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Molecular Monitoring of Acute Graft-versus-Host Disease after Allogeneic Stem Cell Transplantation

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To my parents and the memory of Jan-Erik

Original Papers

This thesis is based on the four papers below, papers that are referred to by their Roman numerals:

I. Increased levels of immune transcript in patients with acute GVHD after allogeneic stem cell transplantation. **Jaksch M.**, Uzunel M., Martinez Cangana G., Remberger M. and Mattsson J. Bone Marrow Transplantation, 2003, 31: 183-190

II. Increased immune transcript levels are correlated with acute GVHD and CMV response after allogeneic stem cell transplantation. **Jaksch M.**, Remberger M. and Mattsson J. Transplantation, 2004, 77(2), January 27: 195-200

III. Molecular monitoring of T-cell chimerism early after allogeneic stem cell transplantation may predict the occurrence of acute GVHD grades II-IV. **Jaksch M.**, Uzunel M., Remberger M., Sundberg B. and Mattsson J. Submitted to Clinical Transplantation

IV. Increased gene expression of chemokine receptors are correlated with acute GVHD after allogeneic stem cell transplantation. **Jaksch M.**, Remberger M. and Mattsson J. Submitted to Transplantation

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List of Abbreviations

AICD	Activation-induced Cell Death
APC	Antigen Presenting Cell
BM	Bone Marrow
Bu	Busulfan
CD	Cluster of Differentiation
cDNA	Complementary DNA
CML	Chronic Myeloid Leukemia
CMV	Cytomegalovirus
CsA	Cyclosporine A
CTL	Cytotoxic T Lymphocyte
CTLA	Cytotoxic T Lymphocyte Antigen
Cy	Cyclophosphamide
DC	Dendritic Cell
DLI	Donor Lymphocyte Infusion
DNA	Deoxyribonucleic Acid
EC	Endothelial Cell
EBMT	European group for Blood and Marrow Transplantation
EBV	Epstein-Barr Virus
ELISA	Enzyme-linked Immunosorbent Assay
FasL	Fas Ligand
G6PD	Glucose-6-phosphate Dehydrogenase
G-CSF	Granulocyte Colony Stimulation Factor
GM-CSF	Granulocyte/Macrophage Colony Stimulating factor
GVHD	Graft-versus-Host Disease
GVL	Graft-versus-Leukemia
GVT	Graft-versus-Tumor
HLA	Human Leukocyte Antigens
HSV	Herpes Simplex Virus
IBMTR	International Bone Marrow Transplantation Registry
IFN	Interferon
IL	Interleukin
IL-1ra	IL-1 Receptor Antagonist
KGF	Keratinocyte Growth Factor
LPS	Lipopolysaccharide
MCP	Monocyte Chemoattractant Protein
MHC	Major Histocompatibility Complex
miH	Minor Histocompatibility Antigens
MIP	Macrophage Inflammatory Protein
M-MLV	Moloney Murine Leukemia Virus
MoAb	Monoclonal Antibody
mRNA	Messenger RNA
MTX	Methotrexate
MUD	Matched Unrelated Donor
NK-cell	Natural killer cell

NO	Nitric Oxide
NST	Nonmyeloablative Stem Cell Transplantation
PBSC	Peripheral Blood Stem Cells
PCR	Polymerase Chain Reaction
PUVA	Psoralen and Ultraviolet Light A
RNA	Ribonucleic Acid
RSV	Respiratory Syncytial Virus
RT-PCR	Reverse Transcription-PCR
SCID	Severe Combined Immunodeficiency
SCT	Stem Cell Transplantation
TBI	Total Body Irradiation
TGF	Tumor Growth Factor
TNF	Tumor Necrosis Factor
VZV	Varicella Zoster Virus
WBC	White Blood Cell

Summary

Acute graft-versus-host disease (GVHD) remains a major barrier to the wider application of allogeneic stem cell transplantation (SCT) for a variety of diseases. GVHD occurs when the transplanted donor T-lymphocytes react to host antigens on antigen-presenting cells and attack host tissues. The donor lymphocytes are introduced into a milieu that promotes their direct attack on target cells, causing tissue damage through perforin, granzyme B, and Fas/Fas ligand (FasL) interactions. In addition, the dysregulated production of inflammatory cytokines, such as TNF-α, IFN-γ, IL-1, and others, may cause direct tissue damage. The immunobiology of acute GVHD is complex and the precise mechanisms by which host tissues are damaged remain unclear. Despite progress in understanding the mediators involved in acute GVHD, treatment has remained frustrating; most patients who develop the severe manifestations of GVHD succumb to it or to complications of its treatment. Today, the only available method of diagnosing acute GVHD relies on clinical observations and clinical judgments. Since plenty of other problems can influence the symptoms seen in patients after SCT and further confuse the picture of the patient's disease, diagnosing acute GVHD can be rather difficult. It is well known that predicting the risk of acute GVHD before its clinical manifestation and early administration of additional therapy may result in less incidence of severe GVHD. Hence, new methods to diagnose acute GVHD are desired. Therefore, the major aim of this thesis has been to find new reliable molecular methods to diagnose acute GVHD after allogeneic SCT.

The gene expression of T-cell effector molecules, granzym B, perforin, FasL, and TNF-α, was evaluated as diagnostic markers for acute GVHD after SCT. Peripheral blood samples from patients were analysed by competitive or real-time polymerase chain reaction (PCR). An up-regulation of the gene expression of was seen in association with acute GVHD diagnosis in 23 of 27, 26 of 27, and 24 of 27 patients diagnosed with acute GVHD for granzyme B, perforin and FasL, respectively. The gene expression of TNF-α did not show the same pattern. Interestingly, we also found that patients with increasing levels during steroid treatment, 2 of 3 in Paper I and 10 of 10 in Paper II, showed persistent or deteriorating acute GVHD.

Using VNTR analysis, chimerism patterns were investigated in 34 patients during the early post-transplantation period. The difference in the clearance rate of host T-cells between day 7 and day 10 was compared. In this study, we found that there was a significantly higher risk for patients with complete donor chimerism on day 7, together with patients with an increase of more than 50% in the donor CD4+ T-cell population between day 7 and 10, to develop grades II-IV acute GVHD. Our data indicate that early monitoring of T-cell chimerism after SCT may be of use for early identification of patients at risk of developing moderate to severe acute GVHD after SCT.

We hypothesized that the gene expression of chemokine receptors in peripheral blood from patients after SCT would reflect the development of acute GVHD since these molecules are involved in the recruitment of activated T-lymphocytes during acute GVHD. Quantitative real-time PCR was used to assess the gene expression of the chemokine receptors CCR5, CXCR3, CCR1, and CCR2. In this study we found that increasing levels of these receptors were associated with acute GVHD. We found increasing levels in connection to acute GVHD diagnosis of all four markers in 35, 33, 35, and 35 of 46 occasions of acute GVHD for CCR5, CXCR3, CCR1, and CCR2,

respectively. Another important finding was that the gene expression levels increased before acute GVHD was diagnosed clinically with the median number of days before diagnosis ranging from 3 to 5 days.

In conclusion, although the pathophysiology of acute GVHD still remains complex, this study shows that different molecular markers involved in this complicated disorder may be used to diagnose and predict acute GVHD and to monitor steroid treatment. This may prevent life-threatening complications and improve the outcome for patients after allogeneic SCT.

General Introduction

Over the past half century, hematopoietic stem cell transplantation (SCT) has evolved from an idea to a well-established therapy used to treat tens of thousands of individuals annually. SCT allows the replacement of an abnormal lymphohematopoietic system with a normal one, making it an effective therapy for nonmalignant diseases such as severe combined immunodeficiency disease (SCID), aplastic anemia, thalassemia, and sickle cell disease. Since hematopoietic toxicity is dose-limiting for many types of chemotherapy and systemic radiotherapy, SCT may be used in the treatment of a variety of malignant diseases to permit the administration of higher and potentially more effective doses of therapy. SCT may also cure malignant diseases by itself through an immunologic attack on cancer cells.

Allogeneic Hematopoietic Stem Cell Transplantation

The early clinical studies on hematopoietic SCT were pioneered by E. Donnall Thomas and colleagues by the end of 1950s (*1*). In 1957, they performed the first allogeneic bone marrow transplantation in humans. Despite poor results, this trial showed for the first time that when grafts were carefully screened before infusion, to remove fat and bone particles, relatively large amounts of bone marrow could be administered to human patients without serious side effects. A major breakthrough in the field of bone marrow transplantation, now known as SCT, was the discovery of the major histocompatibility complex (MHC) and the human leukocyte antigens (HLA) (2). These findings enabled HLA matching of donor and recipient, a technique that contributed to improved results and encouraged further trials of marrow grafting (3). The first successful SCT with HLA-identical donors were performed in 1968 in two patients with immunodeficient disorders (4, 5). By the early 1970s, it had become evident that hematopoietic SCT could cure leukemia, severe aplastic anemia (SAA), and SCID (6, 7).

Allogeneic SCT is one of three types of hematopoietic SCT. In allogeneic SCT the cell source is supplied from a donor that is not genetically identical. It is often performed with stem cells donated from an HLA-identical sibling or from an HLA-matched unrelated donor. The second type of hematopoietic SCT, the syngeneic one, is performed with stem cells from a genetically identical individual, a monozygotic twin. Autologous transplantation, the third type of transplantation, involves removing, cryopreserving, and later reinfusing the patient's own hematopoietic cells to restore hematopoietic function after administration of very high-dose therapy. Unlike allogeneic SCT, autologous transplantation results in neither a risk of graft-versus-host disease (GVHD) nor the potential benefit of a graft-versus-tumor effect. Moreover, there is always a risk that the autologous stem cells may contain viable tumor cells, cells that could cause disease recurrence following reinfusion (8). Because this thesis will be based mainly on allogeneic SCT, the issues concerning this form of therapy will be further discussed.

Conditioning

Before transplantation, a preparative regimen is administered to eradicate the patient's disease and, in case of allogeneic transplantation, to provide adequate immunosuppression to prevent rejection of the graft. In the case of a malignant disease, the treatment is as intensive as possible without causing irreparable damage in other organs. The limiting organs are mainly the lungs and the gastrointestinal tract. The appropriate regimen for any particular patient is determined according to the disease being treated, the source of stem cells, and the patient's age and overall health. One commonly used regiment for the treatment of hematological malignancies combines cyclophosphamide for two days before transplantation followed by total body irradiation (TBI) (9, 10). To enable a higher total dose of radiation, many transplantation centers today give fractionated radiation during a longer time period (11). As an alternative to radiation, busulfan has been used with promising results, especially in patients with acute myeloid leukemia (12-14).

The toxicities associated with conventional myeloablative therapy limit the use of the potentially curative SCT to relatively healthy young patients. To circumvent the age restriction, several transplantation centers are now using a less intensive conditioning therapy, also known as non-myeloablative or "mini-transplant" therapy. These transplants have significantly less tissue damage associated with the reduced preparative regimens that are given. The reduced intensity therapy relies less on chemoradiation therapy and shift the burden of tumor-cell killing to graft-versus-leukemia (GVL) effects (discussed later) (15-17). The goal of the reduced conditioning regimen is a faster immune recovery and a complete elimination or suppression of the patient's tumor-cell population. Several potential advantages are associated with the use of non-myeloablative stem cell transplantation (NST). This approach allows older patients and patients with poor medical conditions to receive allogeneic SCT. Preliminary clinical data for this kind of conditioning therapy are encouraging but need to be confirmed in well-designed prospective controlled trials with direct comparison to conventional allogeneic SCT. A randomized study for patients with AML and CML is now ongoing among the Nordic countries (J. Aschan, personal communications).

Hematopoietic Stem Cell Source

In the past, bone marrow was the source of hematopoietic stem cells for most transplants. Bone marrow cells for transplantation is usually obtained from the donor's anterior and posterior iliac crest with the donor under spinal or general anesthesia (10). However, bone marrow is no longer the only source of stem cells. Both peripheral blood, into which stem cells have been mobilized by the administration of granulocyte colony simulating factor (G-CSF) or granulocyte/macrophage colony stimulating factor (GM-CSF), and placental blood, obtained from the umbilical cord after birth, are sources of stem cells that are being used increasingly (18-22).

Initially, there was reluctance to use peripheral blood for allografting because the number of T-cells in a peripheral blood stem cell inoculum was known to be ten times higher than in bone marrow, and previous experiments in animal models demonstrated a relationship between the number of T-cells infused and the development of GVHD (23). However, this assumption has not been further supported. Indeed, a large randomized trial has recently confirmed that the use of G-CSF-mobilized peripheral blood leads to more rapid engraftment than use of marrow without increasing acute GVHD (24, 25). One reason

for earlier engraftment might be that mobilized PBSC grafts contain three or four times more CD34+ cells, an amount that could hasten the tempo of engraftment. Indeed, preliminary results suggest earlier neutrophil and platelet recovery with PBSC compared to BM (19, 21). The reason for a unaffected incidence of acute GVHD remains unexplained, however, it may be related to the effects of G-CSF skewing the T-cells toward a type-2 cytokine production (26) and/or the suppressive effects on T-cells by the large numbers of monocytes in the PBSC grafts (up to 2-logs more that in marrow grafts) (27, 28). Although stem cells from peripheral blood do not seem to increase the risk of developing acute GVHD the use of peripheral blood does appear to be associated with a trend toward more chronic GVHD, especially if higher numbers of cells are used (29, 30). In patients with HLA-compatible unrelated donors, PBSC can be used safely as an alternative to BM for SCT, with no difference in incidence of acute GVHD (31).

In 1988, a patient with Fanconi's anemia was the first umbilical cord blood allograft transplant recipient (32). Early studies noted that the frequency of hematopoietic stem and progenitor cells is higher in cord blood grafts than in adult BM or PBSC (33). However, the limited volume of cord blood that can be harvested results in an overall 1 log reduction in the total number of nucleated and CD34+ cells transplanted compared with BM and PBSC. Stem cells from cord blood have some advantages over bone-marrow stem cells. Placental-blood stem cells are more tolerant to one or two HLA mismatches, and their donation involves less risk for the donor than either bone marrow aspiration, which is a surgical procedure, or stem-cell mobilization and apheresis of peripheral blood (34, 35). Unfortunately, cord blood has been associated with slower engraftment and an increased risk of graft failure (36, 37). However, the risk of acute and chronic GVHD is lower (18). Unfortunately, the low cell content of most cord blood collections has limited the use of this approach to children and smaller adults.

Major and Minor Histocompatibility Antigens

Major histocompatibility antigens encoded by the MHC genetic loci have a major influence on transplantation and particularly on the biological progress of GVHD. MHC is constituted of three different classes (MHC I, II, and III), that play a central role in both cell-mediated and humoral immune responses. MHC classes I and II are cell surface molecules controlling T-cell recognition and histocompatibility (38, 39). MHC class I antigens (HLA-A, HLA-B, and HLA-C) are widely distributed and are found on all nucleated cells (40). HLA class II antigens (DP, DQ and DR) are found on antigen presenting cells (APC) such as B-cells, dendritic cells, and macrophages. There are hundreds of variant forms of each class I and class II molecule, and even small differences between them can provoke alloreactive T-cell responses that jeopardize the success of transplantation. Hence, matching SCT recipients with sibling donors sharing identical HLA antigens has significantly improved engraftment kinetics and decreased GVHD severity (41-43). However, if individuals are HLA-matched but not identical twins, there will still be differences in many of the endogenous proteins presented by HLA, and T-cells from one person will react, albeit less vigorously, to the "minor" antigens of the other person. Minor histocompatibility antigens (miH) are due to polymorphisms of other non-HLA proteins, differences in the levels of expression of proteins, or genome differences between males and females (44, 45). The miH are critical in matched sibling allogeneic bone marrow graft.

Matching donors and recipients for MHC class I and II molecules not only prevents GVHD but is necessary for the recipient to recover a working adaptive immune system. After transplantation, most patients hopefully become stable chimeras, in which hematopoietic cells are of donor HLA type, but all other cells are of recipient HLA type. For the T-cells of donor type to work effectively in the recipient, they need to recognize peptide antigen that are presented on cells of both donor and recipient HLA type. For example, after infection with a virus, virus-specific donor T-cells stimulated by donor dendritic cells must then be able to kill infected epithelial cells of the recipient. This can only happen if there is a match between donor and recipient MHC class I molecules.

Donors

The preferred donor for any patient undergoing SCT is an HLA-identical sibling. Because the polymorphic HLA genes are closely linked and constitute a single genetic locus, any pair of siblings has a 25% chance of being HLA identical. In practice, the frequency allows approximately one-third of the patients to receive their transplant from an HLA-identical sibling (46). Because the vast majority of patients who might benefit from allogeneic SCT lack an HLA-matched sibling donor, the evaluation of alternative donor sources has been crucial. In order to find a matched unrelated donor (MUD) it has been necessary to develop large donor registries. Volunteer donors have served as source of allogeneic blood cells for more than 20 years. In 1986, the National Marrow Donor Program was developed to facilitate the use of volunteer donors. Currently, more than 9 million normal individuals throughout the world are included in donor registries. The chance of finding a matched unrelated donor is about 80% depending on the ethnic origin of the patient (47). In most studies, both GVHD and graft rejection are more common following MUD transplantations than after transplants involving matched siblings (48). However, at Huddinge University Hospital the incidence of acute GVHD is similar for matched unrelated donor that are identical in HLA-A, -B and –DRB1 as with HLA-identical siblings (49).

Complications

Allogeneic SCT is associated with a number of early and late complications. The risks of these complications are significantly influenced by the degree of matching between donor and recipient for HLA. In addition, GVHD is associated with a severe immunological deficiency, and treatment with immunosuppressive agents further aggravates immunosuppression. Not surprisingly, therefore, infections are frequent and often life threatening. The main complications are briefly summarized in the three sections below and in Figure 1 on page 10.

Graft Failure

Occasionally, following transplantation, marrow function either does not return or is lost after a period of recovery. The main reason for graft rejection is that the conditioning treatment before transplantation has not been effective enough. A sufficient number of immunocompetent cells remain in the patient despite the conditioning given and have

the ability to reject the transplanted stem cells. In the early days rejection was a common problem, but with more intense conditioning, better immunosuppressive therapy, and the use of histocompatible donors, rejection became less common. Today, graft rejection is seen more commonly in recipients of HLA-mismatched or T cell-depleted marrow (50, 51).

Graft failure can also occur when the patient's own cells reappear. Relapse of the underlying disease is a major cause of treatment failure after allogeneic SCT. Generally, leukemic relapses occur in recipient-derived cells, indicating that the original malignant clone survived chemoradiotherapy and escaped from the immunological anti-tumor effects of the graft (52). Relapse rates are lower among recipient of allograft compared with autograft (53), increased in syngeneic compared to allogeneic SCT (54, 55), increased in patients receiving T-cell-depleted allografts compared with unmanipulated allografts (56), and there is an increased risk of relapse in patients without GVHD compared to patients with acute and chronic GVHD (57, 58). Although randomized studies have been performed that compare PBSC with BM, no studies have shown clear differences in relapse risk between the two sources of stem cells (24, 59, 60). As more refined diagnostic methods have been developed to detect minimal residual disease, relapses can be detected at earlier stages (61-63).

Clinical Aspects of Graft-versus-Host Disease (GVHD)

Clinical manifestations of GVHD depend on the degree of donor-host histocompatibility and graft alloreactivity to major host antigens. Matching of MHC antigens speed engraftment and reduces the severity of GVHD (41). Despite HLA identity between a patient and donor, substantial numbers of patient still develop GVHD due to differences in miH antigens (64). Although the molecular pathogenesis of GVHD remains uncovered, there is general agreement that infiltrating T-lymphocytes play a central role in this mechanism (65). In both the chronic and acute form of GVHD, alloreactive donor T-cells undergo activation and expansion, produce cytokines, and mature into effector cells. However, the relationship between chronic and acute GVHD is complex and incompletely understood.

To counter GVHD, patients are treated prophylactically with immunosuppressive treatment after SCT. The combination of methotrexate and cyclosporine A is widely used (66, 67). Both agents inhibit activated donor T-cell proliferation. Cyclosporine A acts by inhibiting IL-2 expression, whereas methotrexate is an antimetabolite that specifically inhibits the first step of purine biosynthesis in stimulated lymphocytes (68, 69). Other agents have also been evaluated, including tacrolimus, mycophenolate mofetil, and rapamycin. The results using tacrolimus suggest that it may be as effective as Cyclosporine A in preventing acute GVHD (70). Lymphocyte depletion of the graft is a very direct way of reducing or eliminating GVHD (71). So far, the potential of this approach has not been achieved because of several major drawbacks. First, the techniques used to remove lymphocytes result in loss of valuable stem cells. Second, relapse rates are higher (72). Third, many trials have seen higher probability of reactivation of viruses such as CMV (73, 74) and EBV (75, 76). Finally, it is associated with increased rates of failed engraftment (51, 77). Therefore, most centers adopt this approach only for extremely high-risk cases such as mismatched transplants.

Acute Graft-versus-Host Disease

Acute GVHD is a rapidly progressing systemic illness characterized by immunosuppression and tissue injury in various organs such as the liver, skin, and intestinal mucosa (78, 79). Apoptosis of cells in these areas results in rash, mucosal denudation, subsequent diarrhea, and biliary stasis. Despite progress in understanding the mediators involved in acute GVHD, effective treatment has remained allusive; most patients who develop the severe manifestation of the disease succumb to it or to complications of its treatment. Acute GVHD can occur within days or as late as more than 2 months after transplantation (9, 10). The incidence ranges from less than 10% to more than 80%, depending on the degree of histocompatibility between donor and recipient, the number of T-cells in the graft, the patient's age, and the GVHD prophylactic regimen (42, 49). There is also an increased risk of developing acute GVHD for a male patient receiving stem cells from a female donor that earlier has been immunized after a pregnancy or blood transfusion (80, 81). The risk factors for acute GVHD in studies performed in our unit include: GVHD prophylaxis with monotherapy (MTX or CsA), seropositivity for several herpes viruses in the donor, and seropositivity for CMV in the recipient before the transplants as well as early engraftment (82, 83).

Acute GVHD is staged within each organ system involved (84). These evaluations are then combined to give an overall clinical stage, ranging from 0 to IV. Within the skin, GVHD is staged by the percentage of the body involved and the severity of the injury, for instance bullous formation. Intestinal disease is judged by the amount of diarrhea. Liver disease is measured by the bilirubin elevation. Although prophylactic treatment to prevent acute GVHD has been given, a number of patients still develop the disease. The most common therapies in these cases are high doses of steroids, cyclosporine A, antithymoglobuline (85, 86) or psoralen and ultraviolet light (PUVA) (87, 88).

Chronic Graft-versus-Host Disease

While the basic understanding of the mechanisms of acute GVHD has increased considerably in recent years, progress in understanding the chronic condition has been slower. One reason for this is the limited number of animal models available. The development of allogeneic animal model for chronic GVHD is difficult and time-consuming because the animal must survive acute GVHD and develop chronic GVHD. However, it is clear from murine studies that chronic GVHD development depend on the continued presence of host-reactive donor T-cells (89). GVHD in its chronic form is a difficult disorder to manage and it can significantly affect the quality of life of long-term survivors following SCT and also lead to mortality (90). It may lead to late death as a direct complication (e.g. bronchiolitis) or by the associated immunodeficiency that increases susceptibility to infections (91). Approximately 40%-60% of long-term survivors of SCT will develop chronic GVHD. This disease may evolve directly from acute GVHD (progressive) which has a grim prognosis, or may follow a period of recovery (quiescent) GVHD, which has an intermediate prognosis. Patients who develop de novo chronic GVHD, i.e., with no history of prior acute GVHD, have a relatively good prognosis. The most commonly used staging system identifies patients as having limited or extensive disease (92). The incidence of chronic GVHD is currently increasing because of the older age of patients receiving transplants, the use of PBSC as the graft source, the use of donor lymphocyte infusion to induce a graft-versus-leukemia (GVL) effect (93), and the use of mismatched and unrelated donors, reviewed in (94). However, the risk of

chronic GVHD increases progressively with increasing severity of acute GVHD. The strongest risk factor for developing chronic GVHD is a previous moderate to severe acute GVHD (95-97). In a study performed at the Karolinska University Hospital in Huddinge significant risk factors for chronic GVHD were high recipient age, acute GVHD grades I-IV, CML, and alloimmune female donor to male recipient (98).

Unlike acute GVHD, chronic GVHD can involve almost any organ, although the skin, liver, and mouth are the most frequent targets. It generally appears after day 100 post transplantation, although patients may develop classic features of chronic GVHD even earlier. The median time of diagnosis is day 201 after HLA-identical sibling transplant, day 159 after mismatched related-donor transplant, and day 133 after unrelated-donor transplant (99). Chronic GVHD is also associated with prolonged immunodeficiency, predisposing to recurrent and sometimes fatal infections (42). Treatment of chronic GVHD mainly consists of a combination of steroids and cyclosporine A. Other treatments include extracorporral PUVA, 1 Gray (Gy) of total lymphnode irradiation (TLI) and more recently, anti B-cell antibodies.

Infections

Breakdown of mucosal integrity created by toxic conditioning regimens, neutropenia resulting from myelosuppressive conditioning, and persistent B- and T-cell dysfunction caused by the pharmacologic immunosuppression required to prevent GVHD, create a state of immunodeficiency ideal for invasive pathogens. Therefore, bacterial infections are one of the major problems soon after SCT (100). Positive blood cultures are found in about one-third of the patients. Gram-positive organisms predominate, but gram-negative organisms and reactivation of herpes simplex virus are also seen. Moreover, invasive fungal infections constitute a major complication after allogeneic SCT (101-103). Strategies designed to improve the early diagnosis, prevention, and treatment of opportunistic pathogens have however enhanced early post-transplant outcomes (104-106).

Persistent immune dysfunction complicates allogeneic SCT and results in late mortality related to infections, particularly in patients with chronic GVHD (107, 108). The most important infections that occur in the interval between successful engraftment and day 100 include aspergillus, cytomegalovirus (CMV), and respiratory syncytial virus (RSV). Approximately 50-90% of the patients show evidence of CMV reactivation post-transplant (109, 110) and about 10-30% of these patients develop symptomatic infections (109, 111). The risk of death from CMV disease has been greatly diminished in the past decade through the use of CMV prophylaxis or preemptive treatment strategies based on sensitive qualitative/quantitative polymerase chain reaction (PCR)-based CMV-detection assays (111-113). Late infections, more than three months post-transplant, usually are due to varicella zoster virus, or in patients with chronic GVHD, recurrent bacterial infections (106, 114).

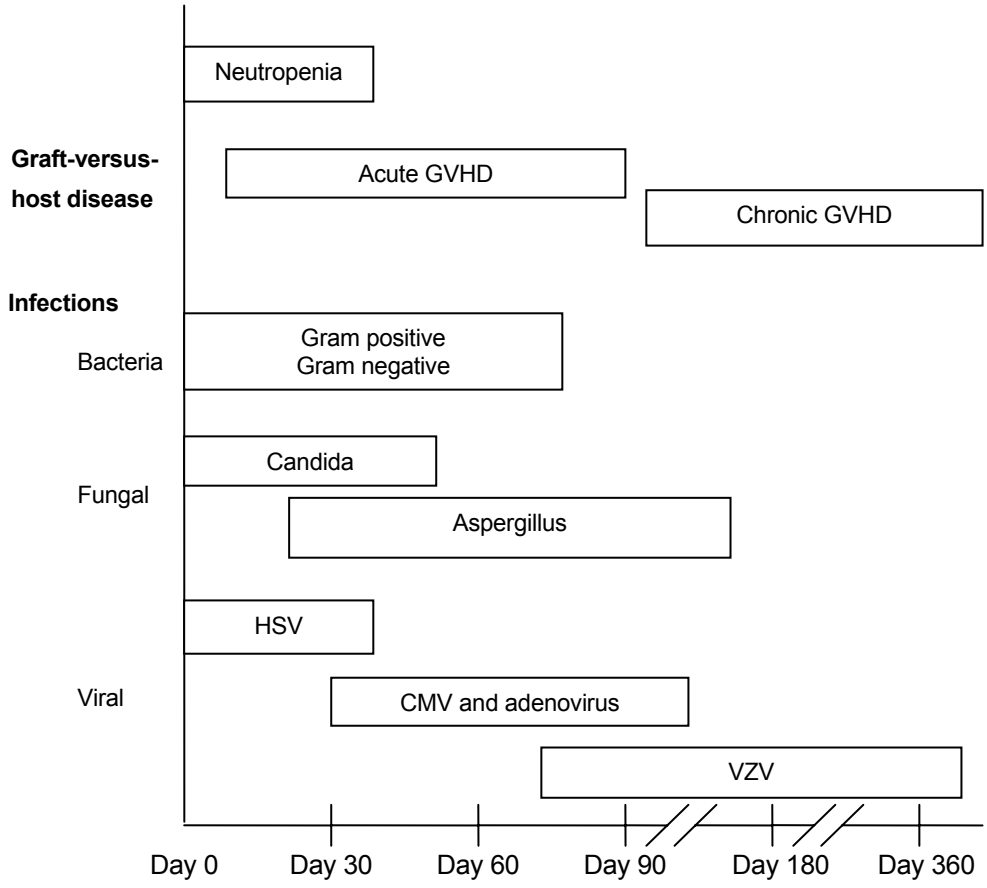

Figure 1. The major syndromes that complicate hematopoietic cell transplantation and the approximate periods in which they develop. Abbreviations: GVHD, graft-versus-host disease; HSV, herpes simplex virus; CMV, cytomegalovirus; VZV, varicella zoster virus.

Graft-versus-Leukemia (GVL)

GVHD is not only affecting allogeneic SCT in a negative way. There is also a positive side to this reaction, the GVL effect, which is an important therapeutic aspect of SCT in eradicating malignant cells. By the end of 1950s Barnes *et. al.* noted that leukemic mice treated with syngeneic marrow were more likely to relapse than mice transplanted with an allogeneic graft (115), and they hypothesized that the allogeneic graft contained cells with immune reactivity. Evidence for a GVL effect in humans first emerged in 1979 with the observation that relapses were markedly lower among patients who developed GVHD than among those who did not (116). It is now well established that alloreactive donor T-lymphocytes react against both the patient's normal hematopoietic cells leading to GVHD and to leukemic cells, known as GVL (Figure 2, page 12). These findings have been further confirmed by the fact that lymphocytes of a transplant donor can prevent tumor growth in the recipient by providing donor lymphocyte infusion (DLI) to induce remission in transplanted patients who have relapsed with chronic myelogenous leukemia (CML) (93, 117, 118). Also, the importance of T-cells in achieving long-term engraftment and in effecting graft-versus-tumor reaction has been showed in patients given T-cell-depleted grafts (72, 119). While these patients had less GVHD, they also had profoundly higher relapse rates.

Since GVHD and GVL are intimately associated, it can be assumed that similar mechanisms are involved in mediating these two reactions. The studies mentioned above all suggest that donor derived T-cells play a central role in the GVL effect. However, donor T-cells may not be the only important effector cells involved in the GVL reactivity. Natural killer (NK) cells are among the first immune cells to recover after SCT (120) and they mediate cytotoxic effects without prior sensitization. In vivo studies in murine models have shown that transplantation of grafts depleted of T-cells but retaining NK cells correlated with reduced relapse rates and minimal incidence of GVHD (121). However, it remains to be seen whether the graft-versus-malignancy response can truly be separated from more generalized alloreactivity (i.e., GVHD) observed clinically. However, indirect evidence for a GVL effect separated from GVHD in patients with acute leukemia has been reported by the EBMT Acute Leukemia Working Party (122) as well as the IBMTR (123). So far, patients with acute GVHD grade I show the highest leukemia-free survival after SCT (124).

Although donor T-cells are likely to be important effector cells for GVL, the target antigens on the tumor cells remain poorly defined. Identifying these targets is of critical importance because understanding the mechanism of tumor-cell recognition may help explain why some leukemias are susceptible to GVL induction and others are not; this knowledge will help in designing better strategies to manipulate the GVL effect for clinical benefit.

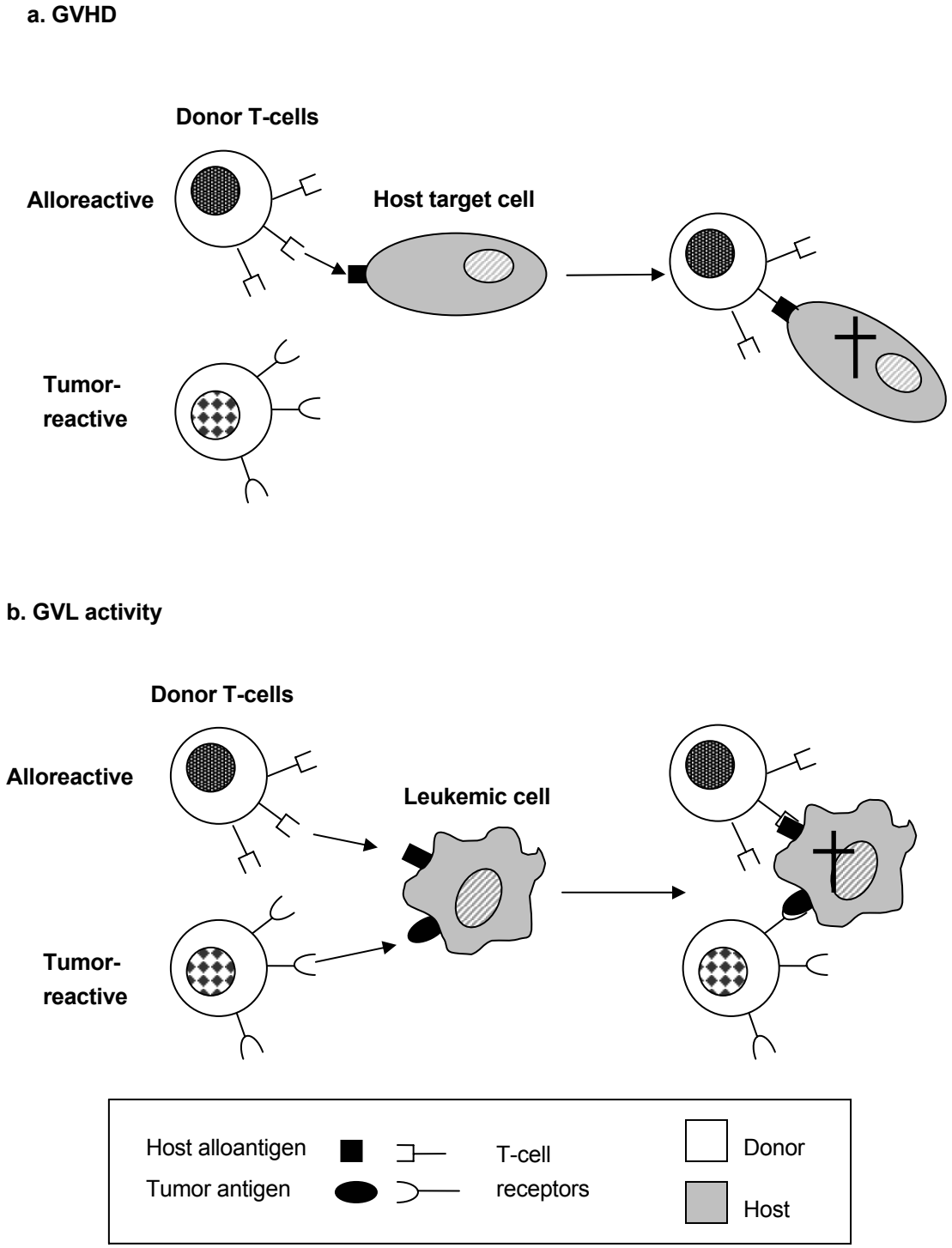

Figure 2. GVHD and GVL activity. a) Donor T-cell populations in the allograft contain alloreactive T-cells, which become activated and proliferate on recognition of specific MHC or minor histocompatibility antigen on host cells. These alloreactive T-cells can have cytolytic activity against host cells, which contributes to the development of graft-versus-host disease (GVHD). **b)** Donor T-cell populations in the allograft can also contain tumor-reactive T-cells, which can recognize tumor-associated antigens and exert cytolytic activity against leukemic cells, the graft-versus-leukemia (GVL) activity.

Acute Graft-versus-Host Disease

Most recipients of an allogeneic SCT experience some degree of acute GVHD. This severe reaction is defined as a progressive, systemic disease that is characterized by immunosuppression and tissue damage to the skin, liver, and intestines in particular (42). The complex pathophysiology involves host tissue damage, which results from the conditioning regimen (chemotherapy and/or irradiation), inflammatory cytokines, and effector cells (125).

The first description of acute GVHD came from rodent experiments documenting hematopoietic reconstitution after marrow lethal radiation (126). Animals that received syngenic stem cell graft recovered from the radiation toxicity (primary disease) and appeared to be normal. Animals that received their stem cells from different strains (with MHC loci differences) recovered from their primary disease but developed "secondary disease," which is now known as acute GVHD (127). In 1966, Billingham outlined the requirements for the induction of GVHD. First, the graft must contain immunologically competent cells. Second, the host must appear foreign to the graft and therefore be capable of stimulating the donor cells. Finally, the host immune system must be incapable of generating an immune response (128). This second part of the thesis will focus on the pathophysiology and mechanisms involved in acute GVHD after allogeneic SCT.

Pathophysiology

Acute GVHD is initiated by allogeneic T-cells (65). After entering the recipient blood circulation, these T-cells home into the secondary lymphoid tissues where the alloreactive T-cells become activated. The activated T-cells proliferate, resulting in an expanded population, and activated alloreactive T-cells migrate from the secondary lymphoid tissues into the peripheral target organs, and initiate the inflammatory process that destroys tissue structures and causes various clinical symptoms (79).

Three-Phase Model of Acute GVHD

Although the interactions of subsets of T-cells and effector cells during acute GVHD via production of cytokines is a rather complex multi-step process, it is by now generally accepted that acute GVHD can be summarized in a three-step process with an afferent and efferent phase (129, 130). Injury to host epithelium and endothelium generates injury signals that recruit donor T-cells (Phase I). In this inflammatory milieu, the donor T-cells recognize alloantigens, resulting in activation and proliferation (Phase II). The first two phases make up the afferent phase of GVHD. Finally, the T-cells cause further injury through both specific and nonspecific mechanisms during the efferent phase (Phase III). The three-phase model of acute GVHD is summarized in Figure 5.

Phase I: Effects of Conditioning

The fist step of acute GVHD occurs during the conditioning phase, a phase that occurs even before donor cells are infused into the host. The conditioning therapy, which

includes irradiation and chemotherapy, is rather toxic to tissues and leads to damage and activation of host tissues, including intestinal mucosa, liver, and other tissues. The injured tissues will respond with the production of factors - such as cytokines, chemokines and, adhesion molecules - that signal to the immune system that injury have occurred (131). Hence, donor T-cells are infused into a host which tissues have already been damaged, by several factors such as the patient's underlying disease and its earlier treatment, infections, and preparative conditioning consisting of high-dose therapy given before SCT. The most commonly secreted inflammatory cytokines that are secreted by activated host cells are TNF-α and IL-1 (132). The presence of inflammatory cytokines during this phase increases the expression of adhesion molecules, co-stimulatory molecules, and MHC antigens (133, 134). This leads to activation of host dendritic cells (DCs) and recognition of host MHC and/or miHs by mature donor T-cells (135). TNF-α also contributes to the intestinal injury by affecting the integrity of the gut mucosa directly (136). Further injury to the barrier results in release of endotoxin, i.e., lipopolysaccharide (LPS), a cell wall component of gram-negative bacteria, which can leak through the damaged mucosa. LPS may subsequently trigger gut-associated lymphocytes and macrophages to additional production of TNF-α and IL-1 (137). Elevated serum levels of LPS have been shown to correlate directly with the degree of intestinal damage occurring after allogeneic SCT (138). The release of LPS and activation of lymphocytes and macrophages may result in the amplification of local tissue injury and further promotion of an inflammatory response. Indirect support for this concept can be found in experimental transplantation and in some clinical settings where transplantation in low bacteria environments strikingly limits the risk of GVHD (137-139). Also, blockade of LPS has shown to prevent GVHD and preserve GVL (140). Moreover, experimental approaches to prevent GVHD include reducing the damage to the gastrointestinal tract by protection of the mucosal barrier. Two unique factors that can shield the gastrointestinal tract from the toxic effects of conditioning therapy, keratinocyte growth factor (KGF) and IL-11, have been shown in experimental models to decrease gastrointestinal toxicity and reduce acute GVHD (141-144). Consistent with the hypothesis that protection of the intestinal mucosa could block the inflammatory cascade of GVHD, KGF prevented the translocation of LPS into the circulation and resulted in a reduction of systemic TNF-α. Also, KGF probably provides its protective effects on acute GVHD by suppression of pro-inflammatory cytokines such as TNF-α and IFN-γ. Unfortunately, IL-11 has proved very toxic, leading to closure of a randomized trial (145). Also, attempts to block the effects of the GVHD-inducing cytokines TNF-α and IL-1 with antibodies have been attractive but these studies have met limited clinical success (146-148).

Phase II: Donor T-cell Activation

Presentation of recipient antigens to donor T-cells, activation of donor T-cells, and subsequent proliferation and differentiation of these activated T-cells are crucial in the second phase of acute GVHD. After infusion of the graft, donor T-cells recognize foreign host antigens presented by activated APCs. The greater the disparity between donor and recipient MHC, the greater the T-cell response. In the case of MHC-mismatched transplantation, the frequency of alloreactive T-cells is at least five times greater than the frequency of peptide-specific T-cells responding to an autoantigen (149). Even single-antigen differences between donor and recipient result in significantly more GVHD than

in HLA-identical pairs. MHC class I differences stimulate CD8+ T-cells, and MHC class II differences stimulate CD4+ T-cells (150). As discussed earlier, in identical pairs, the donor T-cells recognize minor antigen differences.

During the process of donor T-cell activation, APCs play a very important role by digesting large proteins into smaller peptides and present them on the surface in association with MHC molecules. In particular, DCs are uniquely specialized for uptake and presentation of antigen to naïve T-cells. Immature DCs are distributed in tissues, particularly barrier organs, such as skin and bowel, and they are specialized for uptake of antigens via endocytosis. Before, during, and immediately after an allogeneic SCT, hosts are exposed to inflammatory cytokines such as TNF-α and IL-1, pathogen-derived products such as LPS, and necrotic cells that are damaged by recipient conditioning. All these can initiate DC maturation, reviewed in (151). The distribution of APCs may explain the unusual target organ distribution of acute GVHD. For example, a study has shown that selective removal of APCs from a specific organ may reduce GVHD in that target organ but not in other organs (152). Zhang and colleagues suggested that the DCs and macrophages in the secondary lymphoid tissues are critical for the activation and proliferation of donor CD8+ T-cells, whereas APCs in the target organ are required for the recruitment of previously activated donor T-cells to the tissue. These results suggest that the host APCs may not only play a role in the activation phase but also in the recruitment of allogeneic cytotoxic T-cells during the effector phase of acute GVHD. Another study, by Murai and colleagues, demonstrated the importance of the Peyer's patches of the small intestine as key sites of antigen presentation to CD8+ donor T-cells (153). This study showed that if Peyer's patches were absent, or if the donor T-cell migration to Peyer's patches was blocked, lethal GVHD did not occur.

After the preparative regimen, transplant recipients are chimeric for APCs. They have residual recipient-derived APCs that survived the conditioning regimen and they have donor APCs derived from the stem cell graft. Although allogeneic antigens can be presented directly by host-derived and indirectly by donor-derived APCs, host-derived APCs appear to be critical in inducing GVHD across both MiH and MHC mismatches. Murine studies have demonstrated that host APCs alone are both necessary and sufficient to stimulate donor T-cells (154-156). Therefore, recipient APC depletion may be an effective way of decreasing GVHD induced by CD8+ or CD4+ T-cells in MHC-disparate and identical transplants. In addition, the importance of host APCs have recently been confirmed in another interesting study (157). Merad *et. al.* showed that persistent host Langerhans cells, the major APC in the skin, are responsible for cutaneous GVHD. Moreover, the authors reported that ultraviolet (UV) irradiation could deplete host Langerhans cells in mice, a depletion which was shown to be protective against GVHD in the skin. Application of these findings to the clinic could have major implications for the prevention of both acute and chronic GVHD, and may increase the safety and applicability of stem cell transplantation.

Donor T-cell Activation and Co-stimulation

Engagement of the T-cell receptor by peptide presented on a MHC molecule of an antigen presenting cell provides the initiating signal for T-cell stimulation. However, a second co-stimulatory signal is also required for full T-cell stimulation. The outcome of the first signal is regulated by the second signal. Three outcomes may occur: complete

activation, partial activation, or anergy, i.e., a long-lasting state of antigen specific unresponsiveness. A growing number of T-cell-co-stimulatory pathways have been identified (158-160). To date, the most important pathways appear to be mediated by interactions between CD28 with B7 and CD40 with its ligand CD154. In the most completely characterized interaction, B7 bind to T-cell surface receptors CD28 and cytotoxic T-lymphocyte antigen 4 (CTLA-4). CD28 provides a positive signal that lowers the threshold for T-cell activation and promotes T-cell differentiation and survival, whereas CTLA-4 delivers an inhibitory signal (161). The T-cell co-signalling molecules are summarized in Figure 3.

A novel approach to reducing acute GVHD involves the blockade of the T-cell-co-stimulatory signals (162). In order to block these interactions, either *ex vivo* manipulation of the donor T-cells or systemic administration of blocking agents (e.g. the fusion protein CTLA4-Ig or antibodies) *in vivo* has been used. In one animal study on GVHD, blocking of the B7-CD28 interaction inhibited both acute and chronic GVHD (163). Other studies on $CD28^{-/-}$ T-cells, CTLA-4-Ig, and anti-B7 antibodies, have been performed, however, due to variability in strain pairing and transplant conditions it is difficult to interpret the results. The advantage of *ex vivo* treatment is the control and specificity of the conditions. Although blockade of co-stimulatory pathways seem to be a promising way to prevent acute GVHD, this strategy needs further investigation.

	First signal	
	Recognition	
MHC	Class I	CD 8
	Class II	CD 4
Antigen-Peptide		CD 3/TCR
	Co-signal	
	Co-stimulation	
B7-1 (CD 80)		CD 28
B7-2 (CD 86)		CD 28
	Inhibition	
B7-1 (CD 80)		CTLA-4 (CD 152)
B7-2 (CD 86)		CTLA-4 (CD 152)
	Up-regulation of B7 molecules	
CD 40		CD 40L (CD154)

Figure 3. T-cell co-signalling molecules and their immunological role.

Cytokines

IL-2 and IFN-γ

The T-cell activation and proliferation is followed by cytokine and chemokine secretion (164). The activation involves multiple pathways which activate transcription of genes for cytokines, such as IL-2, IFN-γ and their receptors (165). IL-2 has long been considered to be the primary cytokine involved in acute GVHD both because of its central role as a T-cell growth factor and because cyclosporine, a powerful prophylactic agent against acute GVHD, is known to inhibit IL-2 secretion (166, 167). Under the influence of IL-2 and other immune mediators, alloreactive T-cells expand clonally and differentiate into cytotoxic T-lymphocytes (CTL). The importance of IL-2 in the pathophysiology of acute GVHD has been shown by administration of monoclonal antibodies against the IL-2 receptor. In animal and clinical studies administration of monoclonal antibodies early after SCT has in some studies shown to prevent acute GVHD (168, 169). IL-2 has been shown to control and amplify the immune response against alloantigens. When low doses of IL-2 have been administered after allogeneic SCT, the severity and mortality of GVHD have been enhanced (170, 171). However, IL-2 appears to play a rather complex role in GVHD. On the one hand, neutralization of IL-2 has resulted in amelioration of GVHD; on the other hand, administration of high doses of IL-2 have shown to inhibit GVHD (172).

IFN-γ is another crucial cytokine involved in the second phase of acute GVHD. It has been shown that IFN-γ levels are significantly higher in mice with acute GVHD than in those without the disease (173).Together with IL-2, IFN-γ induces further T-cell expansion, induces CTL and NK-cell responses and prime additional mononuclear phagocytes to produce IL-1 and TNF-α. Several experimental studies have shown multiple effects of IFN-γ in the pathophysiology of acute GVHD. As well as other inflammatory cytokines, IFN-γ has shown to induce increased expression of adhesion molecules, chemokines, and HLA-molecules which enhances the recruitment of cells and antigen presentation. Another important effect in the context of acute GVHD is the direct tissue damage caused by IFN-γ (174, 175). It is generally known that the immune system is itself a GVHD target and immunosuppression is a common feature seen during acute GVHD. In several experimental studies IFN-γ appears to mediate this form of immunosuppression through the induction of nitric oxide (NO) and Fas expression (176, 177). Also, by enhancement of Fas-mediated apoptosis IFN-γ plays an important role in regulating cell death of activated donor T-cells (178). Furthermore, IFN-γ also reduces the amount of LPS needed to stimulate macrophages to produce inflammatory cytokines and NO. Interestingly, in this context IFN-γ has two opposing functions. On the one hand, it can mature DCs, prime macrophages to produce inflammatory cytokines, and induce NO secretion, all of which intensify the acute GVHD reaction. On the other hand, IFN-γ can decrease GVHD by inducing the expression of Fas receptors on donor T-cells, causing activation-induced cell death (AICD) and diminishing the donor T-cell response to host antigens (179). Hence, IFN-γ can show both a suppressive and stimulating effect under different circumstances. It is well known that IFN-γ promotes local inflammation; however, at the systemic level it initiates an anti-inflammatory response (180).

IL-18 and G-CSF

IL-18 is a recently discovered cytokine that also influences the pathophysiology of acute GVHD. It is produced by a variety of cells and the major targets of IL-18 include macrophages, NK-cells, T-cells, and B-cells. IL-18 has the capacity of influencing both Th1 and Th2 mediated responses. IL-18 was found to be elevated in acute GVHD, but surprisingly, blockade of IL-18 accelerated acute GVHD mortality in animal models (181). In the study by Reddy and colleagues it was also discovered that administration of IL-18 early after SCT increased the serum levels of IFN-γ, which led to increased expression of Fas receptors on donor CD4+ T-cell. This resulted in a reduction of acute GVHD by induction of Fas-mediated apoptosis of donor T-cells. Administration of IL-18 to the stem cell donor before SCT appeared also to be protective against acute GVHD; however, most likely by an opposite effect, the enhancement of Th2 cytokine production. This study suggests that the timing of administration and the inflammatory milieu may be critical to the eventual outcome of acute GVHD.

As discussed in the general introduction, peripheral blood stem cells are an alternative source of stem cells for allogeneic SCT. Interestingly, it has been demonstrated that G-CSF mobilized donor PBSC reduces the early mortality in acute GVHD after allogeneic SCT in mice (26). It has been suggested that the reason for this is that pretreatment of donor cells with G-CSF may polarize donor T-cells towards Th2 (182, 183). However, when G-CSF is administered to the patient shortly after SCT in order to shorten the neutrophenic phase it has been shown to increase the risk of GVHD and death (184).

Chemokines

A characteristic feature of all inflammatory reactions is the excessive recruitment of leukocytes to the site of inflammation. The process of leukocyte recruitment to target tissue is well-orchestrated and involves several protein families, including proinflammatory cytokines, adhesion molecules, matrix metalloproteinases, and the large cytokine subfamily of chemotactic cytokines, the chemokines (185, 186). Inflammatory chemokines are expressed in inflamed tissues by infiltrated cells, monocytes or macrophages, or by resident cells, epithelial, endothelial, or fibroblastic cells on stimulation by proinflammatory cytokines (e.g. IL-1, TNF-α or IFN-γ) or stimuli (e.g. LPS). This group of chemokines is specialized for the recruitment of effector cells, including monocytes, granulocytes, and effector T-cells. Studies using murine models of acute GVHD have demonstrated the critical role of several chemokines and their receptors (particularly MIP-1α, MIP-2, Mig, MCP-1, MCP-3, and CCR5) by directing T-cell infiltration into target tissues during acute GVHD (187-189). It has earlier been shown that CCR5 expressing T lymphocytes are recruited to the liver during acute GVHD in mice models (187) and that MIP-1α, a ligand for CCR1 and CCR5, also seems to be involved in liver GVHD (189). Recently, Duffer and colleagues showed that the migration of donor CD8+ T-cells to GVHD target organs such as the intestines depends on the expression of CXCR3 and that the presence of this receptor significantly contributed to GVHD damage and overall mortality in mice (190). The role of various chemokines and their receptors in regulating donor T-cell migration to GVHD target tissues in clinical SCT remains unexplored. However, our group has recently shown that increased gene expression of CCR5, CXCR3, CCR1 and CCR2 are seen in connection with acute GVHD after allogeneic SCT (Jaksch et al unpublished data, Paper IV in this

thesis). Therefore, not only may chemokines and chemokine receptors act as potential targets for modulation of acute GVHD but also as diagnostic markers for early detection of the disease.

NK-cells

Although tissue damage in the effector phase of acute GVHD can result from the cytolytic function of CTLs, other effector cells such as NK-cells seem to be involved in the process. NK-cells are MHC-unrestricted cells that are negatively regulated by MHC class I-specific inhibitory receptors, thus HLA-mismatched transplants may trigger donor NK-cell mediated alloreactivity (191). NK-cells, which reconstitute very rapidly after SCT, can be major producers of IFN-γ, TNF-α and NO upon stimulation, and thus can contribute to the tissue damage seen in GVHD (192, 193). However, it has also been suggested that NK-cells may suppress GVH reactions and contribute to GVL effects (194, 195). In murine models of SCT it has recently been shown that activated donor NK-cells prevent GVHD through general elimination of host APCs and/or the secretion of the immunosuppressive cytokine TGF-β (195, 196). In a study by Asai and colleagues, anti-TGF-β completely abrogated the protective effects of activated NK-cells, which indicated the important role for TGF-β in the prevention of GVHD by NK-cells. However, it is not clear whether NK-cells are producing TGF-β or are inducing other cells to make it. The suppressive effect of NK-cells on GVHD has also been confirmed in humans. In a recent studies, with patients receiving haplo-identical transplants, HLA class I differences driving donor NK-mediated alloreaction in the GVH direction, mediated potent GVL effects and produced higher engraftment rates without causing severe acute GVHD (196-198).

Phase III: Cellular and Inflammatory Effector Phase

The third phase of acute GVHD is a complex cascade of multiple effectors. Once donor T-cells are activated and proliferate, they are directly or indirectly responsible for the tissue damage seen in GVHD. Three cytolytic pathways are important in the effector function of T-cells and other cytolytic cells: the perforin/granzyme B, Fas/Fas ligand (FasL), and direct cytokine-mediated injury. The recent use of knockout mice has demonstrated a central role for each of these pathways in the effector stage of GVHD (78, 199-204).

Cell Mediated Cytotoxicity

Although the receptors involved in recognition of target cells differs between CTLs and NK-cells, the mechanism by which they kill are essentially the same. They can mediate their cytotoxicity through two different contact-dependent pathways: Perforin-granzyme B-mediated cytolysis and Fas-FasL-mediated apoptosis (Figure 4) (202, 205). However, even though NK-cells express FasL and most likely use these molecules to kill certain target cells, NK-cells appear to mediate their cytotoxicity primarily through perforin/granzyme-dependent processes (206).

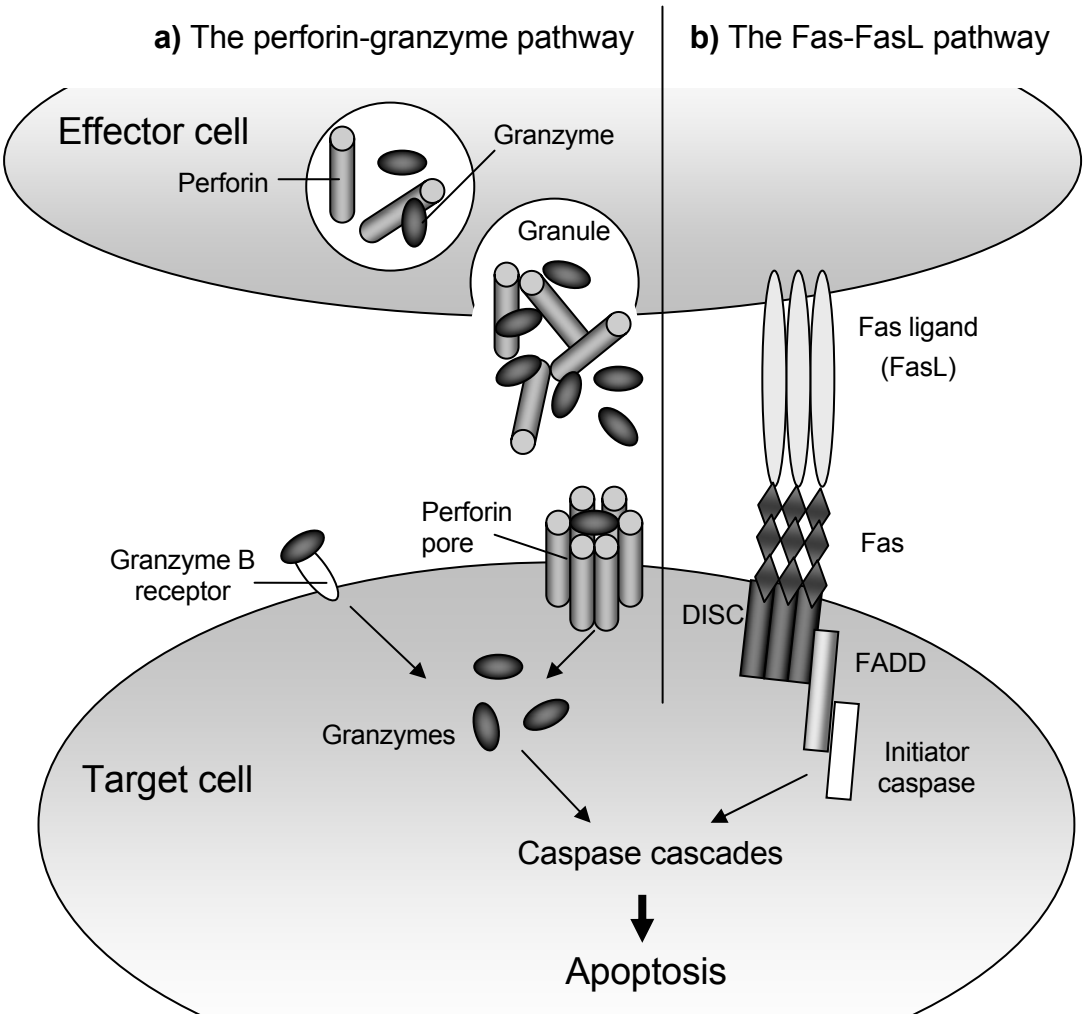

Figure 4. The cytolytic pathways: Perforin-granzyme and Fas-FasL. a) On recognition of the target cell, the granules are released, and perforin monomers insert themselves into the target cell membrane and polymerize into channel-forming aggregates. The perforin pores allow granzymes to enter the target cell. Recent studies suggest that granzyme B also can enter the target cell through binding to specific receptors on the membrane, followed by endocytosis. After entering the cell granzymes induce apoptosis through various downstream effector pathways. **b)** Fas-mediated apoptosis starts with the binding of a FasL trimer on the effector cell surface to a Fas trimer on the target cell membrane, which results in the formation of the death-inducing signaling complex (DISC) around the cytoplasmic chain of Fas. The formation of the DISC results in the interaction of Fas with the Fas-associated death-domain protein (FADD). Subsequently, FADD binds to and cleaves the initiator caspase (pro-caspase 8). This leads to the activation of various downstream effector caspases and results eventually in apoptosis.

The Mechanism of Fas-FasL and Perforin/Granzym Cytotoxicity

The Fas receptor is a tumor-necrosis factor (TNF)-receptor family member. It is expressed in many tissues, which includes the classic target organs of GVHD, and its level of expression can be increased by pro-inflammatory cytokines during inflammation (207). The ligand of the Fas receptor (FasL) also belongs to the TNF family and is expressed predominantly on activated T-cells, macrophages, and neutrophils. Interaction of FasL

with the Fas receptor (on the target cell membrane) results in the initiation of the Fas-mediated apoptosis; this is also known as programmed cell death (208).
The pore-forming molecule perforin is another crucial effector molecule of cytolysis by CTLs and NK-cells. Perforin is expressed mainly by CTLs and NK-cells and is stored in cytotoxic granules together with granzymes and other proteins, reviewed in (209). In the presence of calcium, perforin polymerizes and forms channels in the target cell membrane, which allows the granzymes to pass; however, recent *in vitro* experiments suggest that granzymes may sometimes enter target cells without passing through a perforin channel, but the relative importance if this pathway *in vivo* is currently unknown (210). After entering the target cell, granzymes activate caspase cascades, leading to an apoptotic-induced cell death (211).

Fas/FasL and Perforin/Granzyme in Acute GVHD
Several studies have shown that the expression of both Fas and FasL is increased on CD8+ and CD4+ donor T-cells during acute GVHD in patients and in mice (212-216), and that serum levels of soluble FasL and Fas correlate with the severity of GVHD (217, 218). In experimental mouse models, the role of the Fas-FasL pathways in the development of GVHD have been analyzed by using mice that are deficient for FasL (*gld* mice) as donors. The *gld* mice models show that there is a close relationship between the Fas/FasL system and acute GVHD, especially hepatic and cutaneous acute GVHD (78, 202, 203). Fas-deficient recipients have also been shown to be protected from hepatic GVHD, but not from GVHD in other target organs (219). In addition, administration of anti-FasL antibodies significantly delays but does not completely reduce the mortality of GVHD (201). Simultaneous administration of antibodies against FasL and TNF-α completely protected the mice from GVHD. In this study, Hattori *et al.* verified earlier studies that hepatic GVHD is predominantly mediated by FasL, intestinal GVHD is mainly mediated by TNF-α; and cutaneous GVHD, weight loss, and mortality are mediated by both FasL and TNF-α.

Several groups have created mice deficient for the perforin, granzyme A or B genes (205, 220-223). These mice grow and develop normally and their T-cells still have the ability to undergo activation. Studies using perforin deficient donor T-cells in various murine SCT models with disparity for MHC class I (200), class II (224), and minor histocompatibility antigens (78) have demonstrated improved survival, an indication that GVHD activity can be mediated through the perforin pathway. Interestingly, the use of these donor T-cells did not result in diminished GVHD target organ abnormalities of liver, skin, and intestines (78). The study by Graubert and colleagues also provided evidence that the perforin/granzym pathway is required for class I-restricted GVHD, and that FasL is an important mediator of class II-restricted GVHD. However, CD4+ and CD8+ T-cells are not restricted to the use of only one cytolytic pathway (204, 224). Schmaltz *et al.* also demonstrated that the perforin pathway was important for GVL activity. The importance for the perforin pathway in the GVL effect has also been show by others (203). Experimental murine models also suggest that granzyme B deficient CD8+ T-cells have significantly diminished GVHD induction capability compared to wild-type controls (225).

Inflammatory Effectors

Donor T-cell Derived TNF-α

Although most of the cytolytic activity of CTLs can be accounted for by the classic pathways of perforin/granzyme and Fas/FasL, CTLs deficient for both pathways exhibit residual cytolytic activity. Braun and colleagues demonstrated that mice that received T-cells from donors that were homozygous for nonfunctional perforin and FasL genes did not develop lethal GVHD; however, the CTLs derived from the donors could still display some lytic activity (199). It has been suggested that TNF, which can be expressed and secreted by activated CTLs, could contribute to CTL-mediated cytotoxicity (226). A role for TNF in the pathogenesis of GVHD has been well documented, but most studies have indicated that GVHD-associated TNF is derived mainly from monocytes and macrophages of donor or host origin (227). However, the remaining lytic activity by T-cells deficient for FasL and perforin has been ascribed to TNF in its membrane-anchored or secreted form. One group has recently found evidence for a significant contribution of donor T-cell-derived TNF to morbidity and mortality from GVHD as well as to GVL activity (228).

Macrophage Secreted TNF-α and IL-1

In addition to contact-dependent cytotoxicity secretion of inflammatory cytokines, activated macrophages play a key role in causing tissue damage during the third phase of acute GVHD. Mononuclear phagocytes, primed by IFN-γ, are stimulated by LPS to secrete the inflammatory cytokines TNF-α and IL-1. The central role of cytokines as mediators of acute GVHD has recently been demonstrated in a murine model. In this study severe acute GVHD occurred even in the absence of host alloantigen expression on host target tissues (155).

TNF-α is an inflammatory cytokine that causes a wide variety of biological effects. It activates DCs and enhances alloantigen presentation. By inducing inflammatory chemokines, it recruits effector T-cells, neutrophils, and monocytes into target organs. TNF-α cause direct tissue damage by inducing necrosis of target cells and it can also induce tissue destruction through apoptosis (177). It has been shown that serum levels of TNF-α are increased in patient undergoing GVHD after allogeneic SCT (229, 230) and that administration of anti- TNF-α antibodies markedly reduce the weight loss and mortality in a mouse model of acute GVHD (203, 231). Because TNF-α is thought to be involved in both induction and effector phases of GVHD (79), administration of anti-TNF-α antibodies might diminish not only direct cytotoxic activity of TNF-α , but also T-cell activation responsible for acute GVHD. Some beneficial effects of an anti- TNF-α monoclonal antibody (MoAb) for the treatment of refractory acute GVHD have been obtained in the phase I-II clinical trials, but unfortunately GVHD recurred when therapy was discontinued (146).

The second important cytokine that appears to play an important role in the effector phase of acute GVHD is IL-1. The importance of this cytokine has been verified in mice studies where mice receiving IL1 after allogeneic SCT displayed an increased mortality that appeared to be an accelerated from of GVHD (232). Increased gene expression of IL-1 in mononuclear cells has also been shown during clinical acute GVHD (233). In fact, the use of an IL-1 receptor antagonist (IL-1ra) has shown to reduce acute GVHD in mice

models (234, 235). However, in a recent randomized trial, IL-1ra treatment to prevent acute GVHD was not successful (145).

Nitric Oxide

NO is a short-lived biological mediator that plays an important role in host defense and the anti-microbial and tumoricidal function of macrophages. During the development of acute GVHD, increased production of IFN-γ, combined with entry and accumulation of LPS, results in macrophage activation and release of inflammatory products including TNF-α, NO and IL-1. In addition, exposure to increasing amounts of IFN-γ results in a significant reduction in the amount of LPS needed to trigger macrophage synthesis of inflammatory products (236, 237). As a result of IFN-γ production during the development of acute GVHD, macrophages become primed; therefore, normally insignificant quantities of LPS trigger production of NO and TNF-α (137, 238). In human and experimental animal transplant recipient, the symptoms of GVHD are preceded by an increase in serum levels of NO (239, 240). NO is involved in the effector arm of acute GVHD by inducing immunosuppression and by inhibiting repair mechanisms of target tissue through inactivation of non-heme iron-containing enzymes. This results in inhibition of proliferation of epithelial stem cells in the gut and skin (65, 241, 242) and direct tissue damage (243).

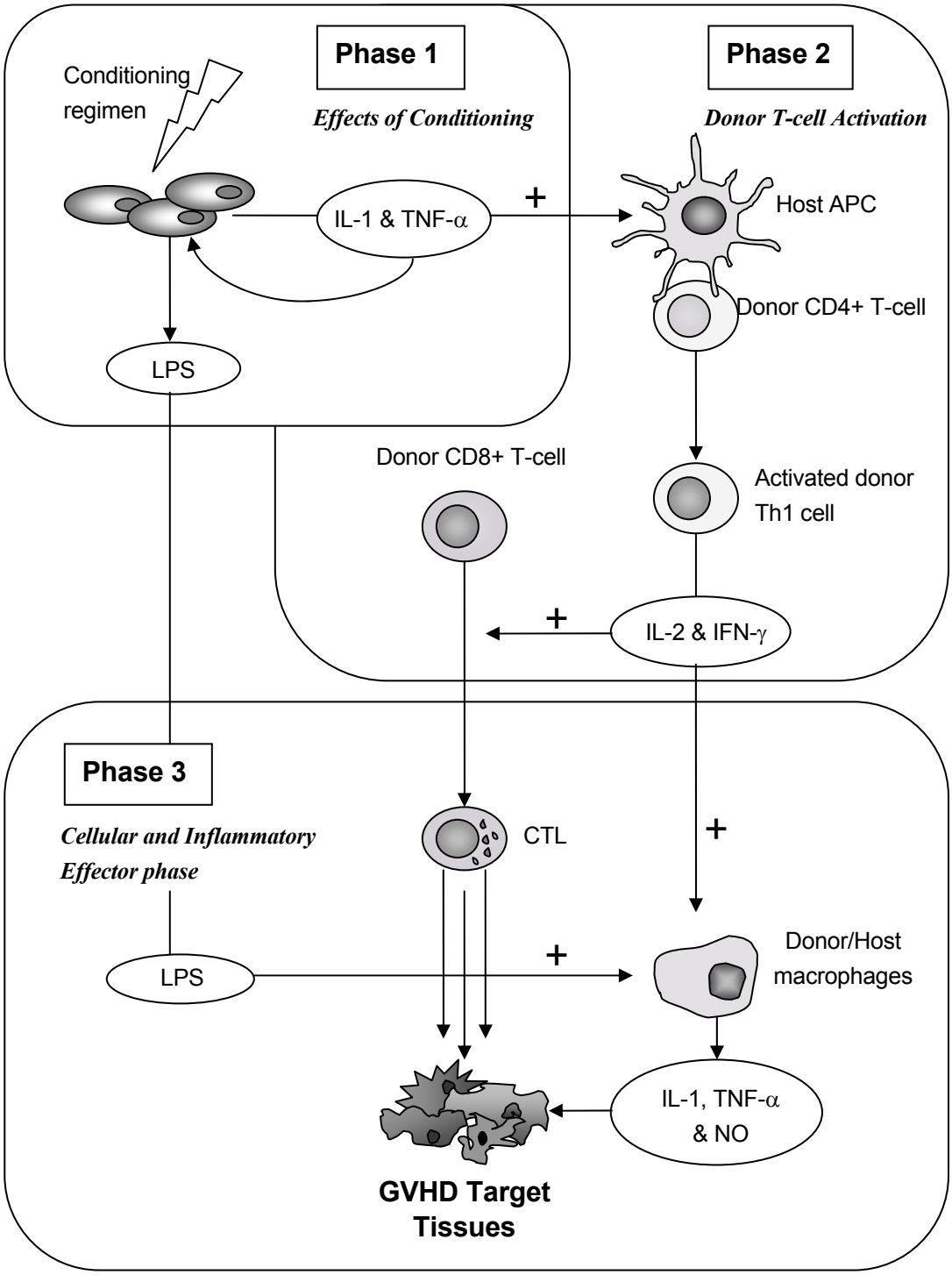

Figure 5. The three phase model of acute GVHD. Step 1) The conditioning regimen (irradiation and/or chemotherapy) leads to damage, activation of host tissues and induction of inflammatory cytokines (TNF-α and IL-1) secretion. Increased expression of MHC antigens and adhesion molecules leads to enhancement of the recognition of host MHC and/or miH by mature donor T-cells. Step 2) Donor T-cells proliferate and secrete IL-2 and IFN-γ. These cytokines induce further T-cell expansion, induce CTL and NK-cells responses and prime additional mononuclear phagocytes to produce TNF-α and IL-1. Also, NO is produced by activated macrophages and it may contribute to the tissue damage seen during step 3. LPS, which leaks through the intestinal mucosa that was damaged during step 1, together with IFN-γ, from step 2, further stimulate macrophages to secrete cytokines and NO. During step 3 CTLs and NK-cells induce target tissue damage through cell-mediated cytotoxicity.

Aims of the Present Study

General Aims

As discussed earlier, GVHD has been the primary limitation to the wider application of allogeneic SCT. To improve the long-term outcome after allogeneic SCT, better understanding of the components involved in the pathophysiology of GVHD is necessary. It has been shown that the survival is poor once GVHD has occurred since the damage caused by the GVHD activates more T-cells, causing more tissue damage, activating more cytotoxic cells. This vicious cycle is hard to disrupt once started since many accessory pathways can keep the reaction going. In addition, it is difficult to clinically separate the toxicity of GVHD from the desired GVL effects. Histological confirmation of tissues is imperative for the diagnosis since many things may cause clinical symptoms similar to GVHD.

There have been several attempts to devise a system that will provide more prognostic information. However, Glucksberg's original staging system is still used. Since early and accurate diagnosis and treatment of acute GVHD are essential to survival of the patient, new molecular diagnostic methods are needed. Therefore, the aims of this thesis were to evaluate molecular methods to diagnose and monitor acute GVHD after SCT without causing any further risks to the patients.

Specific Aims

The specific aims of this thesis are as follows:

Paper I and II. These two studies investigate the correlation between the gene expression of the effector mechanisms of acute GVHD and the diagnosis of the disease.

Paper III. In this paper, we analyzed the importance of the chimerism status early after transplantation for the occurrence of acute GVHD later on.

Paper IV. The aim was to use chemokine receptor gene expression levels as diagnostic markers for acute GVHD after SCT.

Materials and Methods

Most of the materials and methods employed in the present study have been described in detail in Papers I-IV. The following section lists these methods together with some general comments concerning advantages and disadvantages on each method used.

Patients

All studies were approved by the ethics committee at Karolinska University Hospital in Huddinge (DNR 339/00 and 63/96). Patient characteristics are summarized in Table 1.

	I	II	III	IV
No. Of patients	8	53	34	50
Diagnosis				
AML	4	15	7	14
ALL		10	8	10
CML	3	9	7	9
Other hematological malignancies	1	7	5	7
Nonmalignant disorders		3	4	3
Solid tumors		9	3	7
Recipient age (median, range)	39 (11-46)	42 (6-67)	32 (0.5-61)	41 (6-67)
Recipient sex (M/F)	5/3	27/26	22/12	23/27
Donor age (median, range)	32.5 (19-54)	38.5 (4-71)	32 (6-63)	38 (4-71)
Donor sex (M/F)	4/4	27/26	21/13	26/24
Cell source (BM/PBSC)	3/5	14/39	16/18	17/33
Donor (Sib/MUD/MMUD)	3/5/0	28/25/0	14/16/4	25/25
Conditioning				
TBI	3	16	11	16
Bu	5	16	15	15
RIC		21	8	19
GVHD prophylaxis				
MTX+CsA	8	41	30	42
MMF+CsA		12	3	8
CsA			1	
GVHD				
0	3	15	12	14
I	1	19	7	21
II	2	14	11	10
III	1	3	1	2
IV	1	2	3	3

Differences between Mice and Humans

There is no doubt that there are differences between mice and human immunology. These differences have not influenced the results in the studies presented in this thesis. However, one has to keep these differences in mind when the results are interpreted and compared to data obtained in mice models. One important difference between mouse and human studies is that mouse studies are performed under more controlled conditions. The mice are inbred and one or a few parameters can be studied separately. Patients are an outbred population in which various individuals may have severe genetic differences. In addition, differences in treatment regimens influence the results. In clinical research, concomitant variables are difficult to control. Therefore, in clinical studies one should strive at analyzing as homogenous groups as possible. However, in all studies in this thesis the development of acute GVHD was studied exclusively. In this aspect parameters such as conditioning treatment, diagnosis, age and stem cell source did not influence the results markedly. Which patient developed acute GVHD and why was not important in these studies. The only important thing was what was happening on the cellular level during the development of the disease.

Some disparities between mice and humans most likely influence the mechanisms of acute GVHD. One of these differences concerns the Th1 and Th2 paradigm. This polarization is relatively easy to observe in mice; however, the paradigm has never been as clear-cut in the human system. Th1 and Th2 cells can certainly be found in human diseases; however, in many diseases clear distinctions cannot be made and T-cells of both types can often be generated simultaneously (244, 245). For example, in mice, IL-10 is considered to be a Th2 cytokine, whereas in humans both Th1 and Th2 cells can make IL-10 (246). Furthermore, differences have emerged between the murine and human systems of chemokines and chemokine receptors. It is difficult to say what such differences could mean biologically, but one has to be aware of them when animal and patient studies are compared. Another interesting aspect is that both human and mouse endothelial cells (ECs) express MHC class I. However, most human EC in vivo also constitutively express MHC class II molecules, whereas mouse EC do not (247). Moreover, there is now considerable evident that human EC can present antigens to resting memory CD4+ and CD8+ cells, whereas in mice, CD8+ T-cells can be activated by EC, but CD4+ T-cells cannot. It has been suggested that triggering of responses may occur by different mechanisms in mice and humans, involving draining of antigen to lymph nodes in mice, compared with local antigen presentation in humans. Hence, this may influence the pathophysiology of acute GVHD differently.

Samples

Blood Samples

In all papers in this thesis, peripheral blood samples were used as starting material. Blood sampling provides an easy, fast, and non painful procedure for the patients and it is not associated with any complications. As discussed earlier, acute GVHD is today diagnosed clinically or, in more ambiguous cases, by pathological examinations of biopsies. In some cases, however, biopsies may not be diagnostic when other histological changes due to chemoradiotherapy, virus infections, and veno-occlusive disease cannot be distinguished from those seen with acute GVHD. Therefore, the diagnostic system is not completely

satisfactory. Moreover, the use of biopsies in the diagnosis in acute GVHD is associated with a risk for the patients. The liver is the least often biopsied of the major organs that are susceptible to acute GVHD. The major reason for this is an unacceptable risk of post-biopsy hemorrhages due to thrombocytopenia in the early post-transplant period. Another important drawback of the biopsy procedure is that only a few small areas of the affected organ are examined. Hence, false negative results may be obtained. For example, the rectum is the most commonly biopsied of the gastrointestinal organs; however, it may not always accurately reflect the status of the entire gastro-intestinal tract (248). Therefore, the purpose of Paper I, II and IV in this thesis aimed at finding an easy and accurate diagnostic method to diagnose acute GVHD without causing an increased risk of additional complications for the patient. However, one disadvantage of using blood samples may be that the results obtained do not correctly reflect what is actually happening in the target tissues, since gene expression in effector cells nearby affected GVHD target organs would not be included in this assessment.

In Paper I, II, and IV, RNA was extracted from blood "buffy-coats". "Buffy-coats" are composed of all different leukocytes present in the blood. Therefore, it is difficult to know if the constitution of the cells is consistent in samples and over time. Hence, in these studies it had been better to selectively extract desired cells from the peripheral blood sample. For example, CD4+ and CD8+ T-cells could have been selected by antibody-coated magnetic beads as in Paper III. In this case we would have had a more homogenous pool of cells to analyze. In addition, selection of cells specifically involved in the process, may have given a more correct picture of the pathophysiology acute GVHD. However, since the main reason of these studies was to develop a diagnostic method for acute GVHD that was as easy and as fast as possible to perform, the selection was not made. If the purpose had been to elucidate the mechanism involved in the GVHD process it would have been appropriate to analyze each cell type separately.

mRNA versus Protein Expression Analysis

Competitive and real-time RT-PCR was used for quantification of the mRNA expression of effector molecules (Papers I and II) and chemokines (Paper IV). The purpose of all these studies was to develop sensitive diagnostic methods for acute GVHD. We measured gene expression instead of protein expression since PCR offers a robust and sensitive technique that is easy to perform. Detection methods such as ELISA are not sensitive enough for the analysis of proteins expressed at very low levels. This was also seen in Paper I where we compared gene and protein expression by using PCR and ELISA. Many samples were under the level of detection for the ELISA assay used. In addition, assays to measure protein expression rather than gene expression are also influenced by proteases and inhibitory molecules, and they depend on protein specific antibodies for the detection. Another drawback with studies at the protein levels is that membrane bound proteins or proteins stored in intracellular vesicles are difficult to detect. However, one group have recently shown a new promising approach that allows identification of biomarkers for acute GVHD on the protein level (249). In this study, Kaiser *et. al.* showed that proteomic analysis based on capillary electrophoresis and mass spectrometry on urine samples form transplanted patients with acute GVHD was significantly different from those of healthy volunteers. This method seemed to be fast, reproducible and sensitive; however, these results have to be further validated in a prospective study.

One important aspect of gene expression analysis is that the presence of mRNA does not imply that the corresponding protein is translated. After transcription there are also other points of regulation, therefore, it could be argued that not all expressed transcripts will necessarily be translated to active proteins. Hence, by using the gene expression detection method, we assumed that the increase in mRNA is mirrored by the increase in the protein expression. However, since biosynthetic pathways normally do not waste unnecessarily energy, an increase in mRNA expression of a certain gene most likely reflects that the product of this gene is required. However, mRNA degradation and translatation efficiency influence the gene expression based methods.

Quantitative RT-PCR

Polymerase chain reaction (PCR)-based techniques allow us to obtain genetic information through the specific amplification of nucleic acid sequences starting with a very low number of target copies. Many applications in medicine or research require quantification of the number of specific targets in the specimen. In addition, the analysis of the expression on the mRNA level does require quantitative approaches to reverse transcription (RT)-PCR. Up until recently, several methods were used for this purpose. However, recent advances in technology have generated the new powerful method "real-time PCR", which has several advantages over all other methods used. In this thesis, two different quantitative RT-PCR methods were used - first the competitive RT-PCR and later on the real-time quantitative PCR. Some general comments concerning these two techniques will be discussed in this section.

Reverse Transcription (RT)

Analysis of gene expression requires accurate determination of mRNA levels. Since PCR is based on amplification of DNA rather than RNA, the RNA has to be converted to complementary DNA (cDNA), which is done by reverse transcription.

Reverse transcription is based on the ability of the enzyme reverse transcriptase, an RNA-dependent DNA polymerase, to generate a complementary strand of DNA using the mRNA as a template. The reverse transcription can be performed on either total cytoplasmatic RNA or on purified mRNA. In all our studies (I, II, and IV), total RNA was used for reverse transcription. The total RNA was extracted from leukocytes by using a commercially available RNA extraction kit (Qiagene). It is important that no contaminating DNA is present because this will also provide a template for the PCR amplification step later on. Therefore, DNase treatment of RNA samples is a very common procedure after RNA extraction. However, if the assay design includes primers that span an exon/exon junction, it can be avoided. In all our studies, PCR primers specific for cDNA and not for DNA were designed (discussed later).

The next step is to copy the RNA to first-strand cDNA. In our studies, this was done by using the Moloney murine leukemia virus (M-MLV)-based reverse transcriptase (RT) together with random hexamer oligonucleotide primers. This method generated first-strand cDNA of high quality that could be used immediately in both the competitive PCR (Paper I) and real-time PCR (Paper II and IV).

The different RNA transcripts could be reverse transcribed with different efficiencies in the cDNA synthesis. However, in our studies variations in mRNA levels of each respective gene were considered relative to itself at another time point, rather than comparing the absolute transcript numbers between different genes. Therefore, these differences would have had a limited effect on the results.

Competitive PCR

Competitive PCR was used for semi-quantification of mRNA in Paper I. It is a PCR based method where co-amplification of two similar template species is performed. The wild-type template, which has to be quantified and the standard template is co-introduced at a known amount. Because the standard competes with the target cDNA for both primers and enzyme it is referred to as a competitor. The competitor consists of target DNA or RNA that has been slightly modified. In Paper I competitors were generated by the deletion of 68-102 bp from wild-type cDNA. The double stranded wild-type cDNA was introduced into a plasmid vector, which also contained a reference gene. The plasmid used was kindly provided by Cross *et* al, Hammersmith Hospital, London, UK. Advantages of using plasmid vectors are that they can be easily prepared in very large amounts and that they can be stored for long periods at -20° without significant degradation.

Both the target and competitor are primed with a gene-specific primer and the cDNAs are then co-amplified directly in the same tube using a single primer pair. The best way to avoid co-amplification of genomic DNA is to design specific primers sets that do not detect genomic DNA, only cDNA. Therefore, all primers used in Paper I were designed to span an exon/exon-junction. When the primers were optimized, no DNA amplification was observed. In practice several reactions are performed simultaneously with different amounts of competitor cDNA. In Paper I, a constant unknown amount of cDNA was co-amplified with a series of dilutions of competitor plasmid – diluted in steps of half-log of magnitude (from $10\text{-}10^{7}$). The nucleotide deletion in the competing standard template allowed discrimination between the two co-amplified products. The point of equivalence between the co-amplified cDNA of the RNA to be measured and the standard cDNA was determined after electrophoretic analysis of the products on an ethidium bromide-stained agarose gel. The competitive RT-PCR method is also summarized in Figure 6.

Since RNA extraction and reverse-transcription often produce variable yields, the number of transcripts was normalized to a reference gene. In Paper I we used a housekeeping gene called glucose-6-phosphate dehydrogenase (G6PD). To quantify the expression of this gene the same procedure as described above was performed for G6PD. Results were then calculated as the ratio of the amount of detected gene transcript of interest, divided by the amount of detected G6PD in the same sample.

Although careful attention was given to optimization of the competetive PCR technique used in Paper I, there are major drawbacks in using this method. The most important reason that end-point quantification of PCR products is sometimes unreliable, is that PCR reaction generates copies of a DNA template in an exponential fashion. Due to inhibitors of the polymerase reaction found with the template or reagent limitation, eventually the PCR reaction no longer generates templates at an exponential rate, and some reactions will generate more product than others. Hence, it is only during the

exponential phase of the PCR reaction that it is possible to extrapolate back to determine the starting amount of the template. However, when the study for Paper I was performed other quantification methods such as real-time quantitative PCR (RQ-PCR) were unavailable. Therefore, we used the best method available at that time.

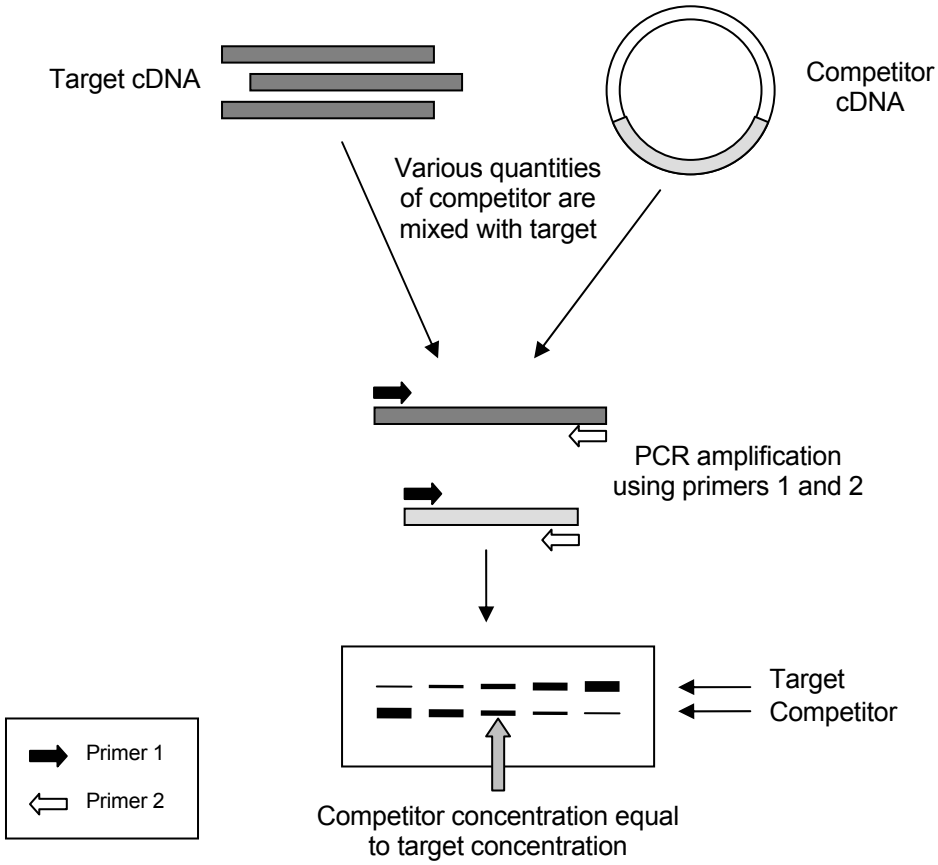

Figure 6. Principle of competitive RT-PCR. A competitor that can be distinguished from the target product upon gel analysis is generated. The RT-PCR reaction is performed by co-amplification of known amounts of competitors (in serial dilutions) with unknown amounts of target. The amount of competitor that gives the same amount of product on the gel as the target sample provides a measure of the amount of target mRNA in the original sample.

Real-time PCR

As described previously, competitive PCR of reversed-transcribed RNA used to be one of the most sensitive methods for molecular quantification of mRNA. However, since end-point quantification of PCR products sometimes is unreliable, a new quantitation method has been developed. RQ-PCR is a very powerful and sensitive method that can be used to quantify DNA levels or mRNA expression levels of genes that are rather low. Another advantage of this method is that no post-PCR steps are required, and since it is performed in a "close tube" chamber it has a decreased risk for PCR cross-contamination. These advantages are of special interest when RQ-PCR is used for diagnostic applications. This

technique uses fluorescent dyes to combine amplification and detection of increase in fluorescence, to permit the monitoring of accumulation of PCR product in "real-time". One major area for application of RQ-PCR assays is the quantification of gene expression. Therefore, in Papers II and IV RQ-PCR was used to quantify gene expression of T-cell effector molecules and chemokine receptors during acute GVHD.

As mentioned briefly above, the detection system in RQ-PCR is based on fluorescent signals generated during the PCR reaction. Fluorescent signals that are proportional to the amount of PCR product can be generated in several ways by using fluorescent dyes that are specific for double-stranded DNA or by sequence-specific fluorescent oligonucleotide probes. The simplest and cheapest principle is based on an interaction between the double-stranded DNA-binding dye SYBR Green I and the double stranded product created during the amplification step. However, since this dye is not specific for a certain sequence, both specific and nonspecific PCR products are detected. Therefore, this assay requires careful optimization of the PCR conditions.

In Papers II and IV, we used the most commonly applied RQ-PCR technique, the TaqMan assay. This method uses the 5´nuclease activity of the *Taq* polymerase to degrade a hybridization probe during the extension step of the PCR amplification. The quantification of nucleic acids (DNA or RNA), in our case cDNA, requires the annealing of three oligonucleotides: a forward primer, a reverse primer and a probe. All of these are specific for the target and are able to bind to it. The forward and reverse primers normally used for PCR amplification define endpoints and length of the amplicon and provide the first level of specificity. The third oligonucleotide is an internal probe, labeled with a reporter dye and a quencher molecule, which is used to generate a fluorescence signal proportional to PCR product. Introduction of the probe further increases the specificity of the quantified PCR product. When the TaqMan probe is intact, the quencher absorbs the fluorescence of the reporter dye, and no signal is generated. The *Taq* exonuclease activity releases the quencher from the oligonucleotide during the primer extension phase of the cycle resulting in an increase in fluorescence in each cycle proportional to the amount of specific product generated. This process occurs in every cycle and does not interfere with the exponential accumulation of PCR product. The increase in fluorescence is measured cycle by cycle by the real-time instrument, in our case the ABI Sequence Detection System (Applied Biosystems, Foster City, CA, USA). The principles of TaqMan RQ-PCR are summarized in Figure 7.

After completion, the sequence detection software constructs an amplification graph by plotting the measured fluorescence against the cycle number. Threshold cycle (Ct) values are then calculated by determining the point at which the fluorescence exceeds a chosen threshold limit, which is based on the variability of the baseline. Ct is reported as the cycle number at this point. Samples with high copy numbers of target will reach the threshold value at an earlier cycle than samples with less target copies, hence, lower Ct values for a higher concentration.

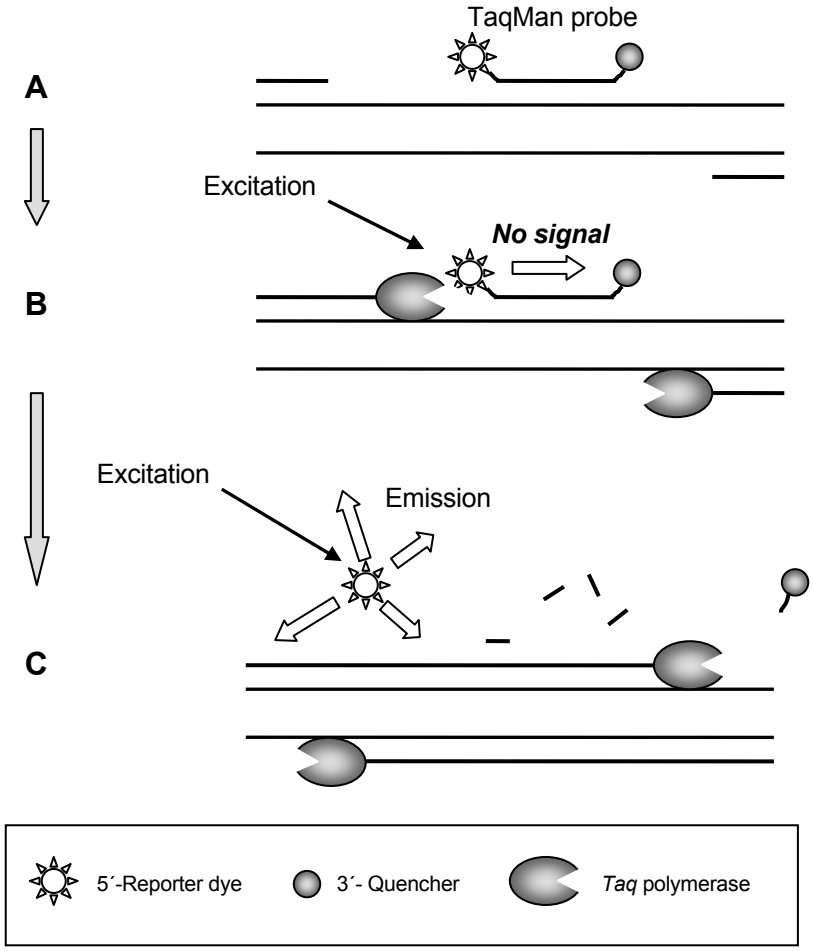

Figure 7. Principle of real-time PCR by using TaqMan assay. A) After denaturation, primers and probe anneal to the target. Fluorescence does not occur because of the proximity between fluorophore and quencher. B) During the extension phase, the probe is cleaved by the 5´→3´enzymatic activity of *Taq* polymerase. C) Thereby quencher and fluorophore are separated, allowing fluorescence emission from the reporter dye.

There are primarily two types of RQ-PCR analysis: "relative quantification" and "standard-curve quantification", also known as "absolute quantification". The standard curve method allows absolute quantification of the target. However, in our studies the main interest was to analyze the relative changes in transcript levels rather than the exact amount of mRNA. Therefore, the comparative threshold method was used in both Paper II and IV. In this method, the quantity of cDNA is usually referred relative to the amount of a housekeeping gene within the same sample. The normalization to a housekeeping gene is currently the most acceptable method to correct for minor variations due to differences in input RNA amount. The critical issues defining the reliability of the obtained data from a RQ-PCR study are the choice of the housekeeping gene. An ideal housekeeping gene has always the same level of expression despite the state of cellular activation. A variety of genes have been used for this purpose. In Paper II we evaluated two different housekeeping genes, G6PD and the Abelson (ABL) gene. In 241 samples, the ABL and G6PD levels correlated with $r = 0.55$, $p < 0.0001$ (Figure 8). Since there was a close correlation between these two control genes, only G6PD was used as housekeeping gene in Paper IV.

In relative quantification the quantification is done relative to the control gene by subtracting the Ct of the control gene from the Ct of the gene of interest, and the resulting difference in cycle number (ΔCt) is the exponent of the base 2 (due to the doubling function of PCR for every cycle), representing the fold difference of template for these two genes.

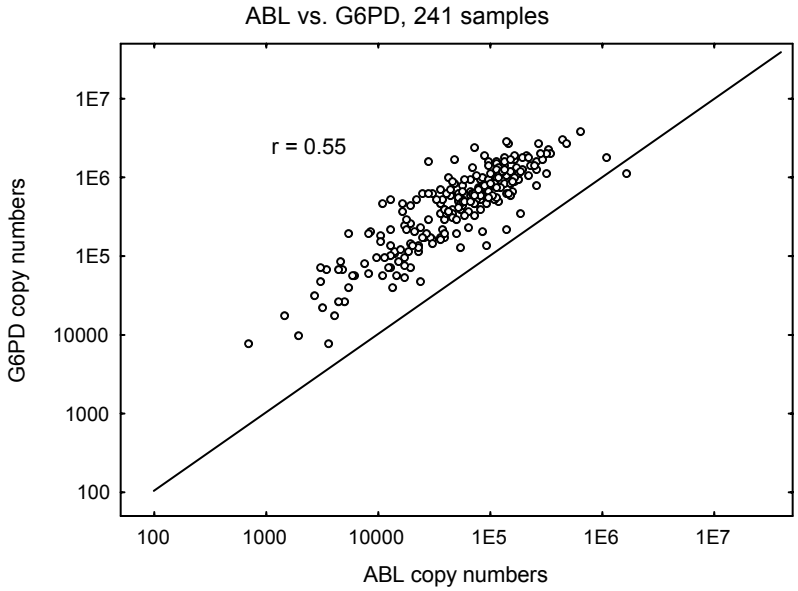

Figure 8. Correlation between the housekeeping genes ABL and G6PD.

Advantages of using this system are that no standards have to be constructed and that all 96 wells in one run can be applied for unknown samples, which saves time and money. A disadvantage, however, is that the efficiency of amplification of housekeeping and target gene have to be similar to obtain reliable results.

Although the RQ-PCR is a very powerful technique, it needs extensive and accurate optimization to be reliable. As for PCR in general, developing an ideal assay includes choosing primer that will result in an amplification product that is specific to cDNA and does not amplify genomic DNA. For all genes in Paper II and IV the primers were designed to target two different exons. To verify that no genomic DNA was amplified we used SYBR Green I in the optimization step of the primers. SYBR Green I allowed specific detection of the amplicon, but in this case more importantly it allowed us to perform a melting curve analysis of each product to ensure that the fluorescent signal observed was from the desired PCR product only. In most cases, nonspecific products have different lengths and therefore deviation melting temperatures. In addition, the PCR products were also verified by gel electrophoresis. Amplicon lengths for RQ-PCR should be chosen as short as possible: preferably between 50 and 150 bp, although in Paper II and IV amplicons up to 160 bp were amplified efficiently.

To make the amplification even more specific, we let the hybridization probes used in our studies span the junction of two adjoining exons. This design made it very unlikely to detect genomic DNA. In addition, the optimized primer sets were run together with the hybridization probes and purified DNA. However, no unspecific amplification of genomic DNA was observed. In both our studies, the fluorescent reporter dye consisted of the commonly used FAM (6-carboxyfluorescein) dye bound to the 5´-end of the probe and

the reporter was quenched by TAMRA (6-carboxytetramethylrhodamine), bound to the 3´-end of the same probe. The primers and probes for all target sequences in Paper II and IV were designed using the computer program Primer Express, a software program specially provided with the ABI 7000 SDS (Applied Biosystems, Foster City, CA, USA), and they were all commercially synthesized (CyberGene Huddinge, Sweden). In addition, to minimize the variability between samples a multi-dispenser pipette was used to add PCR-mix into each PCR well. PCR reagents were bought from the same company throughout all studies and, the same PCR program was used for all PCR reactions. Variations in the duplicate measurements were very low and the intra-assay variability was less than 10% for all markers used.

Chimerism Analysis

The concomitant presence of circulating donor and recipient hematopoietic cells are often investigated after allogeneic SCT. Different methods have been developed to monitor chimerism and one commonly used method is PCR amplification of variable number of tandem repeats (VNTRs), also known as mini-satellites. VNTRs are regions in the genomic DNA that contain specific sequences (10-70 bp) repeated a variable number of times. The number of repeats varies widely between individuals. Hence, PCR amplification of VNTRs results in PCR products of different length depending on the number of tandem repeats. Pre-transplant recipient and donor DNA samples were "screened" with a panel of different VNTRs to find markers that can discriminate patient and donor DNA. In Paper III, we used primer sets for five different VNTRs to be able to find at least one informative locus. Primer sequences and other data concerning the VNTRs used have been published previously (63). If several informative markers were found, the one that generated the shorter PCR product in the patient compared to the donor was chosen. This was done to increase the sensitivity for recipient cells, since in PCR reactions a shorter sequence has shown to be amplified more frequently than a longer one. The chosen primer pair was then used to amplify DNA sequences in the post-transplant recipient samples of interest and patient and donor samples obtained before transplantation. To separate the products from the PCR reaction, a ready-to-use polyacrylamide gel electrophoresis (PAGE) system from Pharmacia Biotech (Uppsala, Sweden) was used. Subsequently, the results from the PCR were analyzed after an automated silver staining procedure. Figure 9 summarizes the principle of PCR amplification of VNTRs.

A semi-quantitative based method was used to estimate the proportion of donor-recipient chimerism. For estimation, the recipient and donor band intensity were compared to a serial 10-step dilution series of mixed patient and donor DNA.

To further increase the sensitivity of this method, cell separation can be performed (63, 250). In Paper III, a selection was performed; however, the main purpose of this selection was not to increase the sensitivity, but rather to study the chimerism in the selected populations exclusively. As discussed in section 3, donor T-cells play a primary role in the development of acute GVHD. Therefore, CD4+ and CD8+ T-cells were of main interest in Paper III and by using immunomagnetic beads these cells were selected for. Although chimerism analysis is not leukemia specific this method is often used as a minimal residual disease (MRD) detection method (63, 251). However, in Paper III, the purpose was not to use this method to detect MRD, but rather to investigate how the

chimerism status influenced the development of acute GVHD. Interestingly, by using this method, we were able to estimate the recipient to donor ratio in blood samples obtained early after SCT when patients are aplastic (< 0.1 x 10^9 WBC/L).

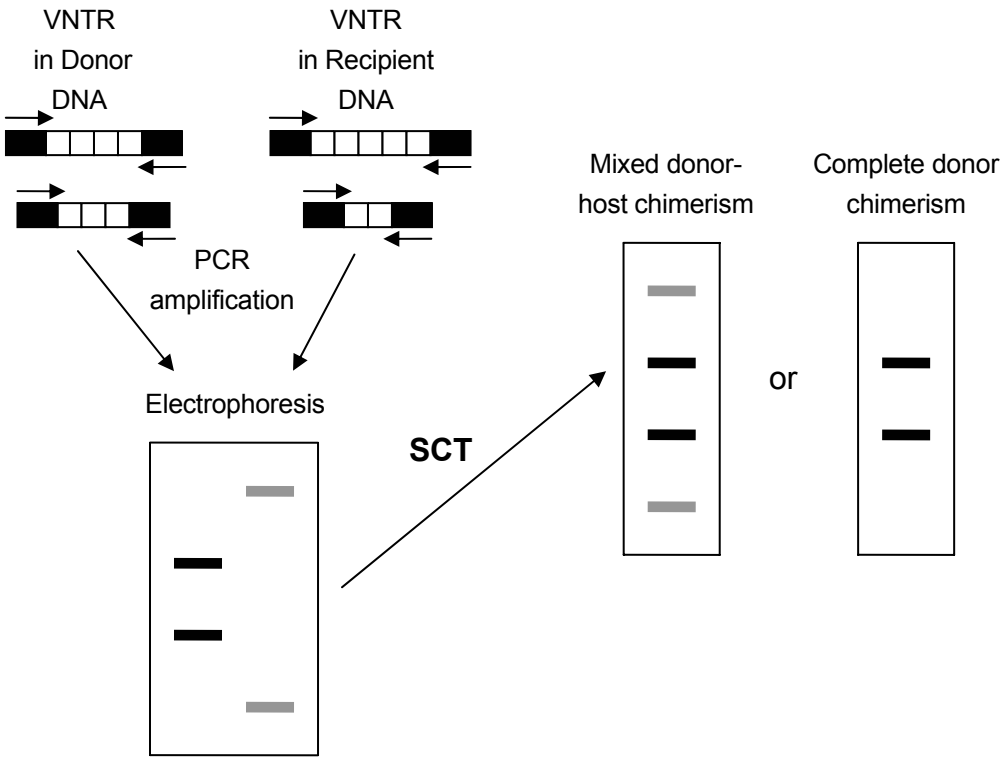

Figure 9. PCR amplification of minisatellite markers. Minisatellite markers (VNTRs) in the donor and the recipient's blood are analyzed by a PCR-based assay. The number of repeats in a given VNTR can differ between one individual to another. This will result in PCR products of different length. After receiving the transplant, the recipient can either experience mixed chimerism, in which the blood system is made up of a combination of host and donor lymphocytes, or complete donor chimerism, where all lymphocytes are donor derived.

Results and Discussion

In all studies included in this thesis, we aimed at finding new diagnostic methods for diagnosis of acute GVHD after SCT. Today, the only available method of diagnosing acute GVHD relies on clinical observations and clinical judgments. Since plenty of other problems can influence the symptoms seen in patients after SCT, diagnosing acute GVHD can be rather difficult. For example, other problems that may affect post-transplant hepatic function include veno-occlusive disease, drug toxicity, viral infections, sepsis, iron overload, and extra-hepatic biliary obstruction. To discriminate a skin rash from being due to GVHD or an allergic reaction to a drug is almost impossible. Furthermore, diarrhea is a common problem after SCT. This may be due to toxicity, GVHD, infection etc. All of these reactions further confuse the picture of the patient's disease. Because many patients are not biopsied, the exact reason for the problems may not always be understood. Although most diagnoses of acute GVHD are usually correct, there are probably cases where a sub-clinical acute GVHD can occur even though it cannot be observed clinically.

Effector Molecules (Paper I & II)

In these two papers, we analyzed the gene expression of the effector molecules granzyme B, perforin, Fas L, and TNF-α in peripheral blood from patients after SCT. The gene expression levels were then correlated to the outcome after transplantation. Although some other immunological events influence the results, we found that there is a correlation between the gene expression of granzyme B, perforin, FasL, and the occurrence of acute GVHD. However, TNF-α showed a more diffuse correlation. Interestingly, in both papers we found that increasing gene expression levels of granzyme B, perforin, and FasL during ongoing GVHD treatment was associated with a deteriorating GVHD. In paper II all 10 patients with increased levels of granzyme B, perforin and FasL during steroid treatment showed a persisting or deteriorating GVHD while only 2 of 28 patients with declining levels showed the same phenomenon. Although not specific for acute GVHD, these results indicate that gene expression levels of granzyme B, perforin and FasL may be used to diagnose and most importantly to monitor GVHD therapy after SCT.

In paper I and II, we used two different quantitative RT-PCR methods, competitive and RQ-PCR. We obtained similar results by using the two techniques; however, the RQ-PCR is to be preferred since it is less time-consuming and more sensitive than the competitive method.

In Paper I, protein expression of TNF-α, soluble FasL (sFasL), and granzyme B was also analyzed. In this limited material the protein levels of granzyme B and sFasL correlated to the gene expression of the corresponding genes. However, it was impossible to evaluate the protein levels of TNF-α since the majority of plasma samples contained protein concentrations under the level of sensitivity for the assay used. One reason for this might be that the measurement of TNF-α in the serum has been particularly difficult to standardize because of the large amounts of soluble circulating TNF-α receptors. Such soluble receptors interfere with the detection of cytokines depending on the specific epitopes recognized by the monoclonal antibodies used in the assay. Therefore, more

studies are needed to determine the usefulness of the assessment of plasma protein levels of granzyme B, sFasL, and TNF-α in diagnosing acute GVHD.

In Paper II, we also observed that an increase in gene expression of the effector molecules studied correlated with the response to CMV infections. We showed that patients with decreased gene transcript levels of granzyme B, perforin and FasL during a CMV infection responded poorly to CMV. We also found that 13 of 17 patients with rising immune transcript levels during CMV reactivation responded well to therapy, and none had a CMV reactivation. Increased immune transcript levels during CMV infection have also been shown previously by Soccal *et. al.* in lung transplant recipients (252). It may therefore be useful to analyze immun transcript levels after SCT in order to evaluate which patient that will respond to CMV therapy. This needs however to be confirmed in a larger patient material.

Changes in gene expression levels have earlier been shown to be useful in predicting rejection after lung and kidney transplantations (252-254). Since the mechanisms involved in organ rejection are quite similar to those responsible for acute GVHD one can assume that elevated gene expression levels of acute GVHD effector mechanisms may be correlated to the graft-versus-host reaction. Apart from our own two studies, a few other groups have shown that increased mRNA expression of granzyme B, perforin, and FasL correlates with acute GVHD in patients after SCT. Lee *et. al.* (212) showed that an increase in the percentage of CD8+ lymphocytes that express Fas and its ligand was correlated to acute GVHD after SCT. Moreover, Das and colleagues (217) showed that elevated levels of FasL in patients after donor lymphocyte infusion were correlated to the clinical development of acute GVHD.

Chimerism (Paper III)

SCT is generally carried out with the intent of complete replacement of host lymphohematopoiesis by donor derived cells. However, it is well known that recipient T-lymphocytes may survive intensive conditioning (255-257). Clinical studies have shown that mixed chimerism, defined as the coexistence of normal donor and host lymphohematopoietic cells, may develop in patient undergoing clinical marrow transplantation. The precise mechanisms leading to mixed chimerism are currently unknown. Judging from findings in murine models, the induction of mixed chimerism may limit the risk of acute GVHD (258). This is also supported by data from some clinical trials (255, 259, 260). However, it is clear that the acute GVHD can occur even in mixed chimeras (261-263). Predicting the risk of acute GVHD before its clinical manifestation and early administration of additional therapy may result in less incidence of severe GVHD. Since T-cells are the prime cell population mediating GVHD, monitoring T-cell chimerism may be important for evaluating the immunological status after SCT.

Chimerism techniques can detect the presence of recipient cells after SCT and allow the relative proportions of host and donor cells to be identified and quantified. Mini-satellites are highly polymorphic regions with a variable number of tandem repeats (VNTR) of nucleotides that are located throughout the genome. PCR amplification of mini-satellite regions is one of the most sensitive and rapid methods of determining chimerism. In a number of studies, molecular monitoring of chimerism has been initiated after the first 4 weeks following transplantation, but analysis during the very early post-

transplant period has rarely been reported even though VNTR analysis enables the analysis of a very small number of cells (264).

In Paper III, we used VNTR chimerism analysis to investigate whether T-cell chimerism during the early post-transplantation period may be used as a diagnostic tool to predict the occurrence of severe acute GVHD after SCT. Acute GVHD may be initiated by either subset or simultaneously by both subsets of CD4+ T-helper cells and cytotoxic CD8+ T-cells (23). Therefore, in the present study chimerism patterns were analysed in the two different cell populations. To increase the chance of detecting the few remaining T-cells of recipient origin that initially exist after SCT we used immunomagnetic beads to sort out the cells of interest. The objective was to evaluate whether the tempo of host hematopoietic cell disappearance between days 7 and 10 after SCT was altered in the event of acute GVHD. In this study, we found that there was a significantly higher risk for patients with complete donor chimerism on day 7 together with patients with an increase of more than 50% in the donor CD4+ T-cell population between day 7 and 10 to develop moderate to severe acute GVHD. Thus, our data suggest that molecular monitoring of the fate of host/donor hematopoietic cells in the early post-transplantation period could be useful in predicting the occurrence of acute GVHD as early as day 10. Previous to our study, Gyger and colleagues have presented similar data (265). In paper III, including a limited number of patients, we could not observe any major differences between CD4+ and CD8+ T-cells. More evaluation is needed to determine whether either one of the cell populations or the two together can be used to identify patients with a higher risk of developing acute GVHD grades II-IV after SCT.

Interestingly, this technique seems to be of special value when limited quantities of DNA are obtained from aplastic patients. In this study, donor/host DNA was detected even though all but 2 patients included in this study were aplastic at the time of assessment.

Admittedly, the development of mixed chimerism after SCT will vary with the disease, GVHD prophylaxis, conditioning regimen, and other factors; however, irrespective of the reason why they become mixed chimera, these results show that the pattern of chimerism on day 7 and 10 post-SCT may help differentiate patients who will subsequently develop acute GVHD from those that will not. However, additional studies, including a more homogenous patient group, are needed to further confirm these findings.

Chemokines (Paper IV)

The inflammatory reaction in acute GVHD begins with T-cell infiltration. Although all nucleated cells express class I MHC molecules on the cell surface (major antigens for alloreactive T-cells) T-cell infiltration in acute GVHD, both in humans and mouse models, does not occur evenly in the body. The reasons for the occurrence of GVHD in these organs are not well understood. However, the discrepancy may be due to the factors that influence T-cell migration. Of these factors, chemokines play a central role by determining the direction of T-cell subpopulations in the trafficking and infiltration process.

In Paper IV, we used RQ-PCR to quantify chemokine receptor mRNA profiles in patients after allogeneic SCT. We hypothesized that the gene expression levels of CCR5, CXCR3, CCR1, and CCR2 could be used to diagnose acute GVHD after transplantation. Chemokine receptors, such as CCR5 and CXCR3, have earlier been shown to be

involved in acute rejection after heart transplantation in patients (266, 267) and in mice (268), and acute lung allograft rejection in rats (269). The involvement of chemokine receptors in acute GVHD have so far been shown only in animal models. For instance, studies using murine models of acute GVHD have demonstrated the critical role of several chemokines and their receptors, particularly MIP-1α, MIP-2, Mig, MCP-1, MCP-3, CCR5, and CXCR3, in directing T-cell infiltration into target tissues during acute GVHD (187-190). In Paper IV, we showed that the four chemokine receptors studied correlated with the diagnosis of acute GVHD after allogeneic SCT in patients. In more than 72% of the cases of acute GVHD, the gene expression levels were up-regulated in connection with diagnosis with a median increase ranging from 3 to 12 times for CCR1 and CXCR3, respectively. Interestingly, in many patients we found increasing gene expression a few days before acute GVHD was diagnosed clinically. It is well known that predicting the risk of acute GVHD before its clinical manifestation and early administration of additional therapy may result in less incidence of severe GVHD. In line with this, our findings in this study may be of importance for diagnosing and earlier administration of additional treatment of acute GVHD in the future.

If this study had been conducted today, we would most likely have started off by performing a microarray on cytokine and chemokine gene expression to distinguish the most important ones. Since this was not done, there may be other chemokines that could be of greater importance for the GVHD process than the ones that we have studied here. However, gene expression profiles using microarray gene expression technology have recently provided valuable information about the mechanisms involved in the development of cutaneous and hepatic GVHD in mice (270, 271). Both these studies showed up-regulations of effector molecules (e.g. Fas and granzyme B), chemokines and their receptors (e.g. Mig, MCP-1, MCP-2, CCR1 and CCR2) as well as acute-phase proteins and adhesion molecules. Furthermore, in addition to microarrays, it would have been appropriate to study not only the chemokine receptor gene expression in peripheral blood but also the chemokine expression in target organs. However, to biopsy all target organs of acute GVHD would not have been ethically acceptable.

Conclusions

- Gene expression of granzyme B, perforin and FasL in peripheral blood is correlated with acute GVHD after allogeneic SCT.

- Increasing gene expression of granzyme B, perforin and FasL during steroid treatment may result in persisting or deteriorating acute GVHD.

- Increasing gene expression of granzym B, perforin and FasL during CMV infection is associated with a better response to CMV treatment than with decreasing levels.

- A rapid increase in donor T-cells during day 7 and day 10 in patients after transplantation together with patients with complete donor chimerism on day 7 was significantly associated with increased risk of developing acute GVHD grades II to IV after SCT.

- Increasing gene expression of chemokine receptors CCR5, CXCR3, CCR1 and CCR2 is correlated with the occurrence of acute GVHD after SCT.

- The gene expression of CCR5, CXCR3, CCR1 and CCR2 seem to be up-regulated prior to the clinical diagnosis of acute GVHD.

Even though not exclusively specific for acute GVHD, Paper I, II and IV suggest that gene expression of T-cell effector molecules as well as chemokine receptors may have a potential as molecular markers for diagnosing acute GVHD, but most importantly, to monitor its treatment. Paper III shows the importance of T-cell chimerism early after SCT for the development of acute GVHD.
Hopefully, these findings will together with new strategies to prevent and treat acute GVHD, result in a safer treatment modality of SCT. This would in turn make allogeneic SCT more available to patients not considered for SCT today.

Summary in Swedish

Benmärgstransplantation (BMT), eller hematopoetisk stamcellstransplantation (HSCT), är idag en etablerad behandlingsmetod vid en rad sjukdomar som exempelvis leukemier, svåra blodbristsjukdomar, immundefekter samt vissa mer ovanliga enzymbristsjukdomar. Dessa sjukdomar innebär att patienten av en eller annan anledning måste få sin benmärg ersatt av nya friska blodstamceller.

Allogen HSCT innebär att patienten får benmärg/stamceller från någon annan individ, antingen ett syskon eller en obesläktad, frivillig givare. Idag beräknas ca 30 % av patienterna som är i behov av HSCT ha tillgång till ett syskon som passar vävnadstypmässigt. De övriga 70 procenten får förlita sig på att det finns en obesläktad, frivillig givare som passar. Idag finns det mer än 9 miljoner frivilliga givare i register runt om i världen. Det är viktigt att givarens och patientens celler stämmer överens vävnadstypmässigt. På ytan av en människas celler finns vävnadsmarkörer som är specifika för varje individ. Dessa kallas för MHC-molekyler och hjälper de vita blodkropparna att känna igen vad som är ”eget” och ”icke eget”. Celler med ”icke eget” MHC uppfattas som främmande och dödas.

Före själva transplantationen behandlas alla patienter med cellgifter och/eller strålning. Syftet med förbehandlingen är att avlägsna de sjuka cellerna i kroppen och att ta bort det egna immunförsvaret så att den nya, friska märgen inte stöts bort. Donatorns friska stamceller ges därefter till patienten som en blodtransfusion. Trots att givarens och patientens celler tycks vara lika vävnadstypmässigt, d.v.s. MHC är matchade, finns det ändå små skillnader mellan dessa individer som cellerna kan uppfatta som främmande. Detta gör att det nya immunförsvaret (vita blodkroppar från de nya stamcellerna) uppfattar den nya kroppen som ”främmande”, vilket framkallar en immunologisk attack. Denna reaktion, som kallas transplantat-kontra-värd-reaktion (engelska, GVHD), drabbar först och främst kvarvarande blodceller från patientens ”gamla” märg och dödar dessa. För leukemipatienter är detta en mycket önskvärd reaktion, eftersom detta hjälper till att utplåna cancerceller som överlevt förbehandlingen. Tyvärr kan transplantat-kontra-värd-reaktionen även drabba kroppens övriga celler. Om reaktionen blir alltför kraftig kan den bli livshotande för patienten. Denna reaktion är alltså både på gott och ont, eftersom en viss reaktion från givarens celler mot patienten eftersträvas men samtidigt får reaktionen inte bli allt för kraftfull. Det optimala vore att finna ett sätt att bli av med transplantat-kontra-värd-reaktionen samtidigt som den s.k. transplantat-kontra-tumör-effekten bibehålls. Hittills är detta en utopi men detta kommer att vara målet för framtida forskning.

Akut transplantat-kontra-värd-reaktion är en av de svåraste komplikationerna efter HSCT, och den drabbar de flesta patienterna i viss grad efter transplantationen. Reaktionen uppträder vanligtvis inom tre månader efter transplantationen. Tre organsystem i kroppen drabbas huvudsakligen: huden, levern och tarmen. Vanliga symtom är hudrodnad som kan börja i handflator, under fotsulorna eller i ansiktet. I svårare fall kan patienten få hudrodnad på hela kroppen, leverpåverkan och svår diarré. Idag kan diagnosen akut transplantat-kontra-värd-reaktion endast fastställas kliniskt, d.v.s. läkaren gör en bedömning utifrån graden av hudrodnad, tarmpåverkan och leverpåverkan. Svårare transplantat-kontra-värd-reaktion bedöms med hjälp av s.k. biopsier, d.v.s. att man tar vävnadsmaterial från exempelvis levern och tarmen. Biopsitagningen är både en besvärlig undersökning för patienten och framförallt förenad

med en ökad blödningsrisk. När diagnosen akut transplantat-kontra-värd-reaktion ställs idag, har med andra ord den immunologiska attacken mot patientens celler, framför allt hud-, tarm- och leverceller, redan startat och då kan det vara svårt att bromsa upp eller stoppa den helt. Det är en välkänd sanning att det är lättare att förebygga en akut transplantat-kontra-värd-reaktion än att behandla reaktionen. Alla patienter får därför en förebyggande behandling mot akut transplantat-kontra-värd-reaktion efter HSCT. Denna behandling kan dock inte vara alltför kraftfull i och med att det skulle öka infektionskänsligheten ytterligare om immunförvaret försvagas för mycket. Det är därför viktigt att nya metoder för att diagnostisera akut transplantat-kontra-värd-reaktion utvecklas, där den immunologiska attacken kan upptäckas på ett tidigare stadium än vad som är möjligt idag. På det viset skulle endast patienter med en ökad risk för svår akut transplantat-kontra-värd-reaktion få ytterligare immunhämmande behandling. Syftet med den här avhandlingen har därför varit att utveckla nya diagnostiska metoder som bygger på att endast blodprov från den transplanterade patienten analyseras.

Det är sedan tidigare känt vilka strategier vita blodkroppar använder sig av för att döda andra celler. Vissa vita blodkroppar producerar nämligen proteiner som gör att de specifikt kan binda till sina ”målceller” och med hjälp av andra proteiner som de vita blodkropparna tillverkat och via komplexa cellulära reaktioner dödas ”målcellerna”. Vid en transplantat-kontra-värd-reaktion är det därför logiskt att tro att givarens celler skulle tillverka dessa proteiner i större mängd än normalt med avseende att döda celler som transplantatet uppfattar som ”främmande”, d.v.s. patientens egna celler. En ökad proteinproduktion förutsätter att generna för dessa proteiner uttrycks i en ökad grad. Syftet med arbete I och II var därför att avgöra hur mycket av dessa gener som uttrycks av vita blodkroppar i blodprover från benmärgstransplanterade patienter. Ett ökat uttryck av dessa gener skulle då vara ett mått på cellernas aktivitet och i och med detta också transplantat-kontra-värd-reaktionen. I dessa två arbeten visade vi att uttrycket av dessa gener korrelerade med diagnostiseringen av transplantat-kontra-värd-reaktionen. Metoden visar sig framförallt vara betydelsefull när det gäller uppföljning av behandlingen av akut transplantat-kontra-värd-reaktion. Många patienter som visade ett ökat genuttryck för dessa proteiner under pågående behandling, drabbades av en mycket svår akut transplantat-kontra-värd-reaktion. Metoden kan därför underlätta för ansvariga doktorer att tidigt se vilka patienter som är i behov av ytterligare immunhämmande behandling.

Arbete IV påminner mycket om arbete I och II. Istället för s.k. ”effektorproteiner” studerade vi här molekyler som finns på de vita blodkropparna och som gör att dessa celler hittar till de målorgan som är inblandade vid transplantat-kontra-värd-reaktionen. Vi fann även här att genuttrycket av dessa molekyler ökade i samband med diagnostisering av transplantat-kontra-värd-reaktionen. Intressant nog fann vi också i många fall ökande gennivåer i blodprover tagna innan diagnosen var fastställd kliniskt. Arbetena I, II och IV visar således att det finns ett samband mellan vissa genuttryck och transplantat-kontra-värd-reaktionen. Tyvärr är dessa markörer inte specifika för just denna reaktion. Även andra typer av komplikationer, t.ex. bakterieinfektioner, har visat sig påverka produktionen av de studerade generna. Trots detta finns det förhoppningar om att denna teknik kan komma att ha ett stort värde inom diagnostiseringen av akut transplantat-kontra-värd reaktion i framtiden.

Även om man med HSCT strävar efter att helt ersätta patientens benmärg med donatorns stamceller förekommer det att kvarvarande patientceller existerar under en kortare eller längre period efter transplantationen. Inom medicinen används ordet

chimärism för att beskriva detta tillstånd. I arbete III studerade vi förekomsten av eventuellt kvarvarande patientceller (blandad chimärism, dvs. en blandning av både patient- och givarblodceller) tidigt efter HSCT. Det har nämligen tidigare visats att patienter som är blandat chimära efter HSCT, har en lägre risk för att utveckla svår transplantat-kontra-värd-reaktion. Vår hypotes var därför att patienter hos vilka andelen donatorceller ökade kraftigt mellan dag 7 och 10 efter HSCT skulle löpa en större risk att senare drabbas av svår transplantat-kontra-värd-reaktion. I det begränsade patientmaterialet i arbete III såg vi indikationer på att denna hypotes skulle kunna vara riktig. Detta måste dock visas ytterligare i studier med ett större antal patienter inkluderade.

Vår förhoppning är att resultaten från dessa studier ska underlätta att tidigare kunna diagnostisera, förutspå och framför allt behandla patienter som drabbas av svår transplantat-kontra-värd-reaktion. Detta skulle ytterligare kunna förbättra resultaten efter allogen stamcellstransplantation och möjliggöra att patienter med andra sjukdomar, som ex autoimmuna sjukdomar, kan bli aktuella för denna terapiform i framtiden.

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