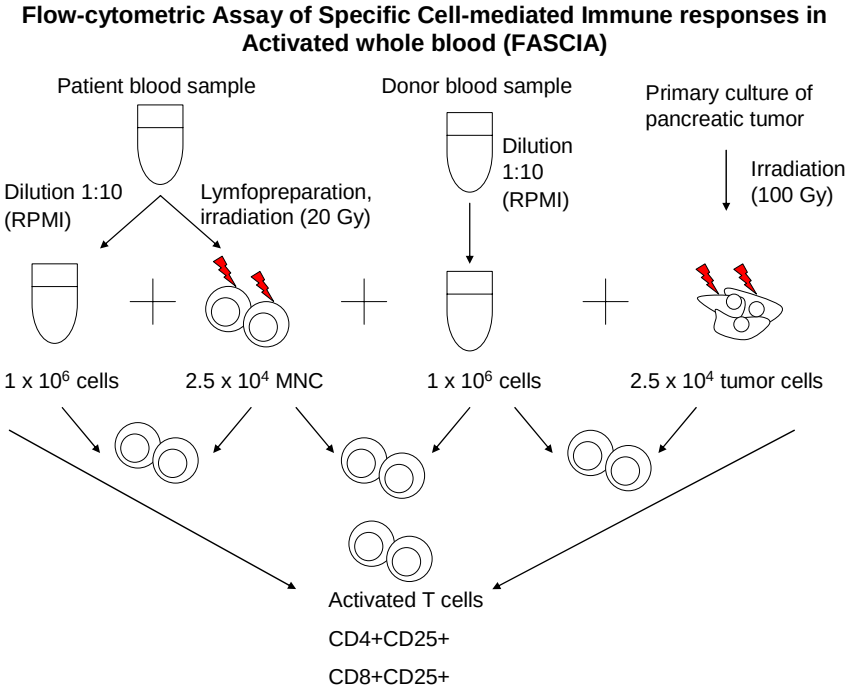

Figure 2. Schematic description of the FASCIA procedure.

4.2 PRIMARY CULTURE OF TUMOR CELLS

In paper IV, fresh pancreatic cancer tissue was obtained from a patient with pancreatic squamous cell carcinoma undergoing pancreatic resection surgery ad modum Whipple. After surgical resection, the malignant phenotype of the tumor was assessed using light microscopy. The tumor represented a squamous epithelial cell malignancy using staining with hematoxyline-eosin demonstrating morphology of squamous cell carcinoma. Immunohistochemical staining showed that the cells were negative for neuroendocrine tumor markers chromogranin A and synaptophysin, thereby neuroendocrine tumor origin was excluded.

After enzymatical digestion of the tumor, tumor cells were incubated and cultured. The initial growth rate was slow. After 3 weeks of incubation and feeding which comprised change of culture medium twice weekly, the tumor cells began to grow and on day 43 these cells were split the first time by trypsinization. Following trypsinization, which removed most stroma derived cell types, the remaining adherent malignant cells were left untouched in the culture flask except for regular media changes. After two months of culturing, the malignant cells had grown to confluency and the majority of the cells appeared to be of epithelial origin and had a malignant phenotype. The prolonged culture period was expected due to the slow growth of the primary tumor and as a consequence of removal of contaminating stromal cells.

The malignant cells demonstrated varying morphology where the majority of the cells had small and round nuclei with clearly visible nucleolis. Multinuclear cells as well as elongated “spindle” shaped cells, possibly representing motile forms, could be observed in the cell culture (Figure 3).

Apo-nec tumor cells were prepared from the patient's tumor cells slightly modified as described by von Euw et al.[261] After gamma irradiation at 100 Gray, the tumor cells were plated onto small culture flasks and incubated for 48 hours to complete the apoptotic process. To confirm that the tumor cells had become apoptotic and necrotic, Annexin V and Propidium iodide binding (Annexin V-FITC Apoptosis Detection Kit, BD Biosciences) and flow cytometric analysis were performed.

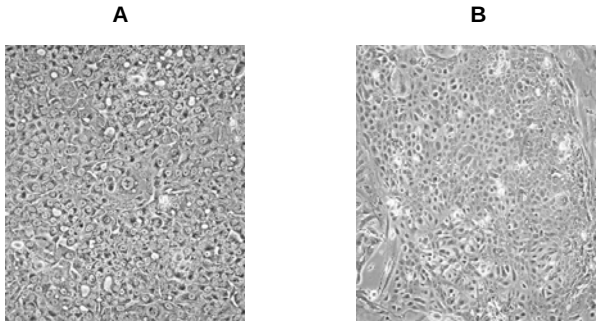

Figure 3. Primary culture of tumor cells from a patient with pancreatic squamous cell carcinoma.
The figure show tumor cells from the patient at 36 (A) and 62 (B) days, respectively.

4.3 CDR3 SIZE SPECTRATYPING

CDR3 size spectratyping is based on amplification of 24 TCR Vβ genes and characterizes the relative usage of each Vβ gene subfamily and also identifies clonal T lymphocyte populations within each subfamily.[262]

This method is sensitive and has the advantage to cover the entire Vβ repertoire as well as to detect changes in T lymphocyte repertoire that can not be performed with flow cytometric analysis. It can be applied for interpretation and identification of clonal T lymphocyte populations responsible for clinical *in vivo* reactions such as GvL/GvT effect and GvHD. The main disadvantage of the method is that it does not reveal the functional specificity of the T lymphocyte clones.

In paper IV, RNA from flow cytometry sorted cells from the patient and his HLA-identical sibling was prepared using the commercially available QIAmp RNA Blood Mini Kit (QIAGEN, Hilden, Germany). cDNA was synthesized from RNA using the high-capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). After cDNA synthesis, reverse transcription (RT)-PCR was performed to amplify the CDR3 of the TCR. A set of 28 Vβ-specific primers spanning all 24 TCR Vβ subfamilies were amplified by a constant 5'-end primer labelled with 6-fluorescein phosphor amidite (6-FAM).[263]

The size distribution of each PCR product was analyzed using capillary electrophoresis on an ABI 3130 Genetic Analyzer (Applied Biosystems) allowing one base pair resolution. The fluorescence intensity of each band was quantified with the Peak Scanner Program (Applied Biosystems) and translated into a histogram displaying the length and intensity of each CDR3 fragment.

4.4 RESPONSE EVALUATION CRITERIA IN SOLID TUMORS

In patients with solid tumors, the tumor load was examined by computer tomography (CT) of the thorax and abdomen before SCT, and following every third month after SCT (paper I, II and III). Evaluation of tumor response was based on the international response evaluation criteria in solid tumors (RECIST).[264]

In paper II, RECIST was not applicable in patients with advanced primary liver cancer because of the lack of pre-transplant metastases. Therefore, these patients were evaluated using clinical parameters based on the results of CT (thorax and abdomen), bone scans and magnetic resonance imaging of liver, bile ducts, and pancreas performed according to the same time schedule after SCT as for the other solid tumor patients, or autopsy examinations.

In paper I, examination of tumor load was also evaluated based on our local method. We used the combined results of CT and autopsy examinations showing a decrease in size and/or number of metastases. Regression, progressive and stable disease were defined separately for each metastatic localization (lung tissue, pleura, lymph nodes and liver). Furthermore, an assessment of the total metastatic load was performed, defined as mixed response, stable disease and progressive disease. Each CT examination was compared with the closest preceding CT examination.

There are both advantages and disadvantages with RECIST and our local method, respectively. One disadvantage of RECIST is the possibility to choose between the evaluation of target lesions or evaluation of both target and non-target lesions (i.e., the best overall response). Another disadvantage with RECIST is the choice of the five largest metastases per organ (and ten largest in total), which implies that smaller metastases in other organs are missed. Therefore, in some patients, the evaluation of the largest target lesions may occur only in one organ, thereby failing to detect the changes of other metastases. In contrast, our local method includes four main metastatic localizations independent of their initial size. Furthermore, an appearance of a new metastasis independently of the organ localization will be evaluated as progressive disease using RECIST, despite the regression of other metastases in the same or other organs. One disadvantage of our local method is that mixed response in some cases was

classified as tumor regression, i.e. despite the fact that progression was not totally absent in the patient. However, our evaluation method reflects the dynamics of the metastases over time because it compares the changes with the previous CT examination instead of the one which was performed pretransplantation as is done according to RECIST.

RECIST has been developed within oncology where it was mainly used after chemo radiotherapy. This therapy initiates cell necrosis of metastases whereas immunological cell therapy triggers inflammation in the tumor. The inflammation appears as increased tumor size or volume estimated with radiological examinations, which is misleading since this will be classified as tumor progression with RECIST. Immunological cell therapy is therefore preferably evaluated when the inflammation has disappeared and the apoptotic/necrotic tumor cells appear as stable disease or regression of the metastases.

4.5 STATISTICAL ANALYSES

In paper I, the two-sided Fisher exact-test was used to compare the cytokine balance to tumor response.

In paper II, the probability of overall survival was calculated according to the Kaplan and Meier method. Time to transplantation-related death, response, acute GvHD, and chronic GvHD were estimated using a non-parametric estimator of cumulative incidence curves. Competing events for transplant-related mortality were death in progressive disease for response, death without response, and death without GvHD. Patients were evaluated for tumor response and chronic GvHD if they survived more than three months. The differences between numbers of days to complete donor chimerism in T and B cells were compared using the Mann-Whitney U test. Analyses were performed using the cmprsk package (developed by Gray, June 2001), Splus 6.2 software, and Statistica software (Statsoft Inc, Tulsa, OK, USA). A p-value <0.05 was considered statistically significant.

5 RESULTS AND DISCUSSION

Allogeneic HSCT using non-myeloablative conditioning has been explored as immunotherapy against progressive disease in patients with different solid tumors. In the beginning of the SCT era, it has become clear that the transplanted immune cells were capable of eradicating tumor cells. This immunological anti-tumor effect or GvT effect has been studied by several groups with slightly varying results. This is most likely due to the different conditioning types, immunosuppression strategies and last but not least, differences in the patients underlying disease. The anti-tumor effect may be strengthened by DLI from the same stem cell donor when tumor progression threatens or mixed chimerism appears. DLI may also contribute to GvHD. The risk of GvHD may be reduced using escalating doses of DLI. However, it seems that donor T lymphocytes mediate both GvHD and GvT effect since the risk of tumor recurrence is higher when the donor T lymphocytes are depleted from the graft. Therefore, a patient having mild acute and/or chronic GvHD has potentially a high chance of the GvT effect.

In the first three studies of this thesis, we investigated the anti-tumor effect in patients with different solid tumors undergoing HSCT. More specifically, in paper I and III, the anti-tumor effect in connection to cytokine release was examined. In paper II, we studied the outcomes after HSCT and reported the most favorable tumor response and the best anti-tumor effect. In paper IV, we showed that it was possible to detect and identify tumor-reactive T lymphocytes from an HLA-identical sibling against tumor cells from a patient with pancreatic squamous cell carcinoma.

5.1 THE RELATION BETWEEN GVT EFFECT AND CYTOKINE RELEASE AFTER HSCT (PAPER I AND III)

Previous studies from our group have revealed allogeneic GvT effect in patients with metastatic colon and renal cell carcinoma.[117, 131] Hentschke et al reported disparate responses of metastases in one patient with colon and two patients with renal cell carcinoma, who had regression of lung metastases but progression of metastasis in the liver.[117] Another study in our group showed higher levels of TNF-α, IFN-γ and IL-10, but lower levels of TGF-β1 in patients with myeloid leukemia who developed acute GvHD II-IV two weeks after SCT in contrast to those with no or mild GvHD.[39]

These results prompted us to examine whether there is a correlation between cytokine release and tumor response in patients with different solid tumors. In paper I, we found a correlation between the two inflammatory cytokines TNF-α and IFN-γ and tumor regression in contrast to the two anti-inflammatory ones IL-10 and TGF-β1 which correlated with disease progression. In paper III, we reported a higher expression of TNF-α, IFN-γ, IL-12 (another inflammatory cytokine) and IL-10 in patients with tumor regression in contrast to those with disease progression during DLI therapy.

Mild acute GvHD grade I-II and chronic GvHD have been associated with the GvL/GvT effect resulting in leukemic/tumor cell regression[126, 219], whereas severe acute GvHD can lead to transplantation related complications. Secretion of the inflammatory cytokines TNF-α and IFN-γ in serum has been reported during the conditioning before SCT and the development of acute GvHD.[37, 39, 45] Antigen presenting cells of the host may activate donor T cells of the graft, thereby enhancing this inflammatory cytokine production.

Previous studies reported high levels of TNF-α in serum and increased TNF-α and IFN-γ producing MNC in patients with hematological malignancies who developed acute GvHD grade I-IV.[40, 52] Similarly, increased TNF-α and IFN-γ levels in serum were found in three of our six patients in association with conditioning and acute GvHD early after SCT. Furthermore, in our patients, who were diagnosed with acute grade II-III and chronic GvHD after DLI, also high expression of TNF-α and IFN-γ in MNC was found during DLI followed by tumor regression. However, in another study decreased IFN-γ producing MNC was reported in patients with hematological malignancies who had grade 0-I acute GvHD.[50]
Since severe acute GvHD grade III-IV is associated with transplantation related complications, more thorough analyses for cytokines in two patient groups, acute GvHD grade I-II and grade III-IV, would be advantageous in order to be able to predict the outcome of DLI administration with escalating doses.

IFN-γ may contribute to an anti-tumor effect by stimulating an immune response since it can up-regulate MHC class I expression on tumor cells.[265] Thus, an increased tumor development mediated by IFN-γ deficiency may occur because of diminished control of target cell growth and apoptosis. High TNF-α and IFN-γ levels in serum found toward the end of the first year after SCT and secretion from MNC found in our studies during DLI therapy may contribute to the GvT effect and are in line with observations in other human studies. Increased expression of IFN-γ in T cells has been reported in patients with CML who achieved clinical remission after DLI.[266] In contrast, decreased IFN-γ in MNC has been found in patients with hematological malignancies who relapsed compared to those with no signs of relapse.[51] DLI containing T cells may contribute to an anti-tumor effect by enhanced IFN-γ production as was seen in some of our patients who showed regression of metastases after DLI.

IL-10 was first described as an anti-inflammatory cytokine, but recent studies have shown that in some cases it acts as an inflammatory one.[267] In some studies immunosuppressive activities were observed as expected, while in others IL-10 augmented immune or inflammatory responses. The results regarding the levels of IL-10 in serum after transplantation and their correlation to the incidence of GvHD are inconsistent. In some studies increased levels of IL-10 after HSCT have been related to low occurrence of acute GvHD.[268, 269] In other studies high levels of IL-10 have been noted before or during acute GvHD.[39, 40, 65, 66, 270-273]

Furthermore, the functional role of IL-10 producing cells in protective immunity has also been demonstrated in kidney[274] and pancreatic islet[275] transplant patients. The ratio of IFN-γ/IL-10 differed between non-rejecting and rejecting transplant patients. Low ratio due to high IL-10 secretion was found in patients with stable graft function.[274, 275]

In our study, high expression of IL-10 in MNC before DLI in patients with favorable outcome of disease response seemed to discern those patients who did not respond with low IL-10 secretion just before DLI. However, Schulz et al reported high circulating levels of IL-10 in patients who failed to respond to DLI, suggesting moderation of the immune system and thereby allowing the leukemic progression.[276] In our study, patients with disease progression showed temporarily increased IL-10 expression in MNC in the samples obtained between each DLI. Thus, IL-10 might have suppressed the cytotoxic effect of donor lymphocytes against leukemic/tumor cells.

The major biological function of IL-10 is to combat inflammatory responses[267] and it is therefore difficult to decide whether IL-10 observed during an inflammatory response such as acute GvHD or GvT effect is due to the response or is secreted as a regulatory mechanism. In the future, we may envisage studies where IL-10 acts in synergy together with inflammatory cytokines supporting immune or inflammatory responses e.g., GvT effect following allogeneic HSCT.

IL-12 is a potent immunostimulatory cytokine and an inducer of Th1 cell activity and IFN-γ production. IL-12 has been reported to play an important role in acute GvHD and GvL effect. High levels of IL-12 in plasma were associated with the development of acute GvHD grade II-IV[72] and with the GvL effect without increasing the risk for GvHD.[73] In our study, high expression of IL-12 in MNC was found in patients with disease response, supporting the anti-tumor effect of this cytokine.

Whether increasing number of infused CD3+ T cells using escalating doses of DLI does influence the balance of inflammatory/anti-inflammatory cytokines needs to be further studied in larger patient groups to determine optimal timing and setting of DLI administration.

Based on our studies, patients seemed to benefit from DLI therapy, (i.e., tumor regression) when their MNC have high capacity to produce IL-10 and the inflammatory cytokines TNF-α, IFN-γ and IL-12. Measurement of the kinetics of these cytokines could predict how the immune response develops after allogeneic SCT. Monitoring these cytokines before administration of DLI could also predict those patients who will respond to DLI therapy. In the future, this monitoring might be useful for directing infusion of donor lymphocytes or NK cells or adoptively transferred tumor specific T cells of stem cell donor origin to intensify the GvT effect after allogeneic SCT against solid cancer.

5.2 OUTCOMES AFTER HSCT (PAPER II)

In 1999, when our centre started with allogeneic HSCT for patients with solid tumors, different non-myeloablative protocols were already in use for patients with hematological malignancies and also to some extent at other centers for patients with solid tumors. Our centre decided to utilize a low intensity conditioning with only 2 Gy TBI and Flu together with the immunosuppressive drugs CsA and MMF. However, in 2001, the high risk of graft rejection prompted us to replace TBI by Cy, thus RIC with Flu and Cy was in use.

Non-myeloablative treatment is suitable for patients with solid tumors since the main emphasis is not to eradicate the hematopoietic cells in the bone marrow, as it is the case with myeloablative treatment for hematological malignancies. This treatment may also be effective for patients with solid tumors older than 50 years, which is the common age, since they could tolerate a transplant better if organ impairment is a difficulty. In this study, we report the outcomes after HSCT based on six years of experience in 48 patients with different solid tumors such as renal cell, colorectal, prostate and advanced primary liver cancer, applying low or reduced intensity conditioning.

There were equal numbers of patients in the two conditioning groups. The age of the donors was lower for patients treated with RIC, which could be explained by a larger proportion of unrelated donors in this group. Because solid tumor patients are often elderly, theirs siblings are also elderly, whereas donor registries worldwide have younger unrelated donors.

Even though, rejection of stem cells occurred in double as many patients in the low intensity group than in the RIC group, no significant difference was found between the two conditioning groups. Our centre uses chimerism analysis of CD3+ cells to define rejection. To better predict rejection, NK cells may also play an important role in chimerism analysis. Less than 50% of donor T and NK cells on day 14 after HSCT has indicated an increased risk for rejection in patients given low intensity conditioning.[277]

Patients receiving low intensity conditioning had a shorter neutropenic phase and earlier development of donor B cell chimerism than patients treated with RIC. Hematopoietic growth factors such as G-CSF have been used after HSCT to accelerate myeloid recovery and shorten the high risk period of bone marrow aplasia.[278] Therefore, patients in the low intensity conditioning group were treated with G-CSF after HSCT which may explain the shorter time to neutrophil engraftment.[279-281] Thus, our finding of higher number of G-CSF-treated patients in the low intensity group was expected since our previous findings of a higher occurrence of acute GvHD grade II-IV in patients with hematological malignancies.[279, 282] This result encouraged us to discontinue G-CSF treatment in patients receiving RIC. However, in this study, we did not find an increased risk of acute GvHD in patients who were treated with G-CSF. The use of G-CSF might also explain the incidence of fewer bacterial infections in the low intensity conditioning group, even though the difference in the two conditioning groups was not significant.

The trend to increased risk of bacterial infections in the RIC group could be explained by a stronger effect of Cy and prolonged Flu treatment that may have caused more effective suppression of the patients' immunocompetent cells as compared to the low intensity group.

There was no significant difference in the incidence of tumor response between the two conditioning groups. Patients with advanced primary liver cancer had the most favorable tumor response compared to all other tumor types (70% vs. 32%).

Furthermore, tumor response was more common in patients receiving adjuvant cell infusions, i.e. DLI or NK/NKT cells, and who developed chronic GvHD either before or after the cell infusions, in the absence of an association with the type of conditioning (75% vs. 34% of all other patients). This was also demonstrated in a larger patient group with renal cell cancer.[126] The effect of DLI and GvHD seems to support the allogeneic GvT effect which has been shown to be associated with CD8+ T cells in patients with renal cell cancer.[46, 283]

Considering these results, we suggest that T and NK/NKT cell functions of DLI may trigger the development of chronic GvHD. This would induce an inflammatory cytokine and chemokine response, supporting the migration of donor lymphocytes towards tumor cells. In this way infusion of donors' NK/NKT cells would be a valuable alternative to DLI. In this study, four patients in the RIC group received *ex vivo* long-term expanded NK/NKT cells from the stem cell donor. None of the patients developed acute GvHD after the cell infusions as reported in more detail by our group.[250] Furthermore, no significant difference was found in the incidence of severe acute GvHD grade III-IV after DLI between the two conditioning groups. However, 32% of patients in the RIC group developed acute GvHD grade II after DLI compared to none of the patients receiving low intensity conditioning. This may imply that acute GvHD grade II is desirable for the allogeneic GvT effect in patients with solid tumors, reflecting the tendency towards prolonged survival in patients given RIC.

A tendency for prolonged overall survival was found in the RIC group compared to the low intensity conditioning one (30% vs. 17% at 2-years). Patients with advanced primary liver cancer had the longest overall survival which might be due to the fact that these patients are younger (median 48 years) than patients with renal cell (median 58 years) and colorectal cancer (median 60 years). However, the overall survival for patients with renal cell and colorectal cancer was longer than that achieved with even the most modern combinations of oncological treatments for metastatic disease. The transplantation-related mortality rate was 65% in the low intensity conditioning group and 52% in the RIC group. Although progress has been made in the field of allogeneic HSCT for patients with solid tumors with a tendency for prolonged survival, the majority of patients, irrespective of tumor type, still die in progressive disease. Previous results on tumor response vary between 17-40% in patients with renal cell,[113, 114, 126] breast[115, 118, 120] and ovarian cancer.[136] However, in most studies only sibling donors were considered which implies that the conditioning regimens did not include antithymocyte globulin and caused less immunological imbalance.

Since the major obstacle for HSCT in the treatment of patients with solid tumors is progression of the underlying disease, the results in this study could potentially be improved if patients with low tumor load and good physical condition are selected for HSCT. Allogeneic HSCT could be given only to patients with stable disease at the time of transplantation. In the future, there might be a possibility for infusion of donor-derived immune or tumor-specific cells instead of DLI after SCT in patients with tumor progression to exert a stronger GvT effect.

5.3 TUMOR-REACTIVE T LYMPHOCYTES FOR PANCREATIC CANCER (PAPER IV)

In this study, we detected and identified tumor-reactive T lymphocytes from an HLA-identical sibling against tumor cells from a patient with pancreatic squamous cell carcinoma. Using the FASCIA technique we could activate T lymphocytes in whole blood from the HLA-identical sibling against patient-derived tumor cells, identify the phenotype and sort activated T lymphocytes based on their expression of activation markers. Using the CDR3 size spectratyping of TCR genes we could distinguish tumor-reactive T lymphocytes from activated T lymphocytes against peripheral blood MNC from the patient. Using this method, previous studies have demonstrated the presence of T lymphocyte populations associated with GvT effect and GvHD.[262, 284, 285] It has been shown that expansion of some clones within the TCR Vβ repertoire appeared early after DLI, and together with clinical responses this suggests that these clones mediate the GvL and GvT effect in patients with CML[285] and multiple myeloma[262], respectively. In the study by Orsini et al other T lymphocytes clones appeared at later time points when GvHD was developed. Michalek et al identified and monitored an alloreactive T lymphocyte clone associated with GvHD in a patient with AML.

Based on the results of this study, we suggest that allogeneic HSCT should be applied to a larger extent for patients with pancreatic adenocarcinoma since these patients' tumor load is limited. Furthermore, pancreatic adenocarcinoma is a solid cancer with poor prognosis where the only curative strategy is surgery. At the time of diagnosis the majority of patients have an advanced disease which is inoperable; therefore they cannot be offered a curative but rather a palliative treatment.[286] Patients, who can be treated with surgery and who show no signs of remaining tumor, have an expected 5-year survival of 10-25%.[287]
Until today, three centres in Japan apply HSCT for patients with unresectable pancreatic adenocarcinoma using HLA-identical sibling donors.[139-141] In one clinical trial they reported tumor response of 23% and median survival of 139 days on 22 patients (15 patients with metastatic disease).[142] At our centre, two patients with resectable pancreatic adenocarcinoma without metastasis underwent HSCT with HLA-identical sibling donors in 2007. These two patients are alive, and with no signs of disease recurrence, three years after HSCT compared with the five control patients who all have died from their disease (unpublished data).

Combining FASCIA and CDR3 size spectratyping might be a way to identify tumor-specific T lymphocytes as well as to isolate and expand these cells for infusion of tumor-specific or leukemia-specific DLI to patients with tumor progression or disease relapse. The methods could be evaluated also for infection-specific DLI to cure viral and bacterial infections in transplanted patients with suppressed immune responses. Another approach would be to identify and deplete GvHD-specific T lymphocytes clones in patients with GvHD development.

In the future, there might be a possibility to enhance the GvT/GvL effect by *in vitro* expansion and infusion of anti-tumor/leukemic specific donor T lymphocytes from donors instead of ordinary DLI after SCT in transplanted patients with signs of tumor progression or disease relapse.

6 CONCLUSIONS

- Dominating TNF-α and IFN-γ levels in serum correlates with tumor regression in patients with renal cell and colon cancer after allogeneic HSCT using our local method on examination of tumor load.

- Increased expression of TNF-α, IFN-γ, IL-12 and IL-10 in MNC was found in patients with solid tumors and hematological malignancies with favorable outcome of disease response after DLI therapy.

- In patients with different solid tumors receiving low intensity conditioning, engraftment and development of donor B cell chimerism occurred earlier than in patients given RIC.

- Patients with advanced primary liver cancer who had previously undergone orthotopic liver transplantation had the most favorable tumor response.

- The most favorable tumor response was more common in patients receiving DLI or NK/NKT cells, and who developed chronic GvHD either before or after the cell infusions.

- A tendency for prolonged overall survival was found in patients receiving RIC compared to the low intensity conditioning group.

- Tumor-reactive T lymphocytes from an HLA-identical sibling against tumor cells from a patient with pancreatic cancer could be detected and identified using FASCIA. Using CDR3 size spectratyping tumor-reactive T lymphocytes could be distinguished from T lymphocytes activated against peripheral blood MNC from the patient.

These findings might give together with new strategies a better treatment for patients after allogeneic HSCT aiming at a more effective tumor response. For example, monitoring cytokines before adjuvant cell infusions could predict those patients who will gain from immunotherapy. Furthermore, combining FASCIA and CDR3 size spectratyping might be a way to identify, isolate, *in vitro* expand and infuse tumor/leukemia-specific T lymphocytes from stem cell donors in order to intensify the GvT/GvL effect when signs of tumor progression or disease relapse threaten.

7 FUTURE PERSPECTIVES

Because donor T lymphocytes mediate both GvHD and GvT effect, the main goal in this field is to design methods where we can distinguish between the cells responsible for GvHD and those that mediate the GvT effect. Thus, suppression of GvHD with maintenance of GvT effect is a desirable outcome for clinical allogeneic HSCT.

One way to achieve this would be co-transplantation of expanded natural CD4+CD25+ regulatory T lymphocytes (Treg) with donor T lymphocytes. In a murine study, it was shown that co-transplantation of these lymphocytes inhibited the development of GvHD while preserving the GvT effect.[288] The authors claimed that massive proliferation of alloreactive T lymphocytes in the graft was inhibited by Treg, which was associated with the onset of GvHD without affecting their function. Thus, Treg could separate GvHD from GvT activity mediated by conventional donor T lymphocytes. The expansion of Treg was performed by stimulating CD4+CD25- T lymphocytes with IL-12 in the presence of Treg. The CD4+CD25- T lymphocytes proliferated and expanded while they expressed the activation marker CD25.

Another way would be infusions of Th17 lymphocytes of donor origin instead of ordinary DLI to enhance the GvT effect. It has been shown that Th17 lymphocytes can promote anti-tumor immune responses indirectly through the recruitment of DC and cytotoxic effector cells and by promoting effector T and NK cells trafficking to, and retention within the tumor microinvironment (reviewed in Zou et al).[18]

Another possible treatment to intensify the GvT effect may be the generation of specific CTL against viral peptides to treat solid tumors as reported by Takahashi et al, where the target antigen for CTL in renal cell cancer appeared to be a human endogenous retrovirus.[140]

In the future, we may envisage generation, expansion and infusion of Th17 lymphocytes or specific CTL against viral peptides to control tumor progression in order to intensify the GvT effect. Furthermore, co-transplantation of expanded Treg at the time of allogeneic HSCT may also contribute to a favorable tumor response.

Finally, the use of FASCIA and CDR3 size spectratyping methods might be a way to identify tumor-specific donor CTL against different solid tumors as well as to isolate and expand these cells for infusion of tumor-specific or leukemia-specific DLI. These methods could be evaluated also for infection-specific DLI to cure viral and bacterial infections in transplanted patients with suppressed immune responses.

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(Goethe)

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10 PAPERS