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METHODS AND BIOMARKERS FOR OUTCOME PREDICTION AFTER ALLOGENEIC HEMATOPOIETIC STEM CELL TRANSPLANTATION

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"If you do not overcome your tendency to give up easily, your life leads to nothing."

Masutatsu Ōyama

To my parents

ABSTRACT

Allogeneic hematopoietic stem cell transplantation (HSCT) is a potent immunotherapeutic procedure but its usability is limited by a high risk of serious complications. A prerequisite for timely initiation of preventive measures is the availability of predictive methods. This thesis aims to evaluate techniques that may potentially be used to assess the risk of some of these complications on the individual level.

Defective function of the pattern recognition receptor NOD2, due to naturally occurring gene polymorphism, has been indicated as a risk factor for graft-versus-host disease (GVHD). We investigated the potential influence of NOD2 on clinical outcome after HSCT in a retrospective study of 198 patients. Contrary to previous reports, we found no association between NOD2 mutations and acute GVHD, transplant-related mortality (TRM) or overall survival. We conclude that NOD2 genotyping is not a pertinent analysis before HSCT.

Leukemic relapse is a major cause of death after HSCT. Donor lymphocyte infusion (DLI) is one of the few therapeutic options remaining in these situations. Previous studies have shown varying results regarding treatment efficacy against acute leukemia. We aimed to investigate if the use of molecular techniques for relapse monitoring could improve the clinical outcome after DLI. Through retrospective analysis of 118 patients treated with DLI we showed that those with acute leukemia or myelodysplastic syndrome, who had received DLI based on the result of molecular methods, had a better survival rate than those treated during hematologic relapse (16% vs. 43%, $p<0.006$). Non-hematological relapse and chronic GVHD were identified as independent predictors for response to DLI in multivariate analysis. The overall incidence of severe acute GVHD was only 8.5% and was acceptable (14%) in the cohort treated before 100 days post-HSCT. Our conclusion is that early administration of DLI to patients with acute leukemia, based on changes in cell lineage-specific chimerism and MRD analysis can significantly improve relapse-free survival after HSCT.

Adaptive immunity is compromised after HSCT, mainly due to defective T-cell function. Reconstitution of the T-cell population is dependent on thymic function. We quantitatively assessed thymic function in 260 patients during a two-year period following HSCT. Levels of T-cell receptor excision circles (TRECs) in separated T-cells were measured with real-time quantitative PCR and used as a surrogate marker for thymic function. We found that low TREC levels 3-6 months after HSCT was correlated to inferior survival, increased TRM, and higher incidence of cytomegalovirus reactivation. We could also for the first time show that the use of bone marrow grafts and anti-thymocyte globulin had a negative effect on TREC levels, as did mesenchymal stromal cells when co-infused with umbilical cord blood grafts. We conclude that TREC analysis appears to have a high predictive value concerning outcome parameters after HSCT, and that factors related to the transplant procedure may significantly affect thymic function.

Finally, we present the results of a prospective pilot study in which we sought to design a functional, individualized strategy for assessing the risk of acute GVHD. Peripheral blood mononuclear cells were collected from patients and donors before HSCT and co-cultured in a mixed lymphocyte reaction (MLR) in the GVHD direction. Cells were phenotypically characterized by flow cytometry before and after MLR. We found that donors corresponding to patients who later developed acute GVHD grades II–IV had significantly higher levels of γδ T-cells and NKT-cells in peripheral circulation. We could also demonstrate a possible correlation between a high proportion of naïve $CD4^+$ T-cells in the allogeneic MLRs and occurrence of acute GVHD *in vivo*. We conclude that flow cytometric analysis of donor cells for phenotype and allogeneic reactivity may be used to predict acute GVHD before HSCT.

LIST OF PUBLICATIONS

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Leukemia lineage-specific chimerism analysis and molecular monitoring improve outcome of donor lymphocyte infusions.
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III. Michael Uhlin, **Darius Sairafi,** Sofia Berglund, Sarah Thunberg, Jens Gertow, Olle Ringden, Mehmet Uzunel, Mats Remberger, Jonas Mattsson
Mesenchymal stem cells inhibit thymic reconstitution after allogeneic cord blood transplantation.
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IV. **Darius Sairafi,** Jonas Mattsson, Michael Uhlin, Mehmet Uzunel
Thymic function after allogeneic stem cell transplantation is dependent on graft source and predictive of long term survival.
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V. **Darius Sairafi,** Jens Gertow, Jonas Mattsson, Michael Uhlin
Donor cell composition and reactivity predict risk of acute graft-versus-host disease after allogeneic hematopoietic stem cell transplantation
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LIST OF ABBREVIATIONS

ALL	Acute lymphoblastic leukemia
AML	Acute myeloid leukemia
APC	Antigen presenting cell
ATG	Antithymocyte globulin
BM	Bone marrow
BMT	Bone marrow transplantation
Bu	Busulfan
CAR	Chimeric antigen receptors
CB	Cord blood
CBT	Cord blood transplantation
CLL	Chronic lymphocytic leukemia
CML	Chronic myeloid leukemia
CMV	Cytomegalovirus
Cy	Cyclophosphamide
DC	Dendritic cell
DLI	Donor lymphocyte infusion
EPCs	Endothelial progenitor cells
G-CSF	Granulocyte colony stimulating factor
GI	Gastrointestinal
GVHD	Graft-versus-host disease
GVL	Graft-versus leukemia
GVT	Graft-versus-tumor
HHV6	Human herpes virus 6
HLA	Human leukocyte antigen
HSCT	Hematopoietic stem cell transplantation (refers to *allogeneic* HSCT in this text if not stated otherwise)
HSV	Herpes simplex virus
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
iNKT	Invariant Natural killer T (cell)
IPS	Interstitial pneumonia syndrome

KIR	Killer-cell immunoglobulin-like receptor
MC	Mixed chimera
MDS	Myelodysplastic syndrome
MHC	Major histocompatibility complex
MiHA	Minor histocompatibility antigen
MM	Multiple myeloma
MM	Mismatch
MRD	Minimal residual disease
MSC	Mesenchymal stroma cell
MTX	Methotrexate
MUD	Matched unrelated donor
NK	Natural killer (cell)
NKT	Natural killer T (cell)
NOD2	Nucleotide-binding oligomerization domain-containing protein 2
PBSC	Peripheral blood stem cell
PCR	Polymerase chain reaction
RIC	Reduced intensity conditioning
RQ-PCR	Real-time quantitative PCR
RSV	Respiratory syncytial virus
SAA	Severe aplastic anemia
SCID	Severe combined immunodeficiency
SNP	Single nucleotide polymorphism
TBI	Total body irradiation
TCD	T-cell depletion
TCR	T-cell receptor
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TREC	T-cell receptor excision circle
TRM	Transplant related mortality
VOD	Veno-occlusive disease
VZV	Varicella zoster virus

1 THESIS SUMMARY

Allogeneic hematopoietic stem cell transplantation (HSCT) is a potent immunotherapeutic procedure but its usability is limited by a high risk of serious complications. A prerequisite for timely initiation of preventive measures is the availability of predictive methods. This thesis aims to evaluate techniques that may potentially be used to assess the risk of some of these complications on the individual level.

NOD2 is one of many pattern recognition receptors that are found in cells of the innate immune system. It has been suggested that defective function of this receptor, due to naturally occurring gene polymorphism, may be a risk factor for graft-versus-host disease (GVHD) and increased transplant-related mortality (TRM) after HSCT. We evaluated the validity of NOD2 mutations as predictive markers for GVHD and TRM in a retrospective study of 198 patients. Contrary to previous reports, we found that the occurrence of NOD2 variants did not significantly affect incidence of acute GVHD, TRM or overall survival. Based on these results we conclude that NOD2 genotyping is not a pertinent analysis before HSCT.

Recurrence of malignant disease is a major cause of death after HSCT in patients treated for leukemia. Donor lymphocyte infusion (DLI) is one of the few therapeutic options remaining in these situations. Previous studies have shown varying results regarding treatment efficacy against acute leukemias and consensus guidelines are currently lacking. We aimed to investigate if the use of existing molecular techniques for relapse monitoring could improve the clinical outcome after DLI. Data on 118 patients with hematological malignancies who had undergone DLI treatment at Karolinska University Hospital were analyzed retrospectively. We could show that patients with acute leukemia or myelodysplastic syndrome who had received DLI based on the result of molecular methods had a superior 3-year survival rate of 42% as compared to 16% for those treated during hematologic relapse ($p < 0.006$). Nonhematological relapse and chronic GVHD were identified as independent predictors for response. The overall incidence of severe acute GVHD was only 8.5% and was acceptable (14%) in the cohort treated before 100 days post-HSCT. Our conclusion is that early administration of DLI to patients with acute leukemia, based on changes in cell lineage-specific chimerism and MRD analysis can significantly improve relapse-free survival after HSCT.

Adaptive immunity is highly compromised after HSCT, mainly due to defective T-cell function, and reconstitution of the T-cell population is dependent on thymic function. To determine the role of the thymus in immune recovery and its potential influence on outcome parameters, we quantitatively assessed thymic function in 260 patients during a two-year period following HSCT. Levels of T-cell receptor excision circles (TRECs) in purified T-cells were measured with real-time quantitative PCR and used as a surrogate marker for thymic function. We found that low TREC levels 3-6 months after HSCT was correlated to inferior survival,

increased TRM, and higher incidence of cytomegalovirus reactivation. We could also for the first time show that the use of bone marrow grafts and anti-thymocyte globulin had a negative effect on TREC levels, as did mesenchymal stromal cells when co-infused with umbilical cord blood grafts. We conclude that TREC analysis appears to have a high predictive value concerning outcome parameters after HSCT, and that factors related to the transplant procedure may significantly affect thymic function.

Finally, we present the results of a prospective pilot study in which we sought to design a functional, individualized strategy for assessing the risk of acute GVHD. Peripheral blood mononuclear cells were collected from patients and donors before HSCT and co-cultured in a mixed lymphocyte reaction (MLR) in the GVHD direction. Cells were phenotypically characterized by flow cytometry before and after MLR. We found that donors corresponding to patients who later developed acute GVHD II–IV had significantly higher levels of γδ T-cells and NKT-cells in peripheral circulation. We could also demonstrate a possible correlation between a high proportion of naïve $CD4^+$ T-cells in the allogeneic MLRs and occurrence of acute GVHD *in vivo*. We conclude that flow cytometric analysis of donor cells for phenotype and allogeneic reactivity may be used to predict acute GVHD before HSCT.

2 INTRODUCTION

2.1 THE HISTORY OF ALLOGENEIC HEMATOPOIETIC STEM CELL TRANSPLANTATION

The research field based on the transfer of blood forming progenitor cells between different individuals was born in earnest in the aftermath of the Second World War. With the development of the atomic bomb and the emerging threat of nuclear warfare, researchers began looking for ways to restore normal function to terminally damaged cells in the bone marrow, the most severe consequence of radiation exposure.

The earliest clinical studies involved the use the patients' own bone marrow cells, which were collected, frozen and reinfused, after the patients had been treated with high-dose irradiation. This procedure is referred to as autografting or autologous transplantation (1). In 1959, E. Donnall Thomas and his group reported two successful attempts transferring bone marrow cells between identical twins (2). Both these patients regenerated their marrow function within two weeks after infusion of donor cells but died later due to leukemic relapse. During the same period, Mathé and coworkers attempted several transplantations using bone marrow derived cells from genetically dissimilar, or *allogeneic*, donors. A few of these patients achieved lasting engraftment but died eventually in a condition referred to as "secondary syndrome", and which later became known as graft-versus-host disease (GVHD) (3, 4).

The concept of histocompatibility was first recognized in 1936, when Peter A. Gorer discovered an association between tissue rejection and antigenic differences on a cellular level in an experimental mouse model (5). In collaboration with George D. Snell, Gorer was able to locate the gene encoding one of these antigens to a locus that they named histocompatibility locus 2, or H-2. To distinguish the H-2 locus from other loci that contained genes encoding weaker antigens, the name was later changed to major histocompatibility locus. When it eventually became apparent that this site contained several different genes with similar function, the term *major histocompatibility complex* (MHC) was adopted.

In 1958 three different groups published papers demonstrating the existence of the human equivalent of MHC, which was termed human leukocyte antigen (HLA) complex (6-8). They did so by using sera from multi-transfused patients or pregnant women and studied their reactions against leukocytes from different individuals. Jean Dausset is generally credited for the discovery of HLA since he was the one who first perceived the significance of the findings. As a conclusion to his paper he wrote: 'Finally, in a more long time perspective, the study of leucocyte antigens might become of great importance in tissue transplantation, in particular in bone marrow transplantation'. Dausset received the Nobel Prize in 1980, together with George Snell and Baruj Benacerraf, for their discoveries regarding the role of histocompatibility antigens in immunological reactions.

The uncovering of the HLA system marked a major breakthrough in the field of clinical bone marrow transplantation. Up until the end of the 1960:s the survival rates for patients undergone this treatment had been less than two percent (9). Early HLA matching techniques enabled the use of matched sibling donors and this resulted in a dramatic decrease in the risk of graft-versus-host disease and graft rejection, the two main causes of death after allogeneic transplantation (10, 11). In the coming years, development of new conditioning regimens and strategies for preventing GVHD would contribute to further improve chances of a favorable outcome (12-14). This was followed by the first trials on the use of allogeneic stem cell transplantation to treat patients with acute leukemia (15, 16). Donnall Thomas's group in Seattle was responsible for much of this early work and he was eventually awarded the Nobel Prize in Physiology and Medicine, which he shared with Robert E. Murphy in 1990. However, despite improvements in treatment, the broader application of allogeneic transplantation was greatly limited by the fact that HLA-identical donors were only available in about a third of all cases. Therefore, efforts were put towards enabling the use of alternative stem cell donors (17-19). With further improvements in HLA-typing techniques and prophylactic treatments for GVHD, it soon became evident that comparable results could be achieved with grafts from matched unrelated donors (20-23). This has led to the establishment of national and international donor registries, which now collectively include more than 18.5 million potential donors. Today, more than half the transplantations performed are with unrelated grafts, and the majority of these are donated from outside the patients' country.

During the last 50 years, the rate of progress within the field of clinical allogeneic hematopoietic stem cell transplantation (HSCT) has been astonishing, making it one of modern medicines fastest expanding disciplines. Advances within the areas mentioned above have markedly reduced the risks, improved outcome, and extended indications of this procedure. However, the incidence and the severity of complications remain high. Thus, HSCT is currently only a valid option for life-threatening conditions and when no alternative treatments are available.

2.2 INDICATIONS FOR HSCT

Initially, the use of HSCT was restricted to acute leukemias, severe aplastic anemia (SAA), and severe combined immunodeficiency (SCID) (10, 11). Through the years this has extended to also include chronic leukemias, lymphomas, myelodysplastic syndromes, multiple myeloma, primary immunodeficiencies, and certain forms of inherited metabolic disorders. HSCT is also being evaluated as a treatment for diseases not conventionally considered for transplant. Some of the conditions, for which clinical studies have shown encouraging results, are neuroblastoma, renal cell carcinoma, sickle cell anemia, beta thalassemia major, and autoimmune disorders (24-28).

Clinical HSCT is a rapidly changing field with new methods and treatment modalities frequently introduced in the routine practice. Guidelines regarding the diagnoses eligible for transplantation and the timing of treatment initiation must

be continuously re-assessed. The European group for Blood and Marrow Transplantation (EBMT) and its American counterpart, Center for International Blood and Marrow Transplant Research (CIBMTR), regularly publish updated recommendations regarding current practice of and indications for HSCT (29, 30). However, it remains up to each center to adapt these recommendations to better match their own specific circumstances, depending on available resources, expertise, and techniques.

2.3 CONDITIONING THERAPY

The term conditioning refers to the preparative treatments that patients receive before the actual transfusion of the hematopoietic stem cells. The original purpose of the conditioning regimens was to prevent rejection of the graft by suppressing the host immune system. When HSCT later was evaluated as a treatment for malignant disorders, there was an additional need to eradicate remaining leukemic cells. At that point, total body irradiation (TBI) and high dose cyclophosphamide (Cy) had been used as two separate approaches but were now combined with the intention of reducing the risk of relapse (16, 31). The results were promising; more than half of the initial patients remained disease-free five years after transplant (32). The introduction of the alkylating agent busulfan (Bu) offered an alternative to the logistically more demanding TBI-based regimens (33). The initial difficulties associated with the hepatotoxic and proconvulsive side-effects of this drug were overcome by individual dose adjustment according to serum levels and prophylactic administration of anti-convulsants (34).

During the 1980s, efforts were made to further reduce relapse and rejection either through dose elevation or by addition of a third chemotherapeutic agent. However, such attempts towards more intense protocols were generally followed by a significant increase in toxicity, higher transplant related mortality (TRM), and did not improve the overall survival of patients (35-38). The main adverse effects of conditioning regimens include interstitial pneumonitis, stomatitis, veno-occlusive disease (VOD) and irreversible damage to the central nervous system (39-42).

Researchers were able to show as early as the late fifties that transplantation of allogeneic stem cells provided an additional anti-leukemic effect than the one delivered by the myeloablative conditioning (43, 44). It eventually became clear that the sustained disease remission after HSCT was highly dependent on an ongoing reaction between the allogeneic immune system and malignant cells of recipient origin (45-47). Based on this concept, new preparative regimens were composed, which mainly aimed to enable engraftment through suppression of the host immune system rather than to completely eradicate all remaining tumor cells (48-51). These non-myeloablative or reduced-intensity conditioning (RIC) protocols were associated with significantly lower risk of TRM due to reduced organ toxicity. This development made HSCT available for a new category of patients for whom the treatment had previously not been considered a safe option, i.e. older patients or those with co-morbid conditions.

Inhibiting polyclonal antibodies against T-cells are sometimes administrated to patients in conjunction with the conditioning regimen (52). Their main effect is to prevent rejection by inhibiting the host immune response but also to reduce the risk of GVHD through a delayed suppressive effect on donor T-cells. This approach is used in situations with particularly high risk of graft failure, e.g. in cord blood transplantation (CBT), previously alloimmunized patients, and certain RIC protocols, or when the risk of GVHD is high. Despite the proven effectiveness of these agents, their use is limited by the associated increase in infections and relapse.

2.4 INFECTIOUS COMPLICATIONS

The time after HSCT is characterized by a state of profound immunodeficiency, during which the patients are at considerable risk of opportunistic infections. Susceptibility to microbial pathogens is generally most pronounced during the first weeks, decreasing gradually as different parts of the immune system regain their functionality. Three different periods can be distinguished based on the incidence of certain infections after HSCT. The predominance of specific pathogens in each phase is a reflection of different types of immunodeficiencies. Table 1 gives an overview of the most common pathogens in each phase.

Recovery of a functioning immunity occurs in several stages and the rate of this process may be influenced by several factors including patient age, stem cell source, conditioning therapy, immunosuppression, and the presence of GVHD. There is a general concern that complications connected to delayed immune reconstitution are increasing, as a consequence of higher median age of patients and the use of alternative stem cell sources such as umbilical cord blood (CB) and haploidentical grafts (53).

There is also a strong association between acute GVHD and increased susceptibility to infections. This is mainly due to an immune modulatory effect of the ongoing systemic inflammation but disruption of epithelial barriers is also a contributing factor (54, 55). In addition, the immunosuppressive agents used for treatment of GVHD contribute to further increase the risk of opportunistic infections. These patients are, therefore, very often in need of additional anti-bacterial and anti-fungal prophylaxis.

Table 1.

Neutropenic phase (days 0–30)	**Intermediate phase** (days 30–100)	**Late phase** (days >100)
Gram positive bacteria	CMV	Pneumococci
Gram negative bacteria	Adenovirus	H. Influenzae
Influenza	VZV	VZV
Candida	Candida	
HSV	Aspergillus	
RSV	HHV-6	

Common causes of infections during the different phases of post-HSCT immune recovery.
CMV, cytomegalovirus; H. Influenzae, Haemophilus Influenzae; VZV, varicella zoster virus; HSV, herpes simplex virus; RSV, respiratory syncytial virus; HHV-6, human herpes virus 6.

2.5 ACUTE GRAFT-VERSUS-HOST DISEASE

GVHD remains one of the major challenges in the clinical management of patients after HSCT and is the cause of significant morbidity and mortality. This condition is the manifestation of an unwanted immunological reaction between transplanted donor lymphocytes and host tissue. It occurs in an acute and a chronic form, each with characteristic symptoms and distinct pathophysiological mechanisms (56).

GVHD was first recognized in animal models as a combination of symptoms that often occurred after allogeneic HSCT and were referred to as “secondary disease” (57). Billingham stated the required conditions for development of this syndrome in 1966: (1) transfer of immunocompetent cells between two individuals, (2) the individuals must differ immunologically from each other, and (3) the host must be immunosuppressed around the time of cell transfer to avoid rejection (58).

Acute GVHD usually develops within 100 days after transplantation and has a more rapid course with relatively sudden onset of symptoms that may progress within hours–days if untreated. A significant characteristic of an acute GVH reaction is its strong inflammatory component, causing severe destruction of host tissue. Chronic GVHD is often diagnosed later than 3 months after engraftment but can also occur earlier. Its features resemble those of autoimmune disorders such as scleroderma, vasculitis, and Sjögren syndrome (59). A more detailed description on the mechanism and clinical presentation of chronic GVHD is not included here as it exceeds the scope of this thesis.

Pathophysiology of acute graft-versus-host disease

Acute GVHD is a major cause of early mortality after HSCT. The current model for its pathophysiological mechanism is a three-step process involving components of

both the innate and the adaptive immune system. Innate immunity plays a central role in the initial phase of acute GVHD. Host tissue damage, caused by cytotoxic agents and radiation during the pre-transplant conditioning therapy, leads to disruption of epithelial barriers, allowing interaction between innate immune receptors and microbial structures. These so called *pattern recognition receptors* lack the variability of the antigen-specific receptors of the adaptive immune system. They occur on macrophages, granulocytes, and dendritic cells and as secreted molecules. Binding of microbial products causes massive release of cytokines and chemokines from the innate immune cells, promoting an inflammatory response (60). These interactions may also explain why acute GVHD commonly affects organs that are exposed to microbes on their epithelial surface. Another group of receptors, which become activated by binding to damage-associated molecular patterns (DAMPs) in injured tissue appear also to be involved in initiation of GVHD (61).

In the next step, antigen-presenting cells (APCs) become activated by the ongoing inflammatory activity and can in turn activate donor $CD4^+$ T-cells through presentation of host-specific antigens. It was originally considered that host APCs exclusively performed this function but recent studies have shown that donor-derived APCs also have the capacity to induce acute GVHD (62). Although both $CD4^+$ and $CD8^+$ T-cells play a part in the GVHD process, $CD4^+$ T-helper cells seem to be crucial for initiation of acute GVHD (56). It has also been shown that GVHD is induced by naïve T-cells, while central and effector memory T-cells mediate the GVT effect (63, 64). Activated donor $CD4^+$ T-cells undergo clonal expansion and elicit a strong cytokine response, which further promotes antigen presentation and maintains the inflammation (65-68).

In the third and final phase cytotoxic T-lymphocytes (CTL), natural killer (NK) cells and macrophages are recruited to the site due to increased levels of cytokines and chemokines (69-71). These effector cells, in combination with pro-inflammatory cytokines such as interleukin-1 (IL-1), tumor necrosis factor-α (TNF-α), and interferon-γ (IFN-γ), result in the tissue damage observed in acute GVHD (72-74).

Clinical features

The organ systems most commonly affected by acute GVHD are the skin, the liver, and the gastrointestinal (GI) tract. The severity of the condition varies from mild symptoms to extensive tissue damage, resulting in nearly complete loss of organ function. The possible occurence of sub-clinical GVHD has also been discussed.

Glucksberg and co-workers proposed the first algorithm for grading of acute GVHD in 1974 (75). According to this, each organ system is scored from 0 to 4 based on the severity of the symptoms and the results are then used to obtain an overall grade. This classification has shown good predictive value regarding outcome parameters and treatment response, and is still used by most clinicians and researchers (76). Histopathological analysis of tissue biopsies are used to

increase diagnostic accuracy, as the signs and symptoms of GVHD are often hard to distinguish from other reactions observed in patients after HSCT.

Prevention and treatment

After HSCT, most patients receive continuous immunosuppressive treatment during the first 3–6 months after engraftment to prevent excessive allogeneic response. All currently available options for GVHD prophylaxis function by inhibiting donor T-cell reactivity. The first pharmacological approach was monotherapy with Methotrexate (MTX), a cytostatic folic acid antagonist (14). This was later replaced by cyclosporine A (CsA), which blocks T-cell receptor signaling by inactivating the intracellular phosphatase calcineurin (77). Eventually, it was shown that a combination of MTX and CsA was far more effective with few added side-effects (78-80). This combination is currently the most frequently used approach for GVHD prophylaxis.

A more long-term inactivation of donor T-cells can be achieved through administration of neutralizing anti-T-cell antibodies, either *ex vivo* or *in vivo*. These approaches are collectively termed T-cell depletion and have the potential to virtually eliminate the risk of GVHD albeit at the expense of significantly increased risk of relapse. The use of RIC can also reduce the risk of acute GVHD as these regimens cause significantly less damage to host tissue than myeloablative protocols. It is however important to consider that the onset of GVHD may be delayed after non-myeloablative regimens.

In parallel with the development of more effective immunosuppressive protocols, advances in HLA-typing have significantly contributed to reducing the incidence and severity of GVHD, increasing rates of engraftment, and improving overall survival (81-83). The initial methods for histocompatibility testing were based on the detection of antigenic differences between HLA-molecules using antibodies. (84, 85). The introduction of PCR in the 1980s enabled HLA-typing on the genomic level. An early approach involved the use of labeled sequence-specific oligonucleotide probes (PCR-SSO) (86, 87). Further increase in sensitivity was achieved with the development of a method based on PCR amplification of genomic DNA with primers corresponding to known HLA-alleles, referred to as PCR with sequence-specific primers (PCR-SSP) (88-90). More recently automated sequencing techniques are increasingly used for identification of polymorphic HLA alleles and this approach will most likely become the predominant method for histocompatibility testing in the near future (91).

The fundamental treatment option for acute GVHD is systemic administration of high dose corticosteroids in addition to the standard immunosuppression. Prednisolone or methylprednisolone are common choices, usually introduced at a dose of 2mg/kg/day and tapered after 1-2 weeks depending on clinical response. A proportion of patients show incomplete response or progress of symptoms after initiated treatment. The probability of unresponsiveness to corticosteroids increases with delay of treatment and the severity of the symptoms. The addition

of alternative therapies are often tried in these situations, e.g. infusion of anti-T-cell monoclonal or polyclonal antibodies and antibodies against the TNF-α receptor. None of these approaches have, however, shown any convincing effects on long-term survival.

2.6 RELAPSE

The significant improvement in survival rates after HSCT seen over the last decades is mainly a consequence of advances in GVHD prevention and supportive care (92, 93). The risk of relapse-related mortality for patients with hematological malignancies has not changed considerably; incidence of relapse in patients with acute leukemias remains around 20-30% after HSCT and is even higher for those with a more advanced disease (94, 95). Recurrence of leukemia in patients who have undergone allogeneic transplantation normally derives from cells of recipient origin, presumably due to incomplete eradication after chemoradiotherapy and/or inadequate anti-tumor effect of the graft (96). Rare cases of late relapse in donor-derived cells have also been reported (97). Relapse after HSCT is generally correlated with poor prognosis, particularly if it occurs a short time after transplant (98).

There are several ways to define leukemic relapse based on the methods used for detection. The classical definition, referred to as *morphological* or *hematological relapse*, is the presence of significant amount of blast cells (> 5%) when bone marrow (BM) or blood samples are analyzed by light microscopy. The sensitivity of this method is low, which means that patients carry a high tumor load at the time of diagnosis. In malignancies characterized by specific chromosomal abnormalities, relapse can be detected by identifying cells containing these defective chromosomes, a technique called *cytogenetic analysis*. The most common example of such abnormalities is the t(9;22)(q34;q11) translocation of the Philadelphia chromosome, which is seen in chronic myeloid leukemia (CML) and some forms of acute lymphoblastic leukemia (ALL) (99, 100). Cytogenetic relapse typically precedes morphological relapse due to the somewhat higher sensitivity of this method. In *immunophenotypic analysis* flow cytometry and monoclonal antibodies are used to distinguish leukemic cells expressing certain combinations of surface antigens. The sensitivity of this technique is usually 10^{-4}–10^{-5} and depends on the degree of phenotypic distinction between malignant cells and healthy cells (101). Gene translocations and rearranged T-cell receptor and immunoglobulin genes characteristic for certain leukemias can be detected with real-time quantitative PCR (RTQ-PCR). This technique offers superior sensitivity compared to other currently available approaches and can detect malignant cells down to numbers as low as 10^{6} (102-104). RTQ-PCR is also used to investigate the occurrence of residual recipient cells after HSCT, a method termed chimerism analysis. Studies have shown that an increase of host-derived cells within the leukemia affected cell subset strongly correlates with impending disease relapse (105-107).

2.7 GRAFT-VERSUS-TUMOR EFFECT

As previously mentioned, the effectiveness of HSCT against malignant disorders is dependent on an immune response between lymphocytes of donor origin and neoplastic cell clones. This is referred to as the graft-versus-leukemia (GVL) or graft-versus-tumor (GVT) reaction, and explains why HSCT, despite a high risk of severe complications, still offers a survival benefit for patients with some late-stage malignant disorders when compared to other treatment modalities. Several observations have led to the general recognition of the importance of this phenomenon.

Early experiments in mice revealed that irradiation alone was not sufficient to eliminate certain forms of leukemia, but that this could be achieved by combining TBI with the infusion of allogeneic marrow cells (43, 44). In the clinical setting, it was noted that patients who developed GVHD after HSCT had a significantly lower risk of relapse than those who did not show any symptoms of GVHD (46, 47, 108). Analogously, it was shown that discontinuation of immunosuppression, with the resulting occurrence of GVHD, could be used to re-establish remission in the case of relapsed leukemia after HSCT (109-111).

The vital role of alloreactive T-cells in GVT reaction and GVHD is illustrated by the fact that both processes are virtually absent in transplantations with grafts fully depleted of T-cells (112). In addition, they seem to depend strongly upon some degree of histoincompatibility between donor and recipient. This is supported by the high incidence of relapse and the absence of GVHD seen after transplantations between identical twins (syngeneic transplantation), and is further demonstrated in experimental HSCT models using leukemic cells that express known histocompatibility antigens (112-115). There is also evidence suggesting that NK-cells may contribute to the GVT reaction. This seems to be more pronounced against malignant cells of the myeloid lineage and is mediated through the function of both inhibitory and activating NK-cell receptors (116-118).

The GVT effect is often accompanied by GVHD, presumably because of common elements in their mechanisms of action (46, 47, 112, 119). However, this seems only to be the case if the GVH reaction is directed against host-specific antigens expressed by malignant cells as well as healthy host cells (113, 114, 120). It is also reasonable to conclude that these antigens consist of minor histocompatibility antigens (MiHA) rather than HLA since the GVT effect is present in transplantations with HLA-identical siblings and there does not seem to be a significant difference in relapse rate between HSCT with HLA mismatched (MM) and fully matched donors (121). Moreover, some antigens are only expressed on neoplastic cells. These tumor-specific or tumor-associated antigens do not seem to be able to independently trigger an allogeneic reaction but may contribute to the GVL effect once an immune response against MiHA has been established (122-126). One proposed reason for this observation is the known tendency of malignant cells to downregulate the expression of tumor-specific antigens as part of their strategy to evade the host immune system (127-129).

Many investigators believe that the GVT effect may be separated from GVHD and this is currently the subject of active research. There are, however, those who propose that the GVL reaction is simply GVHD directed against host hematopoietic cells, and that this cell type's high susceptibility to an allogeneic immune response is the only reason behind the curative effect against leukemia. This point of view is based on several findings: (1) pancytopenia and BM aplasia are seen after GVHD (130, 131), (2) conversion to full donor chimerism is important for preventing relapse after HSCT, (3) fluctuation in chimerism status is tightly connected to incidence of relapse and re-establishment of remission (105, 106, 132), and (4) a rapid conversion to complete donor chimerism after HSCT may precede GVHD (133, 134).

There are today few ways to actively manipulate GVT reactions in clinical practice. Some studies have shown that it is possible to enhance the GVT effect by immunizing the donors against recipient-specific histocompatibility antigens before HSCT (135, 136). Measures to increase the allogeneic potential of the graft after HSCT include tapering of the immunosuppressive therapy and adoptive transfer of additional effector cells. The latter is a routinely used procedure termed donor lymphocyte infusion (DLI) and entails infusion of T-cells from the original donor. DLI can be given as a single high dose of $10^{7\text{-}8}$ T-cells/kg (bulk dose regimen) or according to a dose-escalating protocol with doses starting at $10^{5\text{-}6}$ T-cells/kg and increased gradually by 0.5–1 log at monthly intervals (137). The dose-escalating approach has been the method of choice at our center during the last decade, usually in combination with frequent monitoring of lineage specific chimerism analysis and/or other means to detect minimal residual disease (MRD).

3 AIMS

HSCT is a powerful and effective treatment modality but despite the recent advances in supportive care, its usability remains limited by the relatively high risk of serious complications. These complications do not affect all patients at the same frequency and most of the methods used for treatment and prevention may themselves entail considerable risks of adverse effects. Thus, the prospect of individually adapting these measures according to the risk profile of each patient would significantly improve the outcome after HSCT. The overall aim of this thesis was to evaluate and develop methods that could be used to predict the risk of some of these complications.

The specific aims of the work presented in this thesis are:

1. To evaluate NOD2 polymorphisms as a predictive marker for transplant-related mortality and graft-versus-host disease in allogeneic HSCT.
2. To assess the efficacy of DLI as a treatment for disease relapse after HSCT and the possible risks associated with this treatment.
3. To investigate if the use of lineage-specific chimerism analysis for directing DLI can improve the treatment results.
4. To assess the value of T-cell receptor excision circle (TREC) analysis as a measure of immune reconstitution after HSCT and the correlation between thymic recovery and outcome.
5. To evaluate the predictive value of phenotypic analysis of donor cells before HSCT regarding the risk of acute GVHD.
6. To develop a functional method for prediction of acute GVHD before transplantation.

4 RESULTS AND DISCUSSION

As mentioned in the introduction, HSCT is a highly potent treatment that is associated with a risk of severe complications. Consequently, eligible patients often suffer from conditions with fatal outcomes for which there are no alternative treatments. The main complications after HSCT are acute and chronic GVHD, graft rejection or failed engraftment, relapse of malignant disease, infectious complications, and drug-related toxicity. These adverse effects are all associated with high risk of morbidity and mortality. A prerequisite for timely initiation of appropriate treatments and preventive measures is the availability of methods that can accurately identify high-risk patients. The studies included in this thesis all aim to evaluate methods that may potentially be used to assess the probability of GVHD, relapse, and/or delayed immune reconstitution on the individual level.

4.1 THE PREDICTIVE VALUE OF NOD2 POLYMORPHISM IN HSCT

In 2004, Holler *et al.* reported the results of a single-center study in which they had investigated the effect of mutations in the NOD2 gene on outcome after HSCT (138). They found significantly higher incidences of TRM, severe overall acute GVHD, and acute intestinal GVHD for patient-donor pairs that carried one of the single nucleotide polymorphisms (SNPs) 8, 12, or 13. The risk of GVHD was higher if a gene variant occurred on the donor side and further increased with simultaneous mutations in both individuals, suggesting an additive effect. In a follow-up multicenter analysis that included patients from five different European centers, they were able to confirm the negative effect of NOD2 gene variants on outcome parameters (139). The cohorts included only patients who had received grafts from HLA-identical siblings and were thus genetically more homogenous. Contrary to previous results, however, the occurrence of SNPs in the donors did not appear to affect survival and TRM, while mutations on both sides still seemed to have a larger effect than those in the recipients alone. In addition, when the material from each center was analyzed separately, only three cohorts showed significant associations with TRM. Almost at the same time as Holler and co-workers published their second study, another German group reported the results of a large analysis of NOD2 genes in patients who had undergone HSCT at their center (140). They found a significant increase in incidence of GVHD when both the recipient and the donor carried NOD2 mutations, but a *reduction* in GVHD when gene variants occurred on the donor side only. They also failed to confirm any effect on OS, on TRM, or on infectious complications.

These inconsistencies in results indicated that the impact of NOD2 dysfunction on the outcome of transplantation might be affected by center-specific factors. In order to investigate the clinical significance of NOD2 polymorphism in our own patient population, we performed a retrospective analysis of 198 consecutive patients who had undergone HSCT as treatment for hematological malignancies (Paper I). Patient and donor DNA isolated from samples were collected before transplantation and screened by real-time quantitative PCR (RQ-PCR) for the

presence of NOD2 gene variants. We found that 7.6% of the recipients and 12% of the donors carried one of the SNPs 8, 12, or 13. There were, however, no significant differences regarding incidence of acute GVHD, TRM, or survival between (1) cases where either the patient or the donor carried a gene variant, and (2) cases where both individuals were of wild-type genotype.

Variation in clinical results between centers is an acknowledged phenomenon, most likely caused by differences in treatment strategies and inherent traits in the patient population. Nonetheless, these findings were unexpected since the previous studies had shown strong correlations. Several possible factors may contribute to the apparent discrepancies in the results. The remaining part of this section will focus on identification of some of these factors by describing recent findings in this area.

One factor that may have contributed to the lack of significance in our analysis was the relatively low frequency of mutations in our patient population. Only 7.6% of the recipients carried the gene variants, as compared to 18% and 21% in the two initial German reports (140, 141). There have in fact been studies performed on patients with inflammatory bowel disease and healthy controls that indicated a lower frequency of NOD2 mutations in the Scandinavian countries than in the rest of Europe (142-144). Moreover, the results presented by Holler *et al*. suggest that the combination of HLA-identical donor graft and NOD2 dysfunction is particularly disadvantageous with regard to TRM and GVHD. The frequency of such patients in our material was significantly lower than in the study from Regensburg (3.5% vs. 14%).

The association between intestinal bacterial flora and acute GVHD of the GI tract was demonstrated as early as almost 40 years ago (145). According to our current knowledge of the pathophysiology of GVHD, translocation of pathogenic bacteria from the gut lumen into the tissue has a role in the initiation and maintenance of mucosal inflammation driven by alloreactive cells. This is part of the rationale behind the GI decontamination regimens given to patients around the time of transplantation. The bacterial spectrum covered by these regimens differs between centers. As mentioned earlier, the product of the NOD2 gene is a receptor that recognizes muramyl dipeptide, a cell wall constituent in both gram-negative and gram-positive bacteria. Thus, patients who receive GI decontamination that covers both types of bacteria would theoretically not be affected by a loss-of-function mutation in the NOD2 gene. This has been suggested as a possible explanation for the lack of positive findings in some analyses (139, 140). However, neutralization of pathogenic bacterial species should not explain the lack of effect of NOD2 SNPs in our material, since the antimicrobial prophylaxis used at Karolinska only covers gram-negative species. To investigate this line of thought further, we performed a separate analysis that excluded patients who were treated with broad-spectrum antibiotics over long periods, but the results were no different.

Currently, there is no international consensus regarding treatment and prevention of GVHD and this is an important factor to consider when comparing study results.

Ex vivo T-cell depletion is used by some centers, often when the expected risk of GVHD is particularly high, e.g. in mismatched (MM) or haploidentical transplantations. This preventive measure markedly reduces the alloreactive potential of the stem cell graft by reducing its T-cell content. There have been two reports published on the effect of NOD2 mutations in T-cell-depleted HSCT (146, 147). Even though both studies involved comparable numbers of HLA-identical transplantations, the results were contradictory. In the earliest of these reports, the investigators found no increased risk of GVHD in the presence of NOD2 variants, but a lower disease-free survival and a higher incidence of death in pulmonary infections in this group. In the more recent report, however, van der Velden and co-workers showed that there was a significant correlation between the presence of NOD2 mutations and both severe acute GVHD (of grades III–IV) and higher TRM. One important difference between these studies, which could offer a possible explanation for the contradictory results, is dissimilarities in the T-cell-depletion protocol. In the second of these two studies, the transplanted grafts are repleted with a median of 0.5×10^6 T-cells/kg. This makes a significant difference regarding the alloreactivity of the graft and the risk of GVHD. Another potentially important disparity between these two studies was differences in conditioning regimens. It is possible that the use of less intensive pretreatment strategies, with a reduced tendency to cause damage to the intestinal mucosa and translocation of bacteria, can diminish the role of NOD2 dysfunction (148).

A variation of *ex vivo* T-cell depletion is the use of inactivating antibodies, e.g. anti-thymocyte globulin (ATG) or alemtuzumab (MabCampath, Genzyme), which act by inhibiting T-cell function *in viv*o (52, 149). This approach has been the method of choice at Karolinska, and Thymoglobuline (Genzyme) has been part of the standard conditioning therapy in all MUD and MM transplantations over the last two decades (150). The majority of patients included in our study had an unrelated donor (118 of 198), and had therefore undergone treatment with Thymoglobuline. To rule out any possible influence of ATG on the effects of NOD2 mutations, we performed a separate analysis on the group of patients who had not received such treatment but again failed to find any association with outcome. However, any potential effect of *in vivo* T-cell-depletion would not be distinguishable from that linked to donor type in this material, since ATG was almost exclusively used in unrelated transplantations. Interestingly, two recently published studies on the role of NOD2 SNPs in HSCT, which both included patients treated with ATG, failed to demonstrate any significant correlation to incidence of GVHD (151, 152). A possible explanation for these findings might be that ATG reduces the activity of donor T-cells early after transplantation, a time when there is ongoing tissue damage and inflammation caused by conditioning. This may be a critical period, during which NOD2 mutations can increase the possibility of induction of GVHD by allowing bacterial translocation and promoting local inflammation.

There is only weak evidence to support a connection between NOD2 function and the GVT effect. Mayor *et al*. found a significant increase in the incidence of relapsed malignant disease in the presence of NOD2 SNPs on the recipient side, and this was also identified as an independent risk factor in a Cox regression

analysis (153). Later, Wermke *et al*. showed a similar association in their single-center study, but this failed to reach statistical significance in a multivariate analysis (151). Like most other investigators, we did not find such a correlation: the incidence of relapse in our material was 27% and 24% for the mutation group and wild-type group respectively, and the difference was not statistically significant.

In summary, since the publication of our report several other groups have confirmed the lack of effect of NOD2 mutations on outcome after HSCT. At the same time, there have been reports showing correlations between NOD2 mutations and incidence of GVHD, relapse rate, risk of infectious complications, and/or increased mortality. Despite the inconsistencies in results between studies, one cannot disregard a possible role of NOD2 polymorphism in the immune responses after HSCT. However, the potential effect of these genotypes on outcome parameters appears to be dependent on several variables related both to the transplantation procedure and to patient/donor characteristics. One may also speculate on the possibility of an interaction with other polymorph genes and their products. In recent years, several new innate immunity receptors have been identified. (154, 155) There is a growing understanding of the diversity and importance of the pathogen recognition systems that enable cells of the innate immune system to react to microorganisms and other disease signals. Not unexpectedly, there appears to be an intricate cross-communication between these pathways, the details of which are incompletely understood. It is, however, reasonable to believe that other gene products with overlapping functions could compensate for a single loss of function in one of these pathways, but that combinations of certain gene variants could be the cause of significant dysfunctions. Further research is needed in both the basic biology and the clinical implications of this aspect of the innate immune response. At this time, though, there is not enough evidence to support inclusion of NOD2 analysis in the pretransplant work-up and donor-selection process.

4.2 ALLOGENEIC TREATMENT OF RELAPSED HAEMATOLOGICAL MALIGNANCIES BASED ON MOLECULAR MONITORING

DLI was first evaluated as a treatment for relapsed disease after allogeneic HSCT in patients with CML. It has since been shown that initiation of DLI treatment during molecular and/or cytogenetic relapse is associated with a superior response that is very often long-standing (156, 157). At the same time, there have been few reports on the effectiveness of DLI on relapsed acute hematological malignancies, and the results were not encouraging (158, 159). The existing data are also often difficult to interpret due to heterogeneous patient populations and differences in approach between centers. This is particularly true for situations where chimerism analysis has been used to monitor disease relapse, since inconsistencies in methods preclude broader comparisons of the results. Lineage-specific chimerism analysis for early detection of relapse has been used to direct treatment at Karolinska University Hospital during the last decade, but its effect on transplantation outcome had not been evaluated before our analysis in paper

II. The main goal of this project was therefore to conduct a retrospective comparative study, with the working hypothesis that the use of molecular-based methods for MRD detection, including chimerism analysis, would have a positive effect on response to DLI treatment. We also wanted to evaluate the possible risk of adverse effects associated with DLI treatment.

Recurrence of malignant disease after HSCT is associated with more rapid growth kinetics and reduced responsiveness to treatment, and even more so if the relapse occurs early after transplantation. Low tumor load may therefore be a particularly strong determinant of successful outcome in these situations, which underscores the importance of frequent MRD monitoring and prompt initiation of treatment during the early period after HSCT. We found evidence supporting this line of reasoning in our analysis: the amount of time elapsed after transplant had a stronger effect on response rate within the group with hematological as compared to the group with molecular/cytogenetic relapse ($p = 0.036$ vs. $p = 0.06$). In a large comparative study published in 2008, the authors analyzed data on 1278 pediatric patients with hematologic malignancies reported to the Center for International Blood and Marrow Transplant Research (CIBMTR). They were not able to detect a significant difference in survival between patients treated with DLI in morphological and/or extramedullary relapse, and those who did not receive DLI. Within this group, none of the patients who relapsed within 6 months from HSCT showed any response to DLI (160).

In addition, we found a positive correlation between chronic GVHD and response to DLI in patients with hematological relapse ($p = 0.007$), while no such association was evident in the group of patients in cytogenetic/molecular relapse. All but one of these patients developed symptoms of chronic GVHD before they showed sign of response to DLI. This illustrates another aspect of the same line of reasoning, by showing that a stronger allogeneic effect is required to achieve control of the disease once it has progressed to the state of hematological relapse. A possible confounding factor here could, however, be that the latter cohort included a higher proportion of patients with CML. This disease has proven to be susceptible to the GVL effect in the absence of GVHD (137, 161, 162). Nevertheless, a separate analysis of our material, that included only patients with acute leukemia and MDS, showed a significantly superior overall survival for those treated during molecular relapse ($p = 0.006$). The majority of these patients had been treated due to MC in the leukemia-affected cell lineage. Non-hematologic relapse was also identified as an independent factor correlated with response to DLI in a multivariate analysis. Similar results were found in a recent publication, even though the cohorts included were not balanced regarding all the potentially confounding factors, and chimerism had not been analyzed in separate cell subsets (163).

The interpretation of chimerism results has been the subject of some controversy. Particularly after RIC transplantation, where there is a slower and gradual transition to donor chimerism, it is often difficult to decide when and if measures are to be taken to prevent full relapse. Studies have shown that the dynamics of MC in the leukemia-affected cell lineage can be of crucial importance in such

situations (105, 164). Increased frequency of analysis, preferably in both blood and BM samples, is therefore imperative when there is a suspicion of impending relapse. As previously shown, BM analysis allows for earlier detection of changes in chimerism status and this is of particularly useful in diseases with fast growth kinetics such as ALL and AML (105). It is however also important to consider the possibility of lower specificity in BM, which may be caused by lingering stromal cells of recipient origin (165).

It has been generally believed that administration of DLI early after HSCT is associated with an increased risk of acute GVHD (166). We could confirm such a relationship in our analysis, predominantly regarding the incidence of acute GVHD of grades I–II, which was 46% and 29% following early and late DLI, respectively ($p < 0.05$). There was also a difference in the incidence of severe acute GVHD of grades III–IV between early and late group (14% and 8%, respectively) but it was not found to be statistically significant ($p > 0.1$). It could be argued that the occurrence of manageable forms of GVHD is desirable in these patients, even though we were not able to detect an influence of acute GVHD on response rate in our analysis (112). However, the important implication of these results is that the incidence of severe acute GVHD does not differ considerably from what is generally seen after HSCT at our center and within acceptable limits for these high-risk patients (167). Thus, our results do not support the exclusion of DLI during the early phase after HSCT on account of an increased risk for GVHD.

Also included in this analysis were seven patients with advanced-stage hematological malignancies (2 MDS-AML, 3 AML, 2 pre-B ALL) who had been treated with prophylactic DLI as part of a pilot study. All patients had received escalating doses of DLI three months after HSCT, in the absence of GVHD or relapse. Interestingly, six of them remained in complete remission, with a median follow-up time of 17 years. It is important to mention that this group was analyzed separately regarding relapse-free survival and the results were not included in the analysis of the larger cohort. Lutz *et al*. presented the results of their study on prophylactic DLI in a group of 26 adult patients with ALL. Disease-free survival in these patients was 62%, despite a relatively high incidence of GVHD (168). There were however, some apparent limitations to the design of this study. DLI was started later than 100 days in the majority of patients, and ten patients received DLI five months or later after HSCT. This increases the likelihood of a selection bias favoring those with less aggressive disease. One patient had suspected morphological relapse with 5% peripheral blasts, and 14 had MC at the time of DLI. Thus, only five of the 26 patients included fulfilled our definition of molecular and morphological remission and had received early DLI treatment, i.e. within four months after HSCT. Also, as the authors themselves pointed out, there were many unbalanced confounding variables between the treatment group and the control group, and this prevented a meaningful statistical comparison.

From the findings in our own study and after reviewing the existing publications, we conclude that early administration of DLI to patients with hematological malignancies, based on changes in cell lineage specific chimerism and MRD analysis can significantly improve relapse-free survival after HSCT. Larger,

prospective, multicenter trials must be conducted in order to confirm these conclusions, and to allow establishment of guidelines to optimize results of this treatment. However, to further improve the outcome for patients with leukemic relapse therapies that are more specific are needed. Several novel approaches, which aim to amplify the anti-tumor effect without increasing risk of GVHD, are currently being evaluated. Treatment with transgenic T-cells expressing *specific chimeric antigen receptors* (CARs) that function in a non-MHC-restricted manner is one example on such an approach (169). Recent clinical studies on the use of *bi-specific T-cell engaging* (BiTE) antibodies have also shown promising results in patients with relapsed ALL after HSCT (170). Adoptive transfer of NK-cells is another possible strategy for cancer immunotherapy. NK-cells have a documented anti-tumor potential and the expected risk of alloreactivity is low since their effect is not mediated by MiHA disparities (171, 172). Thus, directed immunotherapeutic measures, combined with early treatment based on sensitive predictive methods may considerably improve the outcome after HSCT in the near future.

4.3 TREC ANALYSIS FOR ASSESSMENT OF T-CELL RECONSTITUTION AFTER HSCT

Two principally different processes contribute to the reconstitution of the T-cell pool after ASCT: peripheral expansion of naïve and memory donor T-cells transferred with the graft, and *de novo* differentiation of bone marrow-derived early T-cell progenitor (ETP) cells into naïve T-cells. The latter is achieved in the host thymus and is dependent on the interaction of ETP with other cell types, e.g. thymic epithelial cells, dendritic cells, and macrophages. The thymic output of naïve T-cells is essential for maintenance of a broad TCR repertoire with the ability to recognize new pathogens and tumor antigens (173-175). This pathway is also particularly important for reconstitution of the T-cell pool in transplantations with CB and T-cell-depleted grafts, since only limited amounts of T-cells are transferred to the host in these situations.

The rate at which thymic function is regained after HSCT varies, and appears to be dependent on both patient characteristics and treatment-related factors. Analysis of TREC has recently been evaluated as a simple and non-invasive approach for assessing the ability of the thymus to produce new T-cells in the individual patient. We wanted to investigate whether TREC analysis, as a quantitative method for assessment of thymic function, can be used at an early stage to identify patients with a high risk of complications related to deficient T-cell immunity. For this purpose, we performed two separate retrospective analyses in which we measured TREC levels in stored samples collected from patients at regular intervals after HSCT (papers III and IV). The two cohorts consisted of 210 patients who had undergone BMT or PBSCT for hematological malignancies and 50 patients transplanted with allogeneic CB units.

TREC analysis in cord blood transplantation

In the group of patients transplanted with CB grafts, a significant increase in TREC levels appeared around six months after transplantation, which was only slightly later than those who underwent BMT and PBSCT. This agreed with the results of a previous study on 27 adult patients after double CBT, while two other reports showed a more delayed thymic reconstitution lasting 12–18 months (176-178). These inconsistencies were most likely due to the higher median age of patients and lower cell doses in the latter two studies. Based on our findings, and on those reported in other publications in this area, it is evident that thymic recovery after CBT occurs at a faster rate in children and young adults. This is line with what has been shown in BMT and PBSCT, where age has been identified as one the strongest determining factors for thymic function. However, the higher cell dose/kg in pediatric populations can contribute to faster immune reconstitution and potentially confound the results. High age, low TNC dose, and low CD34+ cell dose were all identified as independent negative factors in a multiple regression analysis for TREC levels in our investigation, together with the presence of acute GVHD of grades I–IV. There were also borderline correlations for RIC (HR = 1.36, p = 0.060) and chronic GVHD (HR = 0.66, p = 0.069). Different aspects of the relationship between GVHD and thymic function are discussed later in this section.

In order to address the main goal of our study, which was to evaluate the possible use of TREC analysis for prediction of outcome after CBT, we performed a comparison between patients with TREC above median level and those with TREC below median level six months after transplantation. However, we could only detect a trend of increased OS for individuals in the high-TREC group (p = 0.11). This result differed from the findings in our analysis on BMT and PBSCT, where high TREC levels early after transplantation were identified as a strong independent factor associated with lower TRM and superior OS. The failure to reach significance here could very well have been due to a small patient material, but this must still be confirmed in future trials.

TREC analysis in bone marrow transplantation and peripheral blood stem cell transplantation

Compared to our analysis of patients undergoing CBT at our center, the first increase in TREC levels after PBSCT and BMT was noted slightly earlier, at the 3-months sampling point. A significant increase was however, first evident at 6 months in this group. Factors that correlated with delayed TREC reconstitution were the use of ATG in all patients in addition to TBI-based conditioning, and occurrence of acute GVHD grades II–IV in patients less than 30 years of age. Contrary to what is reported in some other publications (179-181), we found a negative effect of RIC on TREC levels. However, we suspected that age might have played a confounding role in this particular situation. Due to the higher toxicity associated with myeloablative conditioning regimens, these treatment modalities are more often used in younger patients with fewer co-morbid conditions.

Consequently, when the data were stratified for age, no correlation was found between type of condition and TREC levels after HSCT.

Regarding the influence of thymic function on outcome, we found that those with TREC levels below median as early as 3 months after BMT and PBSCT had an OS of 56%, as compared to 80% for those with TREC above median value ($p = 0.002$). This association was also reflected in higher TRM (21% vs. 7%, $p = 0.01$) and higher incidence of fatal infections (11% vs. 2%, $p = 0.01$) in the low-TREC group. No other causes of death, including relapse of malignant disease, showed any statistically significant correlations to TREC levels in our analysis. In addition, patients with CMV reactivation (> 1000 DNA copies/ml of peripheral blood) had lower TREC values at all time points during the first year after HSCT. Thus, it seems reasonable to conclude that the inferior survival rate associated with poor thymic reconstitution in our study population was mainly caused by an increased susceptibility to infectious complications.

Mesenchymal stromal cells and thymic reconstitution

Co-infusion of MSCs during CBT was performed as part of a pilot study conducted at our center between 2005 and 2009. The trial was done with a view to improving engraftment and preventing GVHD, and the rationale behind it was based mainly on the findings of two previously published studies (182, 183). Patients were not selected using systematic randomization, but their inclusion was partly based on the availability of MSC units at the time of transplantation. The MSC group and the non-MSC group were nevertheless balanced regarding age, diagnosis, disease stage, type of conditioning, cell dose, and incidence of GVHD (Table 3, paper III). In our analysis, we found that administration of MSCs was correlated to significantly lower TREC levels at 6 and 9 months after CBT in a multivariate analysis ($p = 0.001$), and that it was also associated with inferior 2-year OS (11% vs. 63%; $p = 0.03$). Based on these results, all attempts at co-infusion of MSCs with CB grafts have been terminated. To date, there have been no published reports showing a similar effect, and we were unable to detect any similar effect in our cohort of patients transplanted with BM or PBSCs. The exact mechanism of an inhibitory effect of MSCs on T-cell differentiation after CBT can only be speculated on at this time. It is possible that a noticeable interaction comes about simply as a result of the low ratio of graft cells to MSCs, but it may also be that this effect is caused by factors related to the phenotype or composition of the cells in the CB units. Another consideration is the timing in relation to the infused graft, which may also be of importance. In these patients, MSCs were transfused at about the same time as the CB unit, while in the case of BMT and PBSCs, administration of MSCs had occurred later. These questions highlight interesting aspects of the immunosuppressive potential of these multipotent cells that certainly warrant further investigation.

Thymic function and immunity to CMV

It was recognized for almost 20 years ago that CMV infection might have a negative impact on immune reconstitution after HSCT, resulting in increased susceptibility to other pathogens (184, 185). We found that reactivation of CMV was strongly associated with lower TREC levels at most time points in our two studies (papers III and IV), and this was significant in both univariate and multivariate analysis. A similar correlation has been found in several other reports that included patients who underwent CBT and conventional HSCT (177, 178, 186, 187). Currently, it is not known whether the observed increase in CMV replication is a consequence of poor thymic function, or whether the virus itself has the ability to specifically inhibit T-cell reconstitution. In their report from 2004, Clave et al. showed that low TREC values *before* transplantation were associated with inferior T-cell reconstitution and increased incidence of CMV and bacterial infections following HSCT. This supports a causative role for poor thymic function in this context. Conversely, CMV is also known to directly inhibit cytotoxic lymphocytes through the function of proteins encoded by its genome. Moreover, it has been proposed that ganciclovir, which currently is the standard treatment for CMV infection, may have a suppressive and antiproliferative effect on immune cells. However, the two latter factors would not necessarily cause a decline in TREC levels, since an overall reduction in peripheral T-cell count should not change the proportion of cells containing TRECs. On the other hand, CMV has also been shown to infect T-progenitor cells, stromal cells, and cells of myeloid lineage, and this could theoretically have a negative effect on the differentiation process in the thymus (188-191). There is, however, no evidence that directly supports this relationship at this time.

The effect of ATG on thymic reconstitution

The influence of T-cell-depletion (TCD) on reconstitution of T-cell subtypes, thymic function, and TCR repertoire after HSCT has been studied previously (181, 186, 187, 192). The results presented in these papers contain some inconsistencies, to which dissimilarities in the TCD protocols may have contributed. In paper IV, we showed that patients who had undergone *in vivo* TCD with ATG had significantly lower TREC counts during the first 6 months after BMT and PBSCT. This correlation remained significant in a multivariate analysis as well as in a separate analysis that included only patients transplanted with MUD grafts. At time points past 6 months TREC levels were comparable between the groups, which indicates that ATG may transiently inhibit T-cell differentiation after HSCT. In the cohort that included 50 patients transplanted with CB grafts, all individuals had received ATG as part of the standard condition regimen for CBT. Thus, the effect of *in vivo* TCD on TREC could not be evaluated in this population. To our knowledge, there are currently no other published reports on the specific effect of ATG on T-cell differentiation and thymic function after HLA-matched HSCT.

Stem cell source and TREC levels

An unexpected finding in our analysis was that patients who had received G-CSF-stimulated PBSC grafts had lower TREC levels from 9 months onwards, when compared to those transplanted with BM grafts (paper IV, Fig. 2B). This association was found to be significant in both univariate and multivariate analysis, and was not caused by lower incidence of GVHD or lower age in the BMT group; these factors were statistically comparable between the two groups. Interestingly, Clave et al. found a similar negative correlation between PBSC grafts and TREC reconstitution in their most recent analysis of 93 patients after ASCT. However, since they were unable to confirm this in a multivariate regression analysis, they attributed the finding to an imbalance in patient age between the study groups (193). In light of our own results, one can speculate whether the smaller sample sizes in their analysis could be an alternative explanation for the lack of statistical significance. As mentioned in paper IV, differences in cell composition between BM and PBSC grafts may account for the apparent long-term difference in thymic output that we observed in our cohort. The possible role of cells with a supportive function in T-cell differentiation, such as MSCs and dendritic cells of BM origin, has been discussed in this context (194-196). Endothelial progenitor cells (EPCs) are another type of cells that may have an important role in thymic reconstitution. These bone marrow-derived cells can restore endothelial function in injured tissue and have been shown to promote thymic-dependent T-cell development in mouse models (197, 198). The presence of these cells in allogeneic BM grafts and their ability to colonize endothelial flow surfaces have been demonstrated in dogs (199).

Graft-versus-host disease and the thymus

In most previous publications acute and chronic GVHD have been shown to have a strong negative effect on thymic function (179, 181, 186, 193, 200-205). There is also considerable evidence that supports direct damage to thymic tissue caused by acute GVHD (206-208). This is probably mediated through IFN-γ-dependent apoptosis of thymic epithelial cells, as has been shown in murine models (209, 210).

The potential deleterious effect of immunosuppressive treatment on thymopoeisis should not be disregarded. It has in fact been shown that high doses of glucocorticoids can also promote apoptosis in thymic epithelial cells (211-213). This effect appears to be reversible, especially in younger individuals, which is in line with our current understanding of the regenerative ability of the thymus (214). We were also able to confirm this relationship in our own analysis by showing that younger patients who had undergone irradiation therapy, or had been diagnosed with acute GVHD of grades II–IV, had significantly lower TREC levels during the first year but not at later time points.

Another important point to consider in this context is the fact that peripheral expansion of T-cells has a diluting effect on the proportion of TREC positive cells in the peripheral circulation. Therefore, the temporal correlation between low TREC levels and ongoing GVHD may be a reflection of increased lymphocyte proliferation rate, secondary to strong immune activation. This is most likely a significant factor in the early phase of the reaction, considering the fact that lymphocytopenia is a known occurrence later in the course of acute GVHD. The setup of the studies that have already been done does not allow quantitative assessment of cell division rate, but this can be achieved by measuring levels of the proliferation marker Ki67 or by flow-cytometric analysis of T-cell subpopulations (215, 216).

The role of the thymus in suppressing allo-reactive and auto-reactive responses has been studied extensively (217-219). In light of this information, it is important to consider the probability of a bidirectional relationship between thymic function and GVHD. This means that if a functioning thymus is needed for achievement of tolerance, then thymic damage might consequently enhance GVHD. Suggested mechanism are decreased production of regulatory T-cells and disruption of the negative selection process (220-225). This line of reasoning is further supported by the results of one of the few studies that document TREC before HSCT. Here the investigators found that low TREC levels, measured in pretransplant samples, were associated with increased incidence of acute and chronic GVHD (226).

Concluding remarks and future aspects of TREC analysis

In recent years, numerous reports have described associations between TREC levels and variables related to the treatment procedure and patient characteristics. Even though many of these analyses have included relatively large cohorts of patients, the specific results often differ from–or even contradict–those found by other investigators. One important consideration that may contribute to these inconsistencies is differences in the way TREC levels have been measured and expressed. In our studies, we calculated TREC as a ratio between copies of signal-joint TRECs (sjTREC) and the house-keeping gene GAPDH, measured in purified CD3+ cells. In other approaches, TREC levels are expressed as copies per volume of blood, or per absolute number of PMBCs in the sample. We believe that our setup improves accuracy, because the end results are not affected by variations in frequencies or concentrations of cells in peripheral blood at the time of sampling. The addition of data on ongoing rate of cell proliferation would increase the accuracy of the analysis even further, by allowing compensation for the diluting effect of peripheral expansion. It is important to reach a consensus about the method used for TREC analysis, in order to achieve results that are comparable between centers. This would enable larger multicenter trials, which would hopefully generate findings with a high level of clinical evidence.

Our results confirm that the source of the hematopoietic stem cell graft may indeed have a significant influence on immune reconstitution after HSCT. Different aspects of this have been illustrated in previous publications. TREC analysis

provides the possibility of quantitatively measuring one part of the immune reconstitution process. It would be of great interest to prospectively investigate the impact of BM, PBSCs and CB on thymic reconstitution in a larger patient material, in order to exclude the possible influence of confounding factors. A detailed analysis of minor cell populations in different graft types may also help to elucidate the mechanism behind our findings. Another question that warrants further investigation is the predictive value of pretransplant analysis of TREC levels. Currently, this has only have been addressed in a single study but it must be confirmed using a larger material, preferably in relation to TREC reconstitution after HSCT.

Finally, based on the results presented here, we come to the conclusion that measurement of TREC after HSCT may provide clinically relevant information that can be used to evaluate patients' current status in the process of reconstituting a functional T-cell immunity. This information appears to have predictive value regarding outcome parameters, such as the risk of severe infections and survival rates. However, it is also evident that the rate and final degree of T-cell reconstitution in each individual are the result of a complex interaction between thymic function and several other factors including GVHD, immunosuppression, conditioning therapy, and viral pathogens.

4.4 FLOW CYTOMETRIC ANALYSIS OF DONOR CELLS FOR PREDICITION OF ACUTE GVHD

The effectiveness of HSCT supersedes that of other treatment modalities when it comes to inducing long-term remission of hematological malignancies. This is considered a consequence of the ongoing immunological reactions mediated by donor immune cells, collectively termed GVT effect. The driving force of this process is most likely the phenotypic disparity between donor and recipient that is the defining characteristic of allogeneic transplantation. In a situation where both individuals are fully matched regarding HLA, these differences are comprised of MiHA, with the possible addition of tumor-associated antigens in the presence of malignant conditions (47, 108, 111, 112, 227). Thus, some degree of incompatibility is required to achieve disease response after HSCT, but this also entails a risk of unwanted immunological responses against healthy host tissues. Currently, GVHD remains one of the main factors limiting the applicability of HSCT. However, it has been shown that clinically significant GVHD is not an absolute requirement for durable remission, and that there is no dose-response relationship between increased degree of mismatch and response rate (76, 121, 228, 229). Whether these findings mean that GVT and GVHD are two separated processes with discrete mechanisms is the subject of ongoing discussion, as mentioned in the introduction of this thesis.

The main basis of the donor-selection process is to achieve a high degree of HLA-matching, while considering known risk factors such as female donor to male recipient, blood group mismatch, age of donor, stem cell source, and previous viral infections. Currently, there are no functional tests available that can be used to

predict the risk of a strong allogeneic reaction between HLA-identical individuals *in vivo*. Up until around the beginning of the previous decade, several tests were evaluated for this purpose but the correlations with clinical outcome were highly variable between different studies (230-246). Most methods were variations of the mixed lymphocyte reaction (MLR) and aimed to quantify reactivity of donor lymphocytes against prospective recipient cells. We reasoned that the inconsistencies in results between these studies might be connected to low sensitivity and specificity of the techniques used for detecting events. Therefore, we wanted to investigate a different approach based on the use of multicolor flow cytometric analysis of cells before and after allogeneic MLR.

In paper V, we present the results of a prospective pilot study that included 28 patients who underwent HSCT at Karolinska University hospital. Peripheral blood mononuclear cells (PBMCs) were collected and stored just before the start of conditioning and in conjunction with the harvest of the grafts from patients and donors, respectively. From this cohort, seven patients who later developed clinically significant acute GVHD were included in the final analysis and assigned to the study group ("GVHD group"). In addition, seven patients without any clinical signs of GVHD were included as controls ("non-GVHD group"). The frequency of lymphocyte subsets in the donor samples, as well as phenotypic distinctions within these populations, was determined by flow cytometric analysis. Next, we repeated a similar analysis of the donor cells after an allogeneic MLR against inactivated recipient cells had been performed. The acquired data was statistically analyzed regarding possible differences between the two patient groups.

We found that unmanipulated donor samples in the GVHD-group contained significantly lower frequencies of T-cells expressing the surface markers CD56, CD94 and CD95 when compared to the non-GVHD group. Donors in this group also had significantly lower levels of γδ T-cells in peripheral circulation at the time of graft harvest. The distribution of cells within the major lymphocyte populations, i.e. NK-cells, B-cells, total T-cells, $CD4^+$ T-helper cells, and $CD8^+$ cytotoxic T-cells, did not differ significantly between the groups. Likewise, the frequencies of different memory T-cell subsets in the pre-transplant donor samples were comparable between the groups.

The finding that donor samples from the non-GVHD group demonstrated a higher content of T-cells expressing the NK-cell markers CD56 and CD94 can have different possible explanations. Cells with a similar phenotype were detected for the first time in the beginning of the 1990s and have since then attracted an increasing amount of interest. They were initially referred to as NKT-cells but it eventually became clear that this classification included several different subsets with diverse functions. The term invariant NKT- (iNKT-) cells was introduced for double-negative, $CD3^+$ cells that, in addition to NK-cell specific surface markers, also expressed the invariant TCR α-chain Vα14Jα18 (247, 248). These cells exhibited immune regulatory functions, as opposed to cytotoxicity, and were able to the attenuate allogeneic responses in murine models (249-251). It was later shown that iNKT-cells could suppress T-helper cell activity through paracrine

secretion of cytokines, which in turn promoted expansion of $CD4^+CD25^+Foxp3^+$ regulatory T-cells (252-255). This mechanism did not seem to affect GVT activity since the cytotoxic function of donor $CD8^+$ T-cells was preserved (256, 257). In a recent publication, Chaidos and co-workers analyzed the effect of iNKT-cell dose on acute GVHD in clinical HSCT setting. They found a strong correlation between low frequency of iNKT-cells in the stem cell grafts and increased incidence of acute GVHD (258). This is in line with our results and confirms what was previously noted in a smaller cohort of patients (259). T-cells may also be induced to express NK-cell markers under certain condition but an inhibitory effect on allogeneic responses has only been shown for the $CD4^-$ subset expressing the invariant TCR α-chain. Our experimental setup did not allow for a specific analysis of this cell type but this variable should be included in future studies. Another interesting aspect to this finding is that iNKT-cells appear to be more prevalent in BM than in peripheral circulation, which would partially explain the differences in GVHD incidence observed between BMT and PBSCT (249). This line of thought is further complicated by findings indicating a possible suppressive effect of G-CSF on iNKT-cell responsiveness (259).

T-cells expressing the T-cell receptor γδ chains are another minor lymphocyte population, which may be involved in the regulation of allogeneic responses. The precise role of these cells remains unclear but some studies indicate that they may have immune modulatory as well as antigen presenting capacities. They have been shown to interact with other lymphocytes directly through cell-to-cell contact and indirectly via cytokine/chemokine production (260-263). Our results suggest that a relatively higher content of γδ T-cells in the graft may be correlated with lower incidence of acute GVHD ($p = 0.026$). Similar findings were shown in a recent clinical study and have previously been reported in mouse models (264-266). However, other publications present data indicating an increased risk of GVHD associated with this T-cell subset (267, 268). These contradicting results may be a consequence of differences in sample size and in variables related to the transplantation procedure. Another potentially important factor may be distinctions regarding states of activity and maturation of the γδ T-cells, which may affect their ability to survive and proliferate *in vivo* (269, 270).

Analysis of donor cells after allogeneic MLR in the GVHD direction revealed significantly higher frequencies of $CD4^+$ T-cells in the GVHD group ($p = 0.026$). Moreover, the majority of these cells were of a naïve phenotype (Fig. 3A-B paper V). The distribution of cells within the major lymphocyte populations was comparable between the two groups before the MLRs. The predominance of naïve cells after MLR may be a consequence of a high rate of cell death in the effector memory population. This could in turn be caused by massive expansion in response to allogeneic stimuli, which could shorten the life span of these cells in the suboptimal *in vitro* conditions.

The major weakness of our analysis is the small number of patients and donors included, and the resulting imbalance in possible confounding factors between the groups. The main challenge has been to identify patients with the more severe forms of GVHD, but also to include those who did not show any clinical signs of

GVHD and who had not received any additional immunosuppression. In addition, a large part of the collected recipient samples resulted in very few cells due to the low peripheral cell-count of the patients. Consequently, the majority of the patients and donors who gave informed consent ultimately had to be excluded from the final analysis. We conclude that detailed flow cytometric analysis of donor lymphocyte composition before HSCT may be used to predict the risk of GVHD. By using flow cytometry to detect changes in frequencies and surface expression of lymphocyte subsets after allogeneic MLR, it may also be possible to assess the alloreactive potential of a prospective donor graft.

5 CONCLUSIONS

- Our study did not show any effect of NOD2 mutations on the incidence of GVHD or any other outcome parameter. There is currently not enough evidence to support inclusion of NOD2 analysis in the pre-transplant work-up and donor-selection process.

- Early administration of DLI to patients with acute hematological malignancies, based on changes in cell lineage-specific chimerism and MRD analysis can significantly improve relapse-free survival after HSCT.

- The risk of severe acute GVHD is not significantly increased after DLI and is within acceptable limits for those treated within 100 days after HSCT. Our results do not support exclusion DLI as a treatment option during the early post-transplant phase on account of increased risk of GVHD.

- TREC analysis appears to have a high predictive value concerning survival and susceptibility to infections after HSCT.

- Factors related to the transplant procedure, such as graft source and *in vivo* T-cell-depletion may significantly affect thymic function after HSCT.

- Flow cytometric analysis of donor cells for phenotype and allogeneic reactivity may be used to predict acute GVHD before HSCT.

6 POPULAR SCIENTIFIC SUMMARY IN SWEDISH

Vårt immunsystem består av ett mycket avancerat nätverk av celler som hela tiden arbetar med att skydda vår kropp mot yttre hot, såsom bakterier, virus och svampar. Immunsystemet har också en viktig roll vid läkning av skador. Men som alla kraftfulla verktyg så kan det också orsaka stor skada om det används på fel sätt. Celler i immunsystemet som slutar fungera eller blir överaktiva kan orsaka många allvarliga tillstånd, såsom olika former av blodcancer, immunbristsjukdomar och reumatiska sjukdomar. Det mest effektiva sättet att bota dessa tillstånd är att helt och hållet ta bort de sjuka cellerna och ersätta dem med nya från en frisk person. En sådan behandling kallas ofta för *benmärgstransplantation,* men ett bättre namn är transplantation av blodbildande stamceller (eller kort och gott *stamcellstransplantation*), eftersom de celler man använder sig av nu för tiden oftast tas direkt från blodet och inte från benmärgen.

Det här är en behandling som började användas redan på 1960-talet, men den höga risken för allvarliga biverkningar gör att den i praktiken bara är ett alternativ vid mycket allvarliga sjukdomar och när det inte finns några andra möjliga behandlingar. De viktigaste biverkningarna är infektioner, avstötning av stamcellerna, skador av de läkemedel och den strålning som ges för att kroppen ska kunna acceptera de nya cellerna samt att det nya immunförsvaret reagerar mot patientens kropp och orsakar en så kallad *transplantat-kontra-värd-reaktion*.

Transplantat-kontra-värd-reaktion är den mest allvarliga av alla de biverkningar som kan inträffa efter stamcellstransplantation. Den beror på att cellerna i det nya immunförsvaret, som ursprungligen kommer från en annan person, upplever de andra cellerna i patientens kropp som främmande och försöker bekämpa dem på samma sätt som om de var infekterade av ett virus. Delar av kroppen som oftast drabbas är huden, tarmarna och levern. Symtomen varierar allt från en lindrigare inflammation som går över av sig själv, till att hela det drabbade organet förstörs. Den svåraste formen av reaktionen svarar sällan på behandling och är nästan alltid dödlig. Vi har idag inga möjligheter att med någon större säkerhet förutsäga vilka som kommer att utveckla en transplantat-kontra-värd-reaktion efter en stamcellstransplantation och än mindre kan vi veta hur pass kraftig reaktionen blir. Alla patienter får de första månaderna efter transplantationen en förebyggande behandling mot transplantat-kontra-värd-reaktion med läkemedel som bromsar immunförsvaret och denna behandling är i princip samma för alla.

Trots att man nu för tiden har blivit väldigt bra på att hitta donatorer vars celler nästan helt överensstämmer med patientens egna drabbas ändå hälften av alla som genomgår en transplantation i någon grad av transplantat-kontra-värd-reaktion. Samtidigt är olikheten mellan patienten och donatorn väldigt viktig vid behandlingen av cancersjukdomar, eftersom det är dessa skillnader som gör att det nya immunförsvaret kan utrota cancercellerna mer effektivt än många av de läkemedel som finns tillgängliga idag. Man har till exempel sett att om patienter med leukemi transplanteras med stamceller som kommer från deras enäggstvilling så ökar risken för att leukemin kommer tillbaka markant.

Analys av NOD2 gener före stamcellstransplantation

Man har nyligen sett att personer som bär på en viss gen skulle kunna ha större risk att utveckla svår transplantat-kontra-värd-reaktion och kan ha en ökad risk för att död i komplikationer efter en stamcellstransplantation. Den här genen, som kallas NOD2, finns hos några få procent av befolkningen och är vanligare bland personer som lider av en typ av inflammatorisk tarmsjukdom (Crohn's sjukdom). I första delarbetet i denna avhandling ville vi undersöka om det fanns en liknande koppling mellan den här genen och risken för transplantat-kontra-värd-reaktion även bland våra patienter. Därför analyserade vi prover från ca 200 av de patienter som transplanterats på vår klinik under de senaste 10 åren. Intressant nog såg vi ingen som helst negativ effekt av denna gen bland våra patienter. En trolig förklaring är att det finns skillnader i behandlingsrutiner som i det här fallet motverkar effekten av genen. Vi kan idag inte med säkerhet säga exakt vilka de här skillnaderna är, men utifrån det här resultatet så anser vi inte att en sådan analys behöver ingå i utredningen av patienter som behandlas på vår klinik.

Tidig behandling av hotande canceråterfall

Risken för återfall av cancersjukdomar är relativt stor även efter en stamcellstransplantation. Ett sätt att behandla ett sådant återfall är att ge patienten ytterligare doser av vita blodkroppar från den ursprungliga donatorn. Framför allt handlar det om en särskild grupp vita blodkroppar som kallas för T-lymfocyter eller T-celler. De här cellerna har förmågan att känna igen sjuka eller främmande celler för att sedan dirigera det övriga immunförsvaret till att göra sig av med dem. Erfarenhetsmässigt är effekten av behandlingen med donators-T-celler väldigt varierande men i många fall kan man få sjukdomen att gå tillbaka helt.

I det andra projektet i den här avhandlingen har vi tittat närmare på de patienter som fått donators-T-celler vid Karolinska universitetssjukhuset Huddinge de senaste 20 åren. Detta är en av de hittills största studierna i sitt slag från ett enskilt center. Vi är också ett av de få sjukhus som regelbundet under närmare 15 år följt alla patienter med en känslig metod som gör att man tidigt kan förutsäga om cancern är på väg tillbaka. Metoden, som kallas för *chimerismanalys*, använder sig av så kallad PCR-teknik (från engelskans Polymerase Chains Reaction), som innebär att man på kemisk väg kan gör många kopior av cellers arvsmassa. På så sätt kan man hitta celler som förekommer i väldigt små mängder i till exempel ett blodprov. Vid chimerismanalys använder man sig av s.k. satellitregioner av arvsmassan som alla individer har, men dessa kan variera i "storlek" mellan olika individer. På det viset kan dessa satellitregioner användas för att skilja två individer åt. Liknande metoder används vid faderskapskontroller och inom rättsmedicin för att binda någon vid ett brott.

I den här studien lyckades vi visa att patienter som behandlas i ett tidigt skede med T-celler från donatorn, d.v.s. redan när man ser stigande nivåer av

cancerceller i chimerismanalys, svarade klart bättre på behandlingen och hade mycket bättre chanser att överleva sjukdomen. Vi kunde även visa att om man behandlar med dessa celler tidigt efter transplantationen så innebär detta inte en ökad risk för transplantat-kontra-värd-reaktion vilket man har trott tidigare.

Thymus funktion efter stamcellstransplanation

Första tiden efter stamcellstransplantation är immunförsvaret kraftigt nedsatt. Detta beror till största delen på avsaknad av fungerande T-celler. Som tidigare nämnts har dessa celler en nyckelroll i immunförsvaret. Nybildningen av T-celler sker i thymus (sv. ”brässen”), och de senaste åren har en metod tagits fram som kan analysera hur väl thymus fungerar. Metoden går ut på att man mäter nivåerna av särskilda cirkelformade bitar av arvsmassa som finns inne i T-cellerna. Dessa bitar av arvsmassa (=TREC) bildas som en sorts biprodukt i samband med att nya T-celler formas. Vi mätte nivåerna av TREC i prover från 260 patienter under flera tillfällen, fram till två år efter transplantationen. Vi kunde se att thymus fungerade klart bättre hos patienter som behandlats med stamceller framtagna från benmärg jämfört med dem som fått celler isolerade direkt från blodet. Detta är intressant eftersom det idag görs många fler transplantationer med stamceller från perifert blod än med benmärgsceller.

Vi såg också att patienter som fått en viss typ av immunhämmande behandling med antikroppar som hämmar T-celler hade sämre fungerande thymus under första halvåret efter transplantation. Det var också tydligt att patienter vars thymus inte fungerade lika bra (som alltså hade dålig förmåga att bilda nya T-celler) inte klarar sig lika bra efter transplantationen på grund av flera allvarliga infektioner.

Sammanfattningsvis visar resultaten i den här studien att thymus funktion har en mycket viktigare roll efter stamcellstransplantation än vad man tidigare har trott. Vi har också funnit två olika faktorer som kraftigt påverkar thymus, nämligen val av immunhämmande behandling och typ av stamcellsprodukt. Baserat på detta kan man också säga att den metod som vi använt oss av kan vara en relevant analys för uppföljning av patienter efter stamcellstransplantation. Metoden är dessutom relativt enkel och kan utan problem utföras rutinmässigt.

Test för att förutsäga transplantat-kontra-värd-reaktion

Syftet med det femte projektet i denna avhandling var att utveckla en relativt enkel metod för att kunna förutsäga risken för transplantat-kontra-värd-reaktion hos den enskilda patienten redan före själva transplantationen. Vi började med att sortera ut vita blodkroppar i blodprover som togs från patienter och donatorer strax före transplantationen. Vi lät därefter cellerna från donatorn reagera mot patientens celler i ett särskilt laboratorietest som efterliknar det som händer i kroppen vid en transplantation. Sedan analyserade vi de här cellerna med hjälp av en metod som kallas *flödescytometri,* vilken gör att man noga kan identifiera

särskilda molekyler på cellernas yta. Totalt letade vi efter ca 30 olika molekyler. Vad vi kunde se var att de celler som tillhörde donatorer till patienter som sedan utvecklade svår transplantat-kontra-värd-reaktion hade en särskild kombination av proteiner på sina ytor, både före och efter att de fick reagera med patientens celler. Det här sättet att testa celler före en stamcellstransplantation skulle kunna användas för att förutsäga risken för transplantat-kontra-värd-reaktion. Men innan vi kan vara säkra på noggrannheten av testet behöver resultatet kontrolleras i en större grupp av patienter.

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