Allogeneic stem cell transplantation:
exploration of new indications and strategies to exploit graft-versus-tumor immunity for cancer treatment

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Colofon

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Contents

List of abbreviations

7AAD	7-aminoactinomycin D
APC	antigen presenting cell
ATG	anti-thymocyte globulin
BM	bone marrow
CCyR	complete cytogenetic response
CFSE	carboxyfluorescein diacetate succimidyl ester
CLL	chronic lymphoid leukemia
CML	chronic myeloid leukemia
CMolR	complete molecular remission
CR	complete remission
CCR	continuous complete remission
CTL	cytotoxic T lymphocyte
CsA	cyclosporine A
CY	cyclophosphamide
DC	dendritic cell
DLI	donor lymphocyte infusion
DNA	deoxyribonucleic acid
EBV	Epstein-Barr virus
ELISA	enzyme-linked immunosorbent assay
EFS	event-free survival
FACS	fluorescence activated cell sorting
FCS	fetal calf serum
FISH	fluorescent in situ hybridization
FITC	fluorescein isothiocyanate
FL	follicular lymphoma

Flu	fludarabine
G-CSF	granulocyte colony-stimulating factor
GM-CSF	granulocyte-macrophage colony-stimulating factor
GVHD	graft-versus-host disease
GVL	graft-versus-leukemia
GVM	graft-versus-myeloma
GVT	graft-versus-tumor
HDM	high-dose melphalan
HLA	human leukocyte antigen
HS	human serum
HMBS	hydroxyl-methylbilane synthase
HSA	human serum albumin
IFN	interferon
IgH	immunoglobulin heavy chain
IL	interleukin
IMDM	Iscove's modified Dulbecco's medium
KLH	keyhole limpet hemocyanin
LCL	lymphoblastoid cell line
MA	myeloablative
MCL	mantle cell lymphoma
MDC	myeloid dendritic cell
MHC	major histocompatibility antigen
MiHA	minor histocompatibility antigen
MFI	mean fluorescence intensity
MM	multiple myeloma
MR	minimal response
mRNA	messenger ribonucleic acid
NHL	non-Hodgkin's lymphoma
NK	natural killer
NST	nonmyeloablative stem cell transplantation
OS	overall survival
PB	peripheral blood
PBMC	peripheral blood mononuclear cells
PBS	phosphate-buffered saline
PBSC	peripheral blood stem cells

PCR	polymerase chain reaction
PD	progressive disease
PDC	plasmacytoid dendritic cell
pDLI	pre-emptive donor lymphocyte infusion
PE	phycoerythrin
PFS	progression free survival
PGE2	prostaglandin E2
PR	partial remission
RCC	renal cell carcinoma
RIC	reduced intensity conditioning
RNA	ribonucleic acid
RT	reverse transcriptase
SCT	stem cell transplantation
SNP	single nucleotide polymorphism
TBI	total body irradiation
TNF	tumor necrosis factor
TRM	transplantation-related mortality
tDLI	therapeutic DLI
UPN	unique patient number
VAD	vincristine, adriamycin dexamethasone
VGPR	very good partial remission

Chapter1

General Introduction

General Introduction

Allogeneic stem cell transplantation is a potential curative treatment modality for hematopoietic malignancies. The therapeutic efficacy of myeloablative SCT is based on high dose chemotherapy often in combination with radiotherapy and alloreactivity of the donor immune system, referred to as the graft-versus-leukemia (GVL) reactivity. Despite its powerful antitumor activity, myeloablative allogeneic SCT is unfortunately accompanied by a high treatment related morbidity and mortality due to infections and graft-versus-host disease (GVHD). As most complications of allogeneic SCT are related to GVHD and the favorable results are related to GVL, the key issue of SCT remains to separate GVL from GVHD.

The graft-versus-leukemia effect of allogeneic stem cell transplantation

Early in the era of allogeneic SCT it was already suggested that allogeneic SCT eliminates leukemic cells through immune-mediated effects. Weiden et al. reported in 1979 that patients developing acute GVHD had a decreased chance for leukemic relapse following allogeneic SCT from an HLA-identical sibling.[1] This was confirmed one decade later by the study from Horowitz et al.[2] showing that patients who did not develop GVHD following transplantation and patients transplanted with a T cell-depleted graft or stem cells from a syngeneic donor had a greater chance of relapse. These observations indicate that alloreactive donor T cells are at least partly responsible for this reaction, which is defined as the GVL effect since then. Because not only leukemia cells are susceptible to these immunocompetent T cells, this immune reactivity is also called graft-versus-malignancy or graft-versus-tumor (GVT) reactivity. Another important observation from this study was that the susceptibility to GVL varies between different hematological diseases. Patients with chronic myeloid leukemia (CML) appeared most susceptible, while patients with acute myeloid leukemia (AML) showed intermediate susceptibility and patients

with acute lymphoid leukemia (ALL) were least susceptible. Further proof of the important role of donor T cells in GVL reactivity came from the observation of disease responses after infusion of fresh lymphocytes from the original stem cell donor (DLI) in patients relapsed after allogeneic SCT. [3]

Although donor T cell responses are strongly associated with GVL, also natural killer (NK) cells are capable in mediating anti-tumor effects. Especially in HLA-mismatched or haplo-identical SCT, alloreactive donor NK cells were found to play an important role in GVL reactivity.[4;5] Furthermore, also antibody responses developing after allogeneic SCT are thought to play a role in GVL and GVHD.[6-9] This introduction and thesis will focus on the role of alloreactive donor T cells and their antigenic targets.

Role of minor histocompatibility antigens in GVT immunity

GVHD and GVL after genotypically major histocompatibility complex (MHC)-matched allogeneic SCT are immune responses directed at polymorphic loci outside the MHC, which are referred as minor histocompatibility antigens (MiHA). These MiHA are MHC-restricted peptides derived from intracellular proteins that differ in amino acid sequence between recipient and donor due to single nucleotide polymorphisms (SNP) in their encoding genes. They are presented by MHC class I and II molecules on the cell surface of recipient cells and can be recognized by donor CD8+ and CD4+ T cells, respectively. MiHA can be classified according the chromosomal location of the encoding gene (i.e. autosomal- or Y chromosome-restricted) or based on tissue distribution (i.e. ubiquitous versus hematopoietic-restricted expression pattern). Ubiquitous MiHA are broadly expressed by normal tissues and/or tumor cells and immune responses against these MiHA can result in both GVHD and GVT. In contrast, MiHA selectively expressed on both normal and malignant hematopoietic cells of the recipient may induce GVT without GVHD. MiHA-specific donor T cell responses have no negative effect on normal blood cell formation since the patient hematopoietic system is replaced by donor hematopoiesis, referred to as complete donor chimerism. Although hematopoietic-restricted MiHA are not expressed by normal tissues, some of these MiHA are aberrantly expressed in non-hematopoietic solid malignancies.[10-13]

T cell depletion as strategy to reduce GVHD

GVHD is a most harmful immunologic complication of allogeneic SCT, in many

ways comparable with auto-immune diseases. GVHD is named acute GVHD if it develops within the first three months after SCT and affects mainly the skin, gastro-intestinal tract and liver. Chronic GVHD per definition has a later onset and also mainly affects skin, gastro-intestinal tract and liver, and in addition other mucosal tissues such as the eyes and lung. GVHD may also be induced after DLI.

An important strategy to reduce the incidence and severity of GVHD is removal of T cells from the graft (i.e. T cell depletion). The major advantage of T cell-depleted allogeneic SCT is the reduced morbidity resulting in a much better quality of life after SCT and lower GVHD-related mortality. However, earlier studies in myeloablative allogeneic SCT have shown that T cell depletion did not result in an improved overall survival due to higher rates of graft failure, relapses and Epstein-Barr virus-associated lymphoproliferative disorders (EBV-LPD).[14-16] Strategies to improve the results of T cell-depleted allogeneic SCT are partial T cell depletion by adding back a fixed number of T cells to the depleted graft or adoptive immunotherapy to enhance GVT reactivity after discontinuation of immunosuppression.

Several T cell depletion techniques have been described, either the positive selection of the stem and progenitor cells (CD34 selection) or the removal of T cells (CD3 depletion), for example by immunomagnetic beads. Also in vivo T cell depletion is frequently applied, for example by the infusion of anti-thymocyte globulin (ATG) or Alemtuzumab. Alemtuzumab, eliminating CD54 positive cells is the most rigorous form of T cell depletion, removing also B cells and NK cells.

From myeloablative to reduced intensity conditioning: allogeneic SCT as immunotherapy

The toxicity of the myeloablative conditioning regimens and the recognition of alloreactive T cell responses to be the predominant mechanism for tumor response resulted in the design of several reduced intensity conditioning regimens (RIC). Nowadays, RIC is the most frequently used form of allogeneic SCT. The reduction in toxicity of the conditioning regimen made it possible to significantly enhance the age limit for allotransplant up to 65-70 years of age. The strategy of RIC-SCT differs for the different hematological malignancies. In most hematological malignancies immunosuppressive chemotherapy is only needed for transplant engraftment. But in some hematological malignancies, such as myelofibrosis, chemotherapy is still needed to reduce the tumor burden, making "space" in the marrow and get the new transplant accepted. In non-hematopoietic malignancies (metastatic solid tumors)

the optimal conditioning regimen and the value of RIC conditioning are still under investigation.

Post-transplant immunotherapy by Donor Lymphocyte Infusions

DLI are used after myeloablative allogeneic SCT to augment the GVT effect, at relapse or pre-emptive, mostly in a T cell-depleted setting. Also after RIC-SCT DLI is used to accelerate the conversion from mixed towards complete donor chimerism, supporting engraftment. The first studies on therapeutic immunotherapy with donor lymphocytes in relapsed leukemia after myeloablative allogeneic SCT were published in 1995 and demonstrated curative potential in CML and myeloid forms of acute leukemia.[3] DLI is used in nearly all malignant diseases for which allogeneic SCT is performed. Main causes of treatment-related mortality after DLI are GVHD and marrow aplasia followed by infections. The rate of GVHD increases when higher T cell doses are infused, although the absolute doses may vary in siblings versus unrelated setting.[17;18]

The application of DLI can be divided in two different strategies, i.e. the therapeutic or prophylactic setting. Therapeutic DLI (tDLI) is administered for the treatment of relapse or for the correction of incomplete donor chimerism. Whereas prophylactic or pre-emptive (pDLI) involves the planned administration of DLI after SCT often in the setting of (partial) T cell-depleted grafts.[19]

DLI in therapeutic setting

In patients relapsing from CML after SCT, complete molecular remissions have been obtained in 70-80% of patients.[3] The response to DLI and probability of aplasia after DLI was dependent on the relapse status at time of lymphocyte infusion. Improved responses and less aplasias were observed in patients with molecular and cytogenetic relapse compared to hematological relapse, so related to the tumor burden. In AML or MDS, tDLI is significantly less effective. In a retrospective study the EBMT analyzed the role of DLI in the treatment of relapsed AML after allogeneic SCT.[20] At relapse, most patients received chemotherapy, but overall survival was higher (2 year survival, 21% versus 9%) in those patients receiving DLI. However, in AML still only a minority of the patients benefits of DLI, especially patients in remission, with more favorable karyotype and with a longer interval of relapse after transplant. Due the retrospective nature of this study both patient populations may be different and a selection bias in the DLI group may have occurred.

The mechanisms responsible for the variation in DLI-mediated anti-tumor responses between the different disease types are still unclear. Differences in tumor characteristics such as growth rate, susceptibility to T cell-mediated cytotoxicity, secretion of immune-modulatory cytokines and variation in the expression of co-signaling molecules as well as the possibility for antigen presentation may have a major impact on the response.[21-24]

DLI in prophylactic setting

Several studies have analyzed the value of pDLI to prevent tumor relapse after T cell-depleted myeloablative SCT.[25-31] Timing of pDLI and the T cell dose differed in these studies. In some studies, a fixed T cell dose was given, while other studies selected the T cell dose based on the risk for GVHD and relapse. Most studies were performed in patients with related donors, and only one study included unrelated donors.[30] Furthermore, one study compared the outcomes of patients treated with pDLI with those patients not treated with pDLI.[26] Relapse rates were lower in the pDLI patient group. The 3-year probability of disease-free-survival was 77% in the pDLI patient group and 45% for the patients in the control-group. These groups included AML, ALL, CML and MM patients.

Prophylactic DLI might play an important role when combining T cell-depletion with RIC-SCT to enhance engraftment, reach complete donor chimerism and to boost GVT reactivity.

Novel strategies

Several methods have been developed to improve the efficacy of DLI without induction of (severe) GVHD. One approach starts with a low dose of DLI followed by dose-escalation if the patient does not reach CR. This strategy is based on the assumption that T cell dose and the induction of alloreactivity differs between GVT and GVHD, and dose-escalation may separate these reactivities. Separation of GVT from GVHD was shown in some patients with CML receiving DLI more than one year after SCT.[32] In a dose-finding study from Bacigalupo et al. with 10 patients, GVHD was observed in 1 patient at a dose of $2x10^6$ T cells/kg and in 6 patients with $2x10^7$ T cells/kg.[33] Retrospective comparison with patients treated with higher doses of DLI at the same institution showed that dose escalation reduced the incidence of severe GVHD and improved outcome. Peggs et al. studied the application of dose-escalated DLI following reduced intensity conditioning SCT in patients with

lymphoid malignancies.[34] In this study, separation of GVT from GVHD was achieved in only a minority of the patients.

Another approach is the transfer of subsets of donor lymphocytes, for example CD8-depleted DLI. Because CD8+ T cells play a role in both GVT and GVHD and CD4+ T cells with NK cells are related to GVT without GVHD, infusion of specific subsets of cells may improve the efficacy of DLI without GVHD. Alyea et al. studied T cell-depleted allogeneic SCT followed by CD4+ DLI in MM patients.[35] Only 58% of the patients actually received DLI, the other patients did not receive DLI because of SCT-related complications or early relapse. The 2 year PFS was somewhat higher in the DLI treated patients 65% versus 41% in the historical controls. The specific role of the CD4 T cell subset in this respect is not evaluable. In the study from Meyer et al., 11 patients with high-risk hematological malignancies received a total of 21 CD8-depleted pDLIs.[36] Two patients with HLA-C mismatched donors developed grade II and III aGVHD followed by limited cGVHD. These prophylactic CD8-depleted DLIs accelerate immune reconstitution, but an effect on disease relapse rate has not yet been proven.

A different approach to control GVHD after DLI is the infusion of donor T cells transduced with the herpes simplex virus thymidine kinase suicide gene, which enables selective in vivo T cell depletion of activated T cells with the virostatic drug ganciclovir in case GVHD. This strategy has been explored in a group of 23 patients with relapsed disease after allogeneic SCT.[37] Eleven patients developed disease response with three patients alive and in CR with a median follow-up of 471 days. Three of these 23 patients were successfully treated for GVHD with ganciclovir.

DLI has also been applied to reconstitute antiviral immunity, particularly in pediatric and haploidentical allogeneic SCT.[38;39] To reduce GVHD after DLI, protocols for physical depletion of alloreactive T cells have been investigated. The principle of these methods is stimulation of donor T cells with recipient-derived antigen presenting cells to activate the alloreactive T cells. Thereafter activated T cells are eliminated by antimetabolic drugs, photodepletion, immunotoxins or magnetic beads to target specific upregulated cell surface molecules. Several activation markers, which are upregulated on activated T cells, have been used to deplete alloreactive T cells, including CD69[40], CD25[41-44], CD134[45] and CD137.[46] Another approach to deplete alloreactive T cells is a photodepletion technique. This TH9402-based photodepletion technique targets activation-based changes in p-glycoprotein that results in an altered efflux of the photosentisizer TH9402. Initial clinical studies

indicate that the concept of reconstitution antiviral immunity is feasible, however the role in antitumor immunity needs to be studied in more detail.[47]

Impact of hematopoietic chimerism on GVT immunity

The term hematopoietic chimerism refers to the presence of hematopoietic cells of donor origin after allogeneic SCT. Complete donor chimerism is defined as the complete replacement of recipient hematopoietic cells by donor hematopoietic cells. Whereas mixed chimerism refers to the presence of both donor and recipient-derived hematopoietic cells post-transplantation. Development of mixed chimerism is observed more frequently after T cell-depleted SCT and RIC-SCT. Several studies have demonstrated an increased incidence of mixed chimerism in patients receiving less intensive conditioning regimens.[48-51] Mixed chimerism is also observed after myeloablative SCT but the clinical relevance of mixed chimerism after myeloablative conditioning has been controversial. Some studies have observed high relapse rates in patients with mixed chimerism after T cell depleted SCT[52-54], however other studies did not show a correlation between mixed chimerism and relapse rate.[55;56]

Chimerism in clinical practice

Measurement of chimerism is important after nonmyeloablative or RIC-SCT and even more relevant after the combination of T cell depletion and reduced intensity conditioning. Monitoring of chimerism in time may influence decisions on discontinuation of immunosuppression and the indication of DLI. Chimerism analysis can be performed in whole blood samples, in bone marrow samples or in subsets of cells (i.e. lineage-specific chimerism). Several techniques have been used to analyze chimerism after allogeneic SCT. The most applied method for quantitative chimerism analysis is polymerase chain reaction (PCR)-based amplification of variable number tandem repeats or short tandem repeats (STRs).[57] Another method is fluorescent in situ hybridization (FISH) with XY-chromosome specific probes; however this method is restricted to sex-mismatched donor-recipient combinations. A new method based on real-time quantitative PCR using single nucleotide polymorphisms (SNPs) as discriminating markers was developed in our transplant center.[58;59] The main advantage of this method is that very small percentages of recipient cells (0.1-0.01%) can be detected among donor cells and vice versa.[58;59]

Clinical significance of complete donor chimerism

After RIC-SCT, alloreactive T cell responses are of major importance to induce or maintain remissions. Most studies demonstrate that complete donor chimerism in T cells is required for full potential of the allo-immune response. For instance, Childs et al. have monitored T cell chimerism and myeloid cell chimerism after nonmyeloablative SCT in patients within a variety of hematological malignancies.[60] In this study, full donor T cell chimerism preceded donor myeloid engraftment, acute GVHD and disease regression. However, Mattsson et al. analyzed clinical outcome and chimerism in patients transplanted for CML and solid tumors and at the time of disease response six out of the 15 patients showed mixed chimerism in the T cell fraction.[61]

Baron et al.[62] studied kinetics of donor engraftment among various peripheral blood cell subpopulations in 120 patients with hematological malignancies who received nonmyeloablative SCT. This study showed that early establishment of donor chimerism in NK cells was associated with improved PFS. Another study from Baron et al. examined the impact of early donor chimerism in T cells and NK cells in 282 patients who received allogeneic SCT after a minimal-intensity conditioning regimen of 2 Gy total body irradiation (TBI).[63] This study indicated that high early donor T cell chimerism was significantly associated with acute GVHD. Conversely, high levels of donor chimerism in NK cells correlated with low risk of graft rejection, low risk of relapse, and high PFS, but not with aGVHD.

Not only chimerism in immune effector cells is important, also chimerism in professional antigen presenting cells (APC) at the time of DLI may play a role in the initiation of allo-immune responses. Murine studies have showed that recipient derived APC play a key role in the initiation of allogeneic CD8+ T cell-mediated GVH and GVL reactivity after MHC-matched SCT.[24;64] In addition, in a MHC-mismatched mouse model it has been shown that DLI administration to mixed chimeras produced improved leukemia-free survival compared to administration of DLI to full donor chimeras.[65] From these data it can be hypothesized that the presence of recipient APC may be an important factor for the induction of DLI-induced GVL reactivity.

The interpretation of chimerism results and the translation to clinical decisions such as discontinuation of immunosuppression or DLI remain difficult. Significance of persisting mixed chimerism after allogeneic SCT is related to the diagnosis, type of conditioning, T cell depletion and donor type.

RIC-SCT in Multiple myeloma

In patients with multiple myeloma (MM) a graft-versus-myeloma (GVM) effect has been observed, which in some patients even results in cure or very-long term remissions. The application of conventional myeloablative conditioning in MM has been abandoned due to a high transplantation-related mortality (TRM) which varied from 17% to 38%.[66;67] Nonmyeloablative or RIC-SCT, following autologous SCT, has indeed reduced the TRM to 10-20%.[68] In most studies the treatment starts with induction chemotherapy followed by high-dose melphalan (HDM) and autologous SCT. After recovery from the autologous SCT and in a state of remission or minimal residual disease RIC-SCT is scheduled 3-6 months after autologous SCT.

Three prospective trials comparing autologous transplantation followed by RIC-SCT versus double autologous SCT showed contradictory results in clinical outcome.[69-71] The study by Bruno et al. showed a superior overall survival (OS) for autologous SCT followed by allogeneic RIC-SCT.[69] However, the results of the double autologous arm were less favorable than results from similar autologous trials published before. Rosiñol et al. observed a trend towards a longer progression free survival (PFS) for patients treated with auto/RIC-SCT, but with no significant differences in event free survival (EFS) and OS.[71] In contrast, the Intergroupe Francophone du Myelome observed no differences in EFS and OS comparing double autologous SCT versus auto/RIC-SCT, although this study was confined to high risk patients. The message from all these studies, either favorable or unfavorable to allogeneic SCT, is that improvement of the GVM effect to obtain long-term disease control, without the toxicity and morbidity of GVHD is a prerequisite to further establish this therapeutic approach. This has become even more important in an era in which new and highly effective drugs, like thalidomide, lenalidomide and bortezomib have greatly improved the PFS and OS of MM patients and newer targeted drug are under development.[72] Key issues in the setting of non T cell-depleted RIC-SCT remain morbidity from GVHD and relapses after RIC-SCT, even despite severe GVHD. Better understanding of GVM resistance and escape mechanisms of tumor cells in this setting is needed to continue allogeneic therapy in MM. The role of upfront RIC-SCT in the treatment of MM has not been established and allogeneic SCT in MM should not be performed outside clinical trials.

RIC-SCT in Lymphoma and Chronic lymphocytic leukemia

In NHL and CLL the prognosis has significantly improved due to new treatment

modalities, such as the monoclonal antibodies. However, despite these new treatment modalities, almost all patients with CLL and low grade NHL will relapse. For these patients high dose chemotherapy followed by autologous SCT, but also allogeneic SCT, are increasingly used. Relapse rates after allogeneic SCT are lower compared with those after autologous SCT which strongly suggests the existence of a graft-versus-lymphoma effect.[73] Furthermore, GVHD is also associated with a reduced relapse rate, relapse rate is increased after T cell depletion and finally, patients with relapsed lymphoma respond to donor lymphocyte infusions. However, it is important to realize that not all types of lymphomas are equally sensitive to alloreactive T cells. Slowly proliferating diseases like follicular lymphoma and CLL are particular sensitive to alloreactive T cell responses while the outcome of SCT in aggressive B-cell lymphomas is much less favourable.[74] The major obstacles of allogeneic SCT in lymphoma have been the higher median age at diagnosis, increasing number of alternative treatment options with long progression free and overall survival and earlier studies reporting high TRM after myeloablative SCT, comparable with the findings in MM. Earlier studies using myeloablative conditioning for low-grade NHL or aggressive NHL were associated with a TRM of 25% to 44%.[75-79] RIC-SCT has offered the opportunity for SCT in patients with comorbidities and in older patients, which makes this approach applicable for more lymphoma patients even those who are heavily pre-treated. Outcomes of RIC-SCT for lymphoma show variable results. A retrospective analysis from EBMT on RIC-SCT for lymphoma reported an OS of 62% at one year but TRM still exceeded 30%.[80] Three other studies on RIC-SCT for lymphoproliferative diseases, of which two used Alemtuzumab for in vivo T cell-depletion, showed a clear reduction of TRM of 11% to 16% with an OS of 68% to 88% at two-years.[81-83]

RIC-SCT has lowered TRM in NHL, but relapse rates remain high, especially in aggressive B-cell lymphomas, heavily pretreated patients and chemotherapy-resistant NHL. Furthermore, the optimal conditioning regimen for allogeneic SCT in NHL is subject of research, especially since many patients have been heavily pre-treated. The optimal timing of allogeneic SCT is another subject to be studied, although it has become clear from all studies that chemotherapy refractory patients should probably be excluded from trials. Most published studies include small numbers of patients and included both aggressive and indolent lymphomas as well as CLL patients. Therefore prospective multicentre studies enrolling sufficient patients with distinct lymphomas are needed to define the optimal conditioning regimen and advance the field of RIC-SCT in lymphomas.

Table1: Reduced Intensity conditioning allogeneic SCT in follicular NHL

	Number of patients	T cell depletion	Conditioning regimen	TRM	Survival	Relapse rate
Robinson et al[80]	52	Partly	Various	31% (2 yrs)	65% (2 yrs)	21% (2 yrs)
Khouri et al[81]	47	No	Flu/Cy +/- Rituximab	11% (2 yrs)	88% (2 yrs)	3% (2 yrs)
Morris et al[82]	41	in-vivo Alemtuzumab	Flu/Mel	11% (3 yrs)	73% (3 yrs)	44% (5 yrs)
Faulkner et al[83]	28	in-vivo Alemtuzumab	BEAM	16% (2 yrs)	74% (2 yrs)	10% (2 yrs)
Corradini et al[84]	53	No	Flu/Cy/TT	18% (3 yrs)	66% (3 yrs)	Nr

RIC-SCT in solid tumors

The first studies evaluating allogeneic SCT in solid tumors were performed in the mid 1990s.[85-87] These studies analyzed myeloablative allogeneic SCT in patients with metastatic breast cancer. Although these trials documented a graft-versus-solid tumor effect, the treatment-related morbidity and mortality were the major drawback. Nonmyeloablative or RIC-SCT was first studied in patients with metastatic renal cell carcinoma (RCC), known to be sensitive to T cell-mediated immunotherapy. In 2000, Childs et al. reported the outcome of 19 patients with metastatic RCC after non-myeloablative SCT.[88;89] Ten out of the 19 patients showed regression of disease. This was due to a GVT effect since the responses typically occurred after the withdrawal of immunosuppression or the development of GVHD. Although this pioneer study showed promising results, response rates after allogeneic SCT for RCC in subsequent series have been highly variable ranging from 0%-57% and the rate of CR was low (*Table 2*).[89-99] In a multi-institutional study investigating nonmyeloablative SCT in patients with advanced RCC no objective responses were observed.[97] This study underscores the importance of careful patient selection for this type of treatment because responses after allogeneic SCT are typically delayed and restricted to tumors with clear cell histology.[100]

Nowadays, most patients with metastatic RCC receive initial treatment with VEGF-targeted therapies including sunitinib, sorafinib or bevacizumab plus interferon-α.[101-103] These treatments with VEGF receptor kinase inhibitors have provided a significant benefit to patients with metastatic RCC, but are not curative. Second-line strategies for patients progressing after VEGF-targeted therapies are inhibitors of the mammalian

target of rapamycin (mTOR), such as everolimus.[104] However, the magnitude of the clinical benefit is limited with an objective response rate of only 1% and a median PFS of 4.0 months. Since the introduction of these VEGF-targeted therapies and mTOR inhibitors, inclusion of RCC-patients in studies on allogeneic SCT has been almost stopped. Recently, Tykodi et al suggested combining allogeneic SCT with an mTOR inhibitor to delay post-transplant tumor progression and favoring a GVT response.[105] Nonmyeloablative and RIC-SCT have also been investigated in other metastatic solid tumors including metastatic breast cancer[87], colorectal cancer[106;107] , ovarian cancer[108] and melanoma.[109] Ueno et al. evaluated the feasibility of RIC-SCT in 23 patients with solid tumors including 8 patients with metastatic breast cancer.[99] The best responses in the 8 patients with breast cancer were 2 CRs and one mixed response. A conclusion regarding a survival benefit for patients with tumor response could not be made because of the low number of patients.

Carnevale et al. described 15 patients with metastatic colorectal carcinoma who underwent nonmyeloablative SCT.[107] One patient experienced a partial remission and three patients stable disease. Although these four responses were encouraging, no long term remissions were reached.

Published data on nonmyeloablative allogeneic SCT in metastatic melanoma are limited to reports with small numbers of patients, two reports with four and two patients respectively.[60;109] In two patients, regression of metastatic disease was observed, compatible with a GVT response.

For hematological malignancies, it is obvious that GVT reactivity is mediated by donor T cells. Studies in patients with hematological malignancies have shown that allogeneic SCT using T cell-depleted grafts resulted in an increased relapse rate [110;111] and patients with a relapse after SCT can successfully be treated with DLI.[3] For GVT reactivity against solid tumors, donor T cells are also considered to play an essential role and this is based on several observations. Regression of the tumor is typically delayed after SCT and occurs mainly after discontinuation of immunosuppression.[89] In most studies, tumor responses are preceded by conversion from mixed to complete donor T cell chimerism.[89] Only one study observed mixed chimerism in T cells at the time of disease response.[61] Furthermore, the occurrence of GVHD is strongly associated with tumor responses[89;112] and tumor responses are observed in patients treated with DLI.[112] An increased number of IFNγ-producing CD8+ T-cells in peripheral blood after SCT correlates with tumor regression, however the specificity of these CD8+ T cells was not defined.[113]

Table 2: Nonmyeloablative and reduced intensity conditioning allogeneic SCT in patients with metastatic renal cell carcinoma.

Study	Year	Patients	aGVHD	cGVHD	TRM	Response (CR and PR)
Childs et al[89]	2000	19	53%	21%	11%	53%
Rini et al[114] Artz et al[90]	2002 2005	18	22%	39%	14%	22%
Bregni et al[93]	2002	7	86%	71%	0%	57%
Pedrazolli et al[96]	2002	7	0%	Na	29%	0%
Blaise et al[92]	2004	25	42%	60%	9%	8%
Nakagawa et al[95]	2004	9	33%	44%	0%	11%
Ueno et al[99]	2003	15	47%	27%	33%	27%
Henstchke et al[94]	2003	10	50%	30%	40%	0%
Massenkeil et al[115]	2004	7	29%	57%	14%	29%
Tykodi et al[98]	2004	8	50%	50%	13%	13%
Barkholt et al[91]	2006	124	40%	33%	16%	32%
Rini et al[97]	2006	22	32%	23%	9%	0%

Antigens involved in GVT reactivity

Although RIC-SCT significantly reduced TRM, GVHD and recurrence of the malignancy remain the major drawbacks of RIC-SCT. Therefore, strategies to boost GVT reactivity selectively are even more important in the reduced intensity setting than after myeloablative allogeneic SCT. Identification of the target antigens of the GVT response is crucial to develop targeted T cell-mediated immunotherapy.

Studies analyzing immune responses after allogeneic MHC-matched SCT showed that MiHA expressed on recipient tumor cells are targets of donor T cells.[13;116;117] Furthermore, the T cell response associated with GVL is polyclonal, thus involving T cells with divers antigenic specificities.[118] More than 35 MiHA involved in GVT responses have been identified and a subset of these MiHA are restricted to hematopoietic cells including leukemic cells/tumor cells but not on GVHD tissues. Especially these hematopoietic-restricted or tumor-restricted MiHA are interesting targets for the development of tumor-specific immunotherapy.

MiHA with a potential for immunotherapy in NHL and MM

Several hematopoietic-restricted MiHA have been discovered targeting alloreactive donor T cells towards lymphoid malignancies, and some of these MiHA have even a

lymphoid or B-lineage restricted expression.

HA-1 is the first described hematopoietic-restricted MiHA. HA-1 was identified as an antigenic peptide encoded by the *KIAA0223* gene and presented in HLA-A*0201.[119] Functional assays based on CTL-mediated lysis of tissue-derived cells demonstrated that expression of HA-1 is restricted to hematopoietic cells, including leukemic and progenitor cells.[120] HA-1 is therefore an important MiHA for immunotherapy in NHL and MM.

LRH-1, a hematopoietic restricted MiHA which was identified analyzing cytotoxic T lymphocytes (CTL) from a patient with CML, was also evaluated for expression in lymphoid malignancies and MM.[116] The hematopoietic restricted MiHA LRH-1 is encoded by the *P2X5* gene and results from a single nucleotide frame-shift polymorphism between recipient and donor cells.[116] Expression of the *P2X5* gene has been demonstrated in peripheral blood T cells, B cells and NK cells as well in myeloid progenitor cells and lymphoid organs. *P2X5* expression was not detected in GVHD-target tissues such as skin, liver, colon and small intestine.[116] In a recent study, high *P2X5* expression was demonstrated in malignant cells from all stages of lymphoid development.[121] In vitro analysis showed LRH-1-specific CTL-mediated lysis of LRH-1 positive tumor cell lines and primary cell from patients with lymphoid malignancies. High *P2X5* expression was also found in MM plasma cells and cell lines resulting in efficient lysis of these MM tumor cells by LRH-1-specific CTL.

Recently, a typical B cell-restricted MiHA $CD19^{L}$ was identified, encoded by a SNP in the B cell lineage-specific CD19 gene.[122] This was the first reported MiHA with expression in HLA-class II molecules. *CD19* is a B cell lineage-specific molecule with constitutive expression in acute and chronic B cell lymphoid leukemias, B cell lymphomas but without expression in pluripotent stem cells [123] and therefore considered as a most interesting target for immunotherapy of B cell malignancies. The CD4+ T cell clone, which was used to identify this MiHA produced significant levels of IFN-gamma when tested against malignant cells from several B-CLL patients.[122] Furthermore, this CD4+ T cell clone lysed HLA-matched and $CD19^{L}$ positive B-CLL samples.

Isolation of various CTL clones from a MM patient treated successfully with DLI for relapse has been described by Kloosterboer et al.[124] One dominant CTL clone that recognized the malignant MM cells from the patient was studied more in detail.[117] The epitope recognized by this dominant CTL was designated LB-ADIR-1F. This epitope is encoded by a frequently occurring SNP in the human *ATP-dependent*

interferon-responsive (*ADIR*) gene. LB-ADIR-1F-specific T cells recognized not only MM tumor cells, but also other hematological malignancies and solid tumor cell lines.[117] Furthermore, the recognition of normal tissues was low under steady state conditions, but activation of the target cells with IFN resulted in enhanced recognition.

Another MiHA with a lymphoid-restricted expression pattern is HB-1.[125;126] HB-1 was identified using a CD8+ CTL clone derived from an allotransplanted patient with B-ALL. The first studies showed that HB-1 is expressed in tumor cells of all B cell-ALL subentities and Epstein-Barr virus (EBV) transformed B-lymphoblastoid cell lines (LCL).

PANE-1 is also a MiHA with a B-lineage restricted expression pattern.[127] The PANE-1 transcript that encodes the MiHA is expressed at high levels in resting CD19+ B cells and B-CLL cells and at significantly lower levels in activated B cells.[127]

Not only MiHA-specific T cell responses have been described after allogeneic SCT, but also donor T cell responses against recipient tumor-specific antigens have been found which may play a role in GVT reactivity.[128] Examples of tumor specific antigens, such as antigens expressed by cancer-testis genes are often found in advanced, more dedifferentiated MM. T- and B-cell responses against these tumor-specific antigens have been detected mainly in MM patients treated with allogeneic SCT.[129]

In vivo characterization of alloreactive T cell responses in patients with solid tumors

Alloreactive T cell responses have been analyzed in patients treated with allogeneic SCT for RCC[98], ovarian carcinoma[108], breast cancer[130] and melanoma.[109] However, the target antigens of these alloreactive T cells were molecularly identified in only two reports. In the first study, an HLA-A*0201-restricted MiHA was identified, which was encoded by the *C19orf48* gene located on chromosome 19q13.[131] This MiHA was identified using CTL clones from two patients with metastatic RCC who were treated with allogeneic SCT and achieved near complete response and stable disease with long term survival, respectively. Assays for gene expression showed that C19orf48-encoded peptide is widely expressed in renal tumors and tumor cell lines derived from AML, ALL, CLL, melanoma, ovarian carcinoma, breast carcinoma, prostate carcinoma, pancreatic carcinoma and colon carcinoma.

In a second study, a new tumor-associated RCC antigen was identified by using PBMCs obtained from a patient with disease regression and prolonged survival after allogeneic SCT for RCC.[132] The target antigen is a 10-mer peptide encoded by

genes from human endogenous retrovirus (HERV) type E. An earlier study showed that some HERVs have expression widely in normal tissues, but HERV-E genes are selectively expressed in RCC.

One study in patients with metastatic colorectal carcinoma (CRC) analyzed tumor-specific T cell activity after nonmyeloablative SCT focusing on T cell responses directed against carcinoembryonic antigen (CEA), a tumor-associated antigen over-expressed by CRC cells.[107] Induction of CEA-specific T cell-responses required the presence of GVHD and but also the presence of CEA-expressing tumor cells.

Hematopoietic-restricted MiHA expressed by solid tumor cells

Several MiHA have been described with expression restricted to hematopoietic cells and no expression in normal tissues. These MiHA are designated hematopoietic-restricted MiHA or tumor-associated MiHA and these MiHA may be useful for adoptive immunotherapy. Interestingly, some of these hematopoietic-restricted MiHA are also expressed in solid tumor cells (*Table 3*).

The first hematopoietic-restricted MiHA with aberrant expression in solid tumors was HA-1. HA-1 mRNA expression was shown in tumor cell lines from different types of carcinomas including breast, melanoma, lung, renal cell, colon and head and neck cancer.[12] In vitro studies demonstrated that epithelial tumor cell lines were lysed by HA-1-specific CTL, whereas normal epithelial cells were not recognized.[12] Moreover, an HA-1-specific CTL has been isolated from a RCC-patient with a partial response after SCT, and also the cytolytic activity of this HA-1-specific CTL against renal cell carcinoma tumor cell lines was demonstrated.[98] These findings indicate that MiHA HA-1 is a suitable target for MiHA-based immunotherapy of both hematological malignancies and solid tumors.

LB-ECGF-1H was identified using tumor-reactive cytotoxic CD8+ T cell clones from a patient successfully treated with DLI for relapsed MM after allogeneic SCT.[13] This MiHA is encoded by the angiogenic *endothelial-cell growth factor-1* (*ECGF-1*) gene and is presented by HLA-B7. Significant LB-ECGF-1 mRNA levels were detected in hematological malignancies and solid tumor cell lines such as melanoma, breast carcinoma, colon carcinoma and ovarian carcinoma.

BCL2A1 is a gene that encodes for two MiHA, ACC-1 and ACC-2.[10] The two CTL specific for these MiHA lyse both normal and malignant hematopoietic cells. Expression of *BCL2A1* was recently demonstrated in melanoma cell lines and primary melanoma cells, with an expression comparable to that of hematopoietic

cells.[133] Melanoma cell lines were efficiently lysed by cytotoxic T lymphocytes specific for ACC-1 and ACC-2.

Furthermore, we have found aberrant *P2X5*-expression in 30% of the solid tumor cell lines tested (including RCC, colorectal carcinoma, breast cancer and brain cancer) and lysis of LRH-1 positive tumor cell lines by the LRH-1-specific CTL.[134]

Table 3: Hematopoietic-restricted MiHA with expression on solid tumor cells

MiHA	MHC Restriction	Gene	Chromosome	Tissue-distribution
ACC-1	HLA-A*24	BCL2A1	15	Hematopoietic
ACC-2	HLA-B*4403	BCL2A1	15	Hematopoietic
HA-1	HLA-A*0201	KIAA0223	19	Hematopoietic
LB-ECGF-1	HLA-B*07	ECGF-1	22	Hematopoietic
LRH-1	HLA-B*0702	P2X5	17	Hematopoietic

MiHA-based immunotherapy of hematopoietic malignancies and solid tumors

Identification of tumor-specific MiHA is crucial for further development of specific GVT post-transplantation immunotherapy. Strategies to employ MiHA are based on vaccination strategies with peptides alone or loaded on dendritic cells or adoptive transfer of MiHA-specific T cells.

Adoptive transfer of MiHA-specific T cells

Two central questions regarding this therapeutic concept have been addressed by Hambach et al. in a murine model.[135] Firstly, they show that CTLs directed against a single MiHA can eradicate human solid tumors in a highly MiHA-specific manner. Secondly, HA-1-specific CTL prevents in vivo human breast cancer metastases in immunodeficient mice. Mutis et al. showed that it is feasible to generate HA-1 and HA-2 specific CTLs from HA-1 and/or HA-2 negative donors using HA-1 and HA-2 peptide-pulsed dendritic cells.[136] These ex vivo-generated HA-1 and HA-2 specific CTL have specific cytotoxic activity against MiHA-positive target cells, including AML and ALL cells, but not against non-hematopoietic cells. Recently, Warren et al.

published a study on the adoptive transfer of donor T cells that recognize recipient MiHA in seven patients with recurrent leukemia after MHC-matched allogeneic SCT. These CD8+ CTL clones recognized MiHA expressed in recipient hematopoietic cells but not recipient dermal fibroblasts. Five of the seven patients reached complete although transient remissions. However, three patients developed pulmonary toxicity, which correlated with expression of the MiHA-encoding genes in lung tissue.[137] This study demonstrates that MiHA-specific T cells can be isolated and expanded resulting in remissions, however the antileukemic effects did not persist possibly due to exhaustion of the T cells.[138] Unfortunately, selecting T cells that recognize MiHA expressed on recipient hematopoietic cells and with no recognition of skin fibroblasts did not prevent pulmonary GVHD. Therefore the use of T cells against characterized MiHA might be safer and better predict separation GVT from GVHD.

Vaccination strategies

DCs are the professional APC of the immune system and have a great potential to initiate T cell responses. DCs can be loaded with antigens, such tumor associated antigens, and administered to patients which is called DC vaccination. Most clinical studies using DC vaccines have been performed in patients with non-hematological malignancies, for example melanoma and colorectal cancer patients.[139;140] These studies have shown that DC vaccination is feasible and safe. However, several questions are still under investigation such as the optimal mode of DC preparation, method and timing of antigen loading, dose and interval of administration and the route of administration.[141]

DC vaccination after allogeneic SCT and DLI offers two new dimensions of this strategy. First, both recipient-derived DC and donor-derived DC can be applied and not only tumor associated antigens but also MiHA can be used as antigen. T cell responses after allogeneic SCT targeting MiHA on malignant cells of the recipient can be induced directly by recipient-derived DC and indirectly by donor-derived DC due to cross-presentation.[142] In the setting of DC-vaccination as post-transplantation immunotherapy, two strategies can be applied. First, boosting GVT immunity by vaccination with donor-derived DC loaded with hematopoiesis-restricted or tumor-restricted MiHA, but this approach is hampered by the limited number of known MiHA and their HLA restriction. Another approach is vaccination with recipient DC, which are not loaded with specific antigens. Since recipient DC and hematopoietic tumor cells are both derived from the hematopoietic system, immune responses

induced by recipient-derived DC may enhance GVT in hematopoietic malignancies. Several strategies have been studied to load DC with antigens, such as electroporation of in vitro transcribed mRNA into DC.[143-145] A recent study demonstrated that DC electroporated with MiHA LRH-1 encoding mRNA can efficiently stimulate CD8+ T cells, both early effector T cells and late memory T cells.[146] Feasibility of MiHA-based DC vaccination strategies after allogeneic SCT will be further explored in clinical studies.

Scope of this thesis

The scope of this thesis is further exploration of allogeneic SCT as immunotherapy for hematological malignancies and solid tumors.

In *chapter 2* we studied the role of recipient-derived professional APC in the development of GVT responses after tDLI for relapse CML. Exploration of the role of APC in inducing GVT immunity may help to develop more effective therapeutic interventions for patients with relapse after SCT. Early after transplant and when patients relapse, recipient-derived APC may be present and direct presentation of antigens co-expressed by recipient APC and malignant cells can enhance GVT reactivity. If the recipient hematopoiesis is completely replaced by donor-derived cells, recipient antigen presentation is taken over by donor-derived APC via cross-presentation. The more sensitive real-time quantitative PCR method using SNP as markers was used to analyze chimerism in subsets of professional APC.

In *chapter 3* we studied the outcome of pre-emptive DLI after partial T cell-depleted myeloablative allogeneic SCT in MM. These patients were treated according the HOVON-24 study[147], however only patients transplanted in our centre received partially T cell-depleted transplants and pDLI. The high TRM has been a major obstacle for myeloablative allogeneic SCT in MM and nonmyeloablative conditioning or RIC regimens have been increasingly used since then. Based on the results described in *chapter 3*, we developed a new approach applying partial T cell-depleted SCT followed by pDLI in the RIC-setting. This study is described in *chapter 4*. Furthermore, as a new approach we incorporated recipient-derived dendritic cell vaccination as post-transplantation immunotherapy aiming at efficient direct antigen presentation to donor T cells. In *chapter 5*, we studied partial T cell-depleted RIC-SCT followed by pDLI in patients with relapsed lymphoma and CLL. Both patients with relapsed aggressive lymphomas (transformed lymphomas) and

indolent lymphomas were included.

In the past years, allogeneic SCT has been studied as immunotherapy in patients with metastatic solid tumors. Although there are clear signs for GVT reactivity, these GVT responses are often accompanied by GVHD. Furthermore, most responses are partial and long-term complete remissions are rare. Further characterization of known MiHA or identification of new hematopoietic or tumor-restricted MiHA may play a role in the development of MiHA-targeted post-transplantation immunotherapy.

In *chapter 6* we studied the aberrant expression of *P2X5*, the gene encoding for the MiHA LRH-1, in solid tumor cells. Expression of the *P2X5* gene was demonstrated before in cells of lymphoid origin and lymphoid tissues.[116] High *P2X5* expression was found in a broad range of lymphoid malignancies, including ALL, CLL, various types of B cell lymphoma and MM.[121] Furthermore, *P2X5* is not expressed in the GVHD-tissues such as skin, liver, colon and small intestine. Based on these observations the *P2X5*-encoded MiHA LRH-1 could be a suitable target for immunotherapy, also for allogeneic immunotherapy in patients with solid tumors. In *chapter 7* we studied the feasibility of partial T cell-depleted RIC-SCT followed by pDLI in patients with metastatic RCC. This approach combined partial T cell depletion for the reduction of GVHD with pDLI to boost GVT responses in the RIC-setting. Furthermore, we aimed at the isolation of RCC-reactive cytotoxic CD8+ T cell clones from these patients and the discovery of a new MiHA.

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Chapter2

Dynamics in chimerism of T cells and dendritic cells in relapsed CML patients and the influence on the induction of alloreactivity following donor lymphocyte infusion

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Abstract

Donor lymphocyte infusion (DLI) after allogeneic SCT induces complete remissions in approximately 80% of patients with relapsed CML in chronic phase, but some patients do not respond to DLI. We studied absolute numbers of dendritic cell (DC) subsets and chimerism in T cells and two subsets of blood DCs (myeloid DCs (MDC) and plasmacytoid DCs (PDC)) in relation to DLI-induced alloreactivity. Based on T cell and DC chimerism, we identified three groups. Four patients were completely donor chimeric in T cells and DC subsets. These patients had an early stage of relapse, and three of the four patients attained complete molecular remission (CMolR) without significant GVHD. Six patients were complete donor in T cells and mixed chimeric in DC subsets. All patients entered CMolR, but this was associated with GVHD in four and cytopenia in three patients. Five patients had mixed chimerism in T cells and complete recipient chimerism in MDC; only two patients entered CMolR. Our data suggest that the combination of donor T cells and mixed chimerism in DC-subsets induces a potent graft-versus-leukemia (GVL) effect in association with GVHD. DLI in patients with an early relapse and donor chimerism in both T cells and DC-subsets results in GVL reactivity without GVHD.

Introduction

Donor lymphocyte infusion (DLI) is used to boost graft-versus-leukemia (GVL) reactivity in patients with a relapse after allogeneic SCT. Following DLI, complete molecular remission (CMolR) has been obtained in 70-80% of patients with a relapse of CML in chronic phase.[1] In contrast, patients with a CML in accelerated phase or blast crisis, patients with relapsed acute leukemia and patients with relapsed or persistent multiple myeloma (MM) respond in only 10-40% of cases.[2] The mechanisms responsible for the different DLI-mediated anti-tumor responses between various disease types are still unclear. Differences in tumor characteristics such as growth rate, susceptibility to T cell-mediated cytotoxicity, secretion of immune-modulatory cytokines and expression of co-stimulatory molecules may have a major impact on the response.[3-5] Another important factor that may contribute to the response rate is the chimerism in immune effector and professional antigen presenting cells (APC) at the time of DLI. Murine studies showed that recipient APC play a key role in the initiation of allogeneic CD8+ T cell-mediated GVH and GVL reactivity after MHC-matched SCT.[6;7] In addition, in an MHC-mismatched mouse model, it has been shown that DLI administration to mixed chimeras produced improved leukemia-free survival compared to administration of DLI to full donor chimeras.[8] From these data, it can be hypothesized that the presence of recipient APC may be an important factor for the induction of DLI-induced GVL reactivity.

Dendritic cells (DC) are the most potent bone marrow-derived APC that play a pivotal role in inducing primary immune responses. In human blood, DC precursors constitute less than 1% of lineage-negative human leukocyte antigen (HLA)-DR+ mononuclear cells, and are commonly divided into two distinct subsets, namely myeloid DC (MDC) and plasmacytoid DC (PDC). MDCs have a monocytoid appearance and express myeloid antigens such as CD11c, CD13 and CD33. In contrast, PDC lack myeloid markers, but express CD123 (interleukin-3 receptor-α), and have a plasma cell-like morphology.[9;10] MDC and PDC can be distinguished in peripheral blood using the monoclonal antibodies BDCA-1 (CD1c) for MDC, and BDCA-2 and BDCA-4 for PDC.[11] Emerging evidence suggests that MDC and PDC play an important role in alloimmune responses after SCT.[12]

In this study, we evaluated the absolute number and chimerism of peripheral blood T cells, MDC and PDC in patients with a relapse from CML before the administration of donor lymphocytes. Our hypothesis, based on animal models, is that the presence of recipient MDC and/or PDC is associated with the induction of a more efficient

GVL reaction.

Patients and methods

Patients and donors

Chimerism of hematopoietic cell populations was studied in 15 CML patients with a relapse after partially T cell-depleted allogeneic SCT. In five patients (two with molecular relapse and three with cytogenetic relapse), remission after SCT was confirmed with a negative quantitative PCR for BCR-ABL. In five patients, PCR was performed, but not on a quantitative level. In five other patients, no PCR was performed between SCT and relapse.

These relapsed CML patients received DLI, and were selected on the availability of cryopreserved peripheral blood mononuclear cell (PBMC) samples obtained shortly before DLI. DLIs were given in a period from January 1996 until November 2003. All patients were treated initially with allogeneic SCT for CML in first chronic phase. The median duration between diagnosis and SCT was 9 months (range=3-17). The median age of patients was 40 years (range=26-51) at the time of SCT. Stem cell donors were HLA-identical siblings in 11 of 15 patients. One patient received G-CSF mobilized PBSCs, and the other 14 patients received bone marrow stem cells. One patient received bone marrow stem cells from a one HLA class I locus-mismatched sibling. Two patients were transplanted with stem cells from HLA-identical unrelated donors and one patient received stem cells from a phenotypically identical father. The median age of the donors was 36 years (range=19-70) at the time of stem cell donation. Clinical characteristics of patients and donors are shown in *Table 1*.

Conditioning regimen and GVHD prophylaxis

The standard conditioning consisted of cyclophosphamide 60 mg/kg body weight intravenously on each of two consecutive days followed by fractionated total body irradiation (TBI) in two equal fractions of 4.5 Gy on 2 days. In nine patients, the conditioning regimen was intensified with the addition of idarubicin to a total dose of 42 mg/m^2 body surface given by continuous intravenous infusion. Two patients transplanted with stem cells from an unrelated donor received anti-thymocyte globulin (thymoglobulin, Genzyme Europe, Naarden, The Netherlands) 2 mg/kg intravenously on each of four consecutive days in addition to the standard regimen. In the patients receiving stem cells from a one locus-mismatched or a haplo-identical

donor, the conditioning regimen consisted of cyclophosphamide, TBI plus total lymphoid irradiation 2 x 2 Gy on each of the two consecutive days. All patients received cyclosporine A for GVHD-prophylaxis. The grafts were partially depleted of T cells by counterflow centrifugation as described before.[13] Further details on conditioning, T cell content and on the quality of the grafts are given in *Table 1*.

Table 1: Transplantation characteristics

	CML (n=15)
Patients	
Recipient male/female	7 / 8
Donor male /female	8 / 7
Median recipient age, years (range)	40 (26-51)
Median donor age, years (range)	36 (19-70)
Conditioning	
Cyc, TBI	2
Cyc, TBI, Ida	9
Cyc, TBI, ATG	2
Cyc, TBI, TLI	2
Graft	
Median CD3+ cells infused x 10^6/kg (range)	0.7 (0.3-0.9)
Median CFU-GM infused x 10^4/kg (range)	10.0 (2.8-21.2)
Median CD34+ cells infused x 10^6/kg (range)	1.6 (0.7-3.2)

Abbreviations: ATG, anti-thymocyte globulin; Cyc, cyclophosphamide; Ida, idarubicin; TBI, total body irradiation; TLI, Total lymphoid irradiation.

Infusion of donor lymphocytes

DLI was performed as described before.[14] Briefly, donor lymphocytes were obtained from the original stem cell donor by leukapheresis using the blood cell separator Baxter CS 3000 (Baxter, Deerfield, IL, USA) or the Fresenius AS 104 (Fresenius, Oberursel, Germany). Lymphocytes were administered to the patient within 3 hours of completion of the leukapheresis procedure. No GVHD prophylaxis was administered after the infusion of the donor lymphocytes. If a patient did not respond to the first DLI, a second or third DLI was given in escalating doses of T lymphocytes. The first dose ranged from 0.1 to 0.7 x 10^8 CD3+ cells/kg for patients

with a hematological or cytogenetic relapse. The second dose ranged from 0.7 to 1.0 x 10^8 CD3+ cells/kg. For patients with an unrelated donor, the first dose ranged from 0.05 to 0.1 x 10^8 CD3+ cells/kg. Median time between relapse and the first DLI was 1.4 months (range=0,2-39.1). There was a long interval of 39.1 months in the patient transplanted with stem cells from a phenotypically identical farther, because this patient had a history of acute and chronic GVHD and aspergillus infection at the time of relapse.

Cytogenetic analysis and quantification of BCR-ABL-positive cells

Chromosome studies on peripheral blood and bone marrow cells were performed after SCT and DLI as described before.[15] In sex-mismatched donor-patient pairs, heterosome determination of 400 interphases with fluorescence in situ hybridization (FISH) allowed additional differentiation between donor and recipient cells. If more than five autologous marrow interphases were found, bone marrow cells were analyzed for the presence of the Philadelphia chromosome using BCR-ABL probes. For quantification of BCR-ABL-positive cells, total RNA from peripheral blood cells was extracted using Trizol (Invitrogen, Carlsbad, CA, USA). Real-time PCR analysis was performed on an ABI/Prism 7700 system (Applied Biosystems, Foster City, Ca, USA) as described previously.[16] Expression of the porphobilinogen deaminase (*Pbgd*) gene was used to normalize BCR-ABL expression. This normalized BCR-ABL expression in patient samples was related to a standard curve obtained from K562 cells diluted into normal bone marrow cells. The limit of detection of BCR-ABL PCR is $1x10^{-5}$.

Quantification of DC subsets

Blood samples were obtained from patients shortly before DLI. PBMC were isolated by Ficoll-Paque (Pharmacia, Uppsala, Sweden), cryopreserved and stored in liquid nitrogen until use. DC subsets were enumerated using thawed PBMC by 3-color immunofluorescence analysis. The following mAbs were used: FITC-conjugated anti-CD45 (J33), anti-CD14 (TÜK4) (Beckman Coulter, Mijdrecht, The Netherlands), PE-conjugated anti-CD1c (BDCA-1, AD5-8E7), BDCA-2, BDCA-4 (AC144 and AD5-17F6, Miltenyi Biotech, Bergisch Gladbach, Germany), Cy5-conjugated anti-CD19 (J4.119), and anti-CD45 (J33) (Beckman Coulter). PBMC were incubated with the appropriate concentration of mAb in phosphate-buffered saline (PBS) supplemented with 20% pooled human serum and 0.1% NaN_3 (4°C for 30 min). Cells were washed in PBS/1% bovine saline albumin and analyzed using a Coulter

XL flow cytometer (Beckman Coulter, Fullerton, CA, USA). MDCs and PDCs were identified as CD45+BDCA-1+CD19- and CD45+BDCA-2/4+CD14- mononuclear cells, respectively. Absolute number was calculated by multiplying the percentage of MDC and PDC with the total number of mononuclear cells per liter.

Purification of PBMC subsets

PBMC subsets were isolated using a two-step flow cytometric cell-sorting procedure that allows purification of five different subsets. PBMC were labeled with a mixture of Cy5-conjugated mAb against the lineage markers CD2 (39C1.5), CD20 (B9E9), CD15 (80H5) (Beckman Coulter), and FITC-conjugated anti-CD11c (KB90, DAKO, Glostrup, Denmark) mAb. Labeled cells were sorted into three fractions, that is CD11c+Lin-, CD11c-Lin- and Lin+ cells, using the Coulter Epics Altra hypersort flow cytometer (Beckman Coulter). These pre-sorted cell fractions were subsequently stained with the appropriate concentration of PE, Cy5 and ECD-conjugated mAb and different subsets were sorted using the Coulter Epics Elite flow cytometer (Beckman Coulter). The Lin+ cell fraction was used to isolate CD3+ T cells (CD3-ECD, UCHT1, Beckman Coulter) and CD13.CD33+ myeloid cells (CD13-PE, SJ1D1 and CD33-PE, D3HL60.251, Beckman Coulter). MDC were isolated from the CD11c+Lin- cell fraction based on expression of BDCA-1 (BDCA-1-PE, AD5-8E7, Miltenyi Biotec) and negativity for CD19 (CD19-Cy5, J4.119, Beckman Coulter). PDCs were isolated from the CD11c-Lin- cell fraction based on expression of BDCA-2 (BDCA-2-PE, AC144, Miltenyi Biotec) and BDCA-4 (BDCA-4-PE, AD5-17F6, Miltenyi Biotec) and negativity for CD14 (CD14-Cy5, RMO52, Beckman Coulter). An aliquot of sorted cells was reanalyzed and purity was more than 95% in all cases.

Quantification of the percentage recipient and donor cells in PBMC subsets

Real-time quantitative PCR of single nucleotide polymorphisms (SNP) and/or the *SMCY* gene was used for the quantification of donor and recipient hematopoietic cells, as described previously.[17;18] Briefly, recipient/donor pairs were screened for discriminating SNPs. Quantification is based on real-time PCR with allele-specific primers for DNA sequences containing the discriminating SNP and target DNA-specific probes. Quantitative analysis is performed by generating calibration functions from cycle thresholds (C_t) obtained by real-time PCR of DNA serially diluted in water. Detection limit for T cells was 0.1 –1 % and for DC subpopulations 1 – 10%, depending on the number of isolated cells.

Definitions

Complete donor chimerism in T cells, myeloid cells and monocytes was defined as <1% recipient cells among donor cells, and complete recipient chimerism as <1% donor cells among recipient cells. For DC subpopulations, complete donor chimerism was defined as <10% recipient cells among donor cells, and complete recipient chimerism as <10% donor cells among recipient cells. Mixed chimerism in T cells, myeloid cells and monocytes was defined as ≥ 1% recipient cells and ≥ 1% donor cells. For DC subpopulations, mixed chimerism was defined as ≥ 10% recipient cells and ≥ 10% donor cells. Higher thresholds were chosen for DC-subsets because the amount of input DNA obtained from MDC and PDC was lower than from T cells, myeloid cells and monocytes. This lower input of DNA resulted in a lower ΔC_t, and for some samples the limit of detection was <10%.

Acute and chronic GVHD were classified grade I–IV and limited or extensive, respectively, according to the criteria described by Glucksberg et al. and Shulman et al.[19;20] GVHD after DLI was classified as acute GVHD for the first three months after DLI. Cytopenia after DLI was defined as a white blood cell count of $<1.0x10^9$/l and/or platelet counts of $<15x10^9$/l, and/or a hemoglobin-level of <6.0 mmol/l.

Hematological remission of CML was defined as disappearance of all signs and symptoms of disease and normalization of blood cell counts and cellularity of the bone marrow. Complete cytogenetic response (CCyR) was defined as the disappearance of the Philadelphia chromosome in the bone marrow. Complete molecular remission (CMolR) was defined as a real-time PCR for the BCR-ABL fusion transcript under the detection level at ≥ 2 consecutive points after DLI. Accelerated phase was defined according to the WHO criteria.

Results

Response of CML following DLI

Nine of the 15 patients had a hematological relapse at the time of first DLI with a median interval of 15 months (range=6-64) after SCT (*Table 2*). Six of the nine patients attained CMolR after first DLI with a T cell dose ranging from 0.1 to $0.7x10^8$/kg. Two patients (UPN 306 and UPN 389) did not respond to the first DLI ($0.1x10^8$ T cells/kg), but obtained CCyR after a second DLI (0.7 and $0.9x10^8$ T cells/kg, respectively). Only one patient (UPN 396) did not respond to DLI ($0.7x10^8$/kg and $1.0x10^8$ T cells /kg).

Six patients were in an accelerated phase, including the non-responding patient and the two patients obtaining CCyR. In four patients, the presence of an accelerated phase is based only on cytogenetic abnormalities, however in three of these four patients we found del (13) which is typical for CML. One patient had 12% blasts in bone marrow and multiple structural cytogenetic abnormalities, and one patient presented with thrombocytosis unresponsive to therapy (treatment with hydroxyurea).

Four patients had a cytogenetic relapse at the time of first DLI with a median interval of 24 months (range=10-37) after SCT. Two of these four patients attained CMolR after first DLI (*Table 2*). Patient UPN 522 did not respond to the first DLI ($0.1x10^8$ T-cells/kg) and progressed to hematological relapse. But, this patient obtained CMolR after a second DLI ($0.7x10^8$/kg). Patient, UPN 336 achieved CCyR after the first DLI ($0.1x10^8$/kg). This patient did not receive a second DLI because of comorbidity. One patient with a cytogenetic relapse had an additional deletion 18 in 3 of the 10 studied bone marrow cells, indicative of acceleration.

Two patients received DLI for a molecular relapse at 10 and 39 months after SCT. Patient UPN 489 did not respond to first DLI ($0.1x10^8$ T cells /kg), but obtained a CMolR after second DLI ($0.7x10^8$ T cells /kg). Patient UPN 681 obtained a CMolR after first DLI ($0.05\ x10^8$/kg).

Overall, 11 of 15 (73%) relapsed CML patients attained CMolR following DLI. Eight patients showed symptoms of GVHD and six patients developed cytopenia. Blood counts normalized spontaneously between one week and 5 months (median=1 month) in four patients, but two patients received a stem cell boost from the original stem cell donor. The three patients reaching CCyR only developed a second relapse of CML, 1 to 4 years after this CCyR.

GVHD following DLI

After DLI, 8 of 15 CML patients developed acute GVHD. Three patients developed grade III, one patient grade II and four patients grade I. Factors known to be associated with the occurrence of GVHD after DLI are T cell dose, donor type and time between SCT and DLI.[21;22] The mean number of T cells/kg (first and eventually second DLI) did not differ between the group of patients with GVHD and without GVHD; $0.5x10^8$/kg versus $0.4x10^8$/kg. But, only one patient developed GVHD grade I after a DLI-dose of $0.1x10^8$/kg. All patients with GVHD > grade I were treated with a high T cell dose. Mean number of T cells/kg in the group of patients with GVHD > grade I was $0.6x10^8$/kg versus $0.4x10^8$/kg in the patients with GVHD ≤ 1 (calculated

on the first and eventually second DLI).

The doses of the first DLI's were low in patients with an unrelated and haplo-identical donor, and these three patients did not develop GVHD. The patient receiving lymphocytes (0.7 x 10^8 T cells/kg) from a one locus-mismatched donor developed grade III GVHD.

For the group with GVHD, the median time between SCT and DLI-1 was 17 months (range=9-37). For the patients without GVHD, the median time between SCT and DLI-1 was 24 months (range=9-64) months (no significant difference, P=0.41 according to the Mann–Whitney test).

Table 2: Disease status at the time of first and second (in non-responding patients) DLI, interval SCT-DLI, T cell dose of DLI and response to DLI.

UPN	Disease status at 1st DLI	Donor	Interval SCT-DLI (months)	DLI dose (x10^8/kg)	GVHD after DLI	Response to DLI
337	Hem Rel, acc	HLA-id	13	0.4	2	CMolR
354	Hem Rel, acc	HLA-id	20	0.2	1	CMolR
406	Hem Rel	Mismatch	23	0.7	3	CMolR
407	Hem Rel	HLA-id	37	0.7	3	CMolR
474	Hem Rel, acc	VUD	22	0.1	0	CMolR
306	Hem Rel	Phen-id Father	64	0.1	0	no
			68	0.9	0	CCyR
389	Hem Rel, acc	HLA-id	10	0.1	0	no
			12	0.7	1	CCyR
396	Hem Rel, acc	HLA-id	9	0.7	0	no
			11	1.0	0	no
387	Hem Rel, acc	HLA-id	9	0.4	1	CMolR
447	Cyt Rel, acc	HLA-id	37	0.1	1	CMolR
459	Cyt Rel	HLA-id	10	0.7	3	CMolR
522	Cyt Rel	HLA-id	24	0.1	0	no
			29	0.7	0	CMolR
336	Cyt Rel	HLA-id	26	0.1	0	CCyR
489	Mol Rel	HLA-id	39	0.1	0	no
			43	0.7	0	CMolR
681	Mol Rel	VUD	10	0.05	0	CMolR

Abbreviations: UPN, unique patient number; Hem rel, hematological relapse; acc, accelerated phase; Cyt Rel, Cytogenetic relapse; Mol Rel, Molecular relapse; HLA-id, HLA-identical; Mismatch, one locus mismatched; VUD, voluntary unrelated donor; Phen. id, phenotypically-identical; Interval SCT-DLI, interval between SCT and DLI in months; DLI dose, number of CD3+ T cells x 10^8/kg; CMolR, complete molecular remission; CCyR, complete cytogenetic remission.

Pre-DLI T cell chimerism and disease response

Previously, we showed that the percentage of T cells from donor origin at the time of DLI correlates with response, and that the presence of significant numbers of recipient T cells is unfavorable.[23] Therefore, we analyzed T cell chimerism in these 15 CML patients.

Five patients had mixed T cell chimerism at the time of first or second DLI (*Table 3*) and only two attained CMolR (40%). The remaining 10 patients had complete T cell donor chimerism at the time of first or second DLI, and all but one obtained a CMolR (90%, P=0.07; Fisher's exact test). These data suggest that in relapsed CML patients the presence of autologous T cells indicates reduced effectiveness of DLI.

Pre-DLI T cell and DC chimerism

To analyze the role of recipient-derived DC, we divided the patients with donor T cells in a group with donor chimerism in MDC and PDC (group 1) and a group with the presence of recipient DC (group 2) (*table 3*). The four patients in group 1 had either a molecular (n=2) or a cytogenetic relapse (n=2), indicating an early stage of relapse. Three of four patients entered CMolR after 1 or 2 DLI's and only one of these four patients developed GVHD grade I. None of these patients developed chronic GVHD nor cytopenia.

The six patients in group 2 were complete donor chimeric in T cells and mixed chimeric in MDC and/or PDC. One patient had a cytogenetic relapse and five patients had a hematological relapse. Despite the advanced stage of disease, all patients entered CMolR. However, four of these six patients (66%) developed aGVHD and three of six patients (50%) developed chronic GVHD. The combination of GVL with GVHD may indicate that the DLI-mediated alloreactivity is stronger in group 2 compared to group 1. Furthermore, three patients developed pancytopenia. The cytopenia may be explained by the eradication of the malignant clone and the slow recovery of the normal donor hematopoiesis. Moreover, cytopenia is indicative for the tumor load and a low number of donor hematopoietic cells.[24]

Interestingly, in one of the patients of group 2 (UPN 406), we could sort enough DC to perform FISH-analysis using probes for BCR-ABL. This patient had mixed chimerism in both MDC (68% recipient derived) and PDC (37% recipient derived). In the MDC subset, 8 of 43 cells (18%) were BCR-ABL positive and in the PDC subset 39 of 164 cells (23%) were BCR-ABL positive, indicating that part of the recipient-derived MDC and PDC originates from malignant CML progenitor cells.

Table 3: Chimerism in T cells, DC-subsets and myeloid cells in the patients prior to first and second (in non-responding patients) DLI.

UPN	T cells	MDC	PDC	Myeloid	Abs MDC x 10^6/l	Abs PDC x 10^6/l	Response	GVHD Acute/ chronic	Cyto-Penia	Relapse	Interval SCT -DLI	T cell Dose
Group 1												
681	D	D	D	D	5,8	3,5	CMolR	0/0		Mol Rel	10	0.05
489	D	D	D	M	15,4	2,5	no	0/0		Mol Rel	39	0.1
	D	D	D	M	11,3	1.0	CMolR	0/0		Mol Rel	43	0.7
336	D	D	D	M	11,6	1,7	CCyR	0/0		Cyt Rel	26	0.1
447	D	D	D	M	9,1	3,3	CMolR	1/0		Acc Rel	37	0.1
Group 2												
337	D	D	M	M	4,4	1,4	CMolR	2/E	yes	Acc Rel	13	0.4
522	D	M	M	M	7.0	1,2	no	0/0		Cyt Rel	24	0.1
	D	M	nc	M	4,6	2,5	CMolR	0/0		Hem Rel	29	0.7
354	D	M	M	M	3,1	2,0	CMolR	1/0	yes	Acc Rel	20	0.2
406	D	M	M	M	10,8	5,9	CMolR	3/E		Hem Rel	23	0.7
407	D	M	M	M	6,9	5,3	CMolR	3/L		Hem Rel	37	0.7
474	D	R	R	M	2,6	3,0	CMolR	0/0	yes	Acc Rel	22	0.1
Group 3												
306	D	R	R	R	5,8	9,9	no	0/0		Hem Rel	64	0.1
	M	nc	R	M	7,7	53	CCyR	0/0	yes	Hem Rel	68	0.9
459	M	D	D	M	3,1	4,6	CMolR	3/L		Cyt Rel	10	0.7
387	M	R	R	R	0,7	0,7	CMolR	1/0	yes	Acc Rel	9	0.4
389	M	nc	D	M	9,7	2,2	no	0/0		Acc Rel	10	0.1
	M	R	D	R	14,6	1,5	CCyR	1/L	yes	Acc Rel	12	0.7
396	M	R	nc	R	4,2	1,4	no	0/0		Acc Rel	7	0.7
	nc	nc	nc	nc	0,7	0,7	no	0/0		Acc Rel	11	1.0

Abbreviations: UPN, unique patient number; MDC, myeloid dendritic cells; PDC, plasmacytoid dendritic cells; Myeloid, myeloid cells; Abs MDC, absolute number of myeloid dendritic cells; Abs PDC, absolute number of plasmacytoid dendritic cells; Interval SCT-DLI, interval between SCT and DLI in months; T cell dose, number of CD3+ T cells x 10^8/kg; D, complete donor chimerism; M, mixed chimerism, R, complete recipient chimerism; Hem rel, hematological relapse; Acc Rel, relapse in accelerated phase, Cyt Rel, Cytogenetic relapse; Mol Rel, Molecular relapse; CMolR, complete molecular remission; CCyR, complete cytogenetic remission; nc, no cells.

The third group consists of five patients with mixed chimerism in the T cells at the time of the first or second DLI. The clinical presentation at the time of the first DLI

was a cytogenetic relapse in one patient and a hematological relapse in four patients. Four patients were complete recipient chimeras in the MDC subpopulation. One patient had donor MDC in combination with mixed chimerism in T cells. Two of these five (40%) patients reached CMolR in combination with GVHD. One patient did not respond to DLI and two patients were temporary responders with a second relapse 1.5 and 4 years after the first DLI.

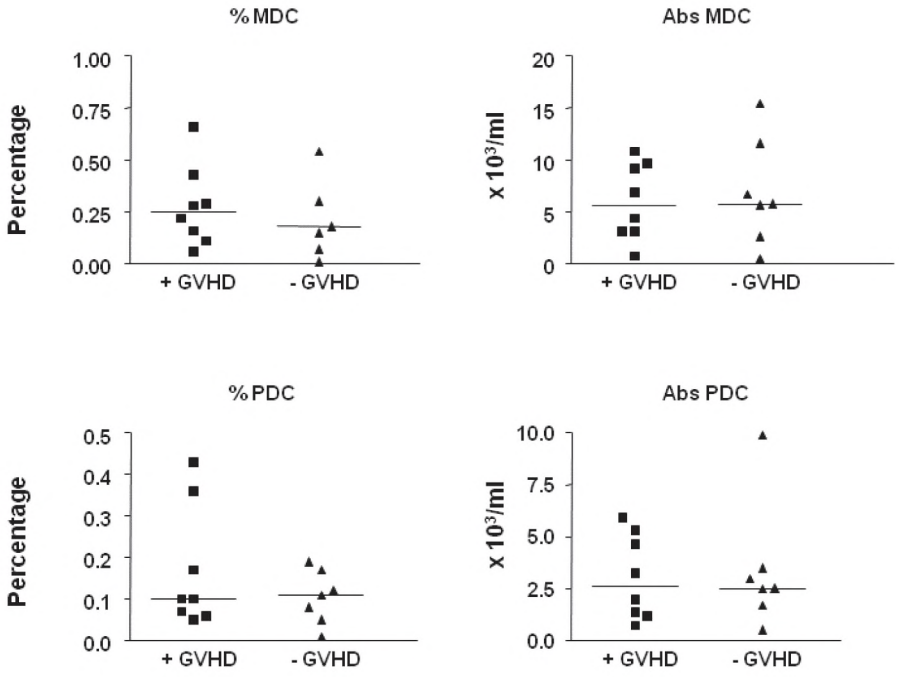

Figure 1: Percentage and absolute number of MDC and PDC in peripheral blood at the time of first DLI in patients with and without GVHD. The median level is shown by the thick line. GVHD, graft-versus-host disease; MDC, myeloid dendritic cell; PDC, plasmacytoid dendritic cell.

Dendritic cell numbers at the time of DLI

To investigate the correlation between the absolute numbers of MDC and PDC and DLI-induced alloreactivity, we performed immunophenotyping analysis of PBMC samples obtained shortly before DLI. The median number of MDC and PDC at

the time of first DLI was $5.8x10^6$/l (range=0.7-$15.4x10^6$/l) and $2.5x10^6$/l (range=0.7-$9.9x10^6$/l), respectively. The median numbers of MDC and PDC did not differ between the patients with and without CMolR or with and without GVHD after DLI (*Figure 1*). The median number of MDC was $10.6x10^6$/l (range=5.8-$15.4x10^6$/l) in patients with a molecular relapse, $8.1x10^6$/l (range=3.1-$11.6x10^6$/l) in patients with a cytogenetic relapse and $4.4x10^6$/l (range=0.7-$10.8x10^6$/l) in patients with a hematological relapse. Median numbers of PDC did not differ between the groups based on the stage of relapse. These results suggest that absolute MDC numbers in relapsed CML patients may decrease with progression of disease.

The patient who did not respond to DLI (UPN 396) and two patients with a second relapse after the initial CCyR (UPN 336 and 389) ultimately progressed to an accelerated or blast phase. At the time of CML progression to an accelerated phase, MDC and PDC numbers were very low (<0.01%).

Discussion

Complete remission after DLI is strongly associated with the occurrence of GVHD, indicating that alloreactive T cells largely mediate GVT reactivity. Studies in MHC-identical murine transplantation models showed that recipient APC are absolutely required to initiate alloreactive CD8+ T cell responses mediating GVHD.[6] But once initiated, GVHD can be intensified by donor-derived APC that are capable to cross-present recipient antigens to the primed alloreactive CD8+ T cells.[7] The same mouse model shows that donor APC do not contribute to the induction of CD8+ T cell-mediated GVT reactivity against chronic-phase CML.[6;7] These studies clearly show the importance of recipient APC in the induction of allo-immune responses after transplantation. However, little is known about the initiation of alloreactive CD8+ T cell responses following DLI in the human setting. In a MHC-mismatched mouse model it has been demonstrated that DLI in mixed hematopoietic chimeras produced improved leukemia-free survival compared to DLI in full donor chimeras.[8]. This suggests that recipient APC present in mixed chimeras at time of DLI may exert more optimal presentation of recipient antigens leading to superior alloactivation of infused donor T cells, whereas absence of recipient APC in full donor chimeras may result in non-responsiveness. This lead to our hypothesis that the presence of peripheral blood MDC and PDC from recipient origin at the time of DLI is associated with the induction of an efficient GVL reaction.

Based on chimerism analysis, we identified three groups. Group 1 was complete

donor chimeric in T cells and DC subsets. They had an early stage of the relapse and three of the four patients obtained CMolR after DLI without significant GVHD. Group 2 was complete donor chimeric in T cells and mixed chimeric in MDC and/or PDC. The six patients in the second group had a more advanced stage of relapse: five had a hematological relapse and one patient had a cytological relapse. All six patients entered CMolR. In this group, the GVL reactivity was associated with clinically overt GVHD in four patients. From these results we speculate that the combination of donor T cells with donor DC induces mild alloreactivity, which is sufficient for the eradication of early stage of relapse. In contrast, the combination of full donor T cell chimerism with host MDC and/or PDC induces stronger alloreactivity leading to potent GVL response associated with GVHD. The combination of mixed T cell chimerism with recipient-derived MDC is less favorable as shown in the third group. This suggests that in patients with an early relapse GVL can be separated from GVHD by DLI with low T cell dose in the presence of donor chimerism in T cells and DC subsets. Therefore, these data only partly support our initial hypothesis that recipient-derived DC are necessary for induction of a DLI-mediated immune response. However, the presence of recipient-derived DC subsets in patients with a cytogenetic or hematological CML relapse may be favorable to induce more potent GVL reactivity for the eradication of higher tumor loads. Further evolution of chimerism to mixed chimerism in T cells is unfavorable and only two of the five patients reach CMolR. Moreover, evolution of chimerism to mixed T cell chimerism is associated with declining absolute MDC numbers indicating that both are related with tumor load of the CML.

Our data suggest that the balance between T cells and DC subsets may be important in the initiation of an alloimmune response after DLI. However, chimerism of APC present in lymphoid tissue or GVHD target organs such as skin or gastro-intestinal tract may also play a role in the initial priming and intensification of the alloimmune response.[25] Furthermore, recipient-derived monocytes and/or myeloid (progenitor) cells may exert antigen-presenting function.[21] In all but one patient, the myeloid cell fraction was mixed chimeric or recipient derived, indicating that myelopoiesis had turned to host origin. Another factor is the sensitivity of the real-time PCR for chimerism determination and we might fail to identify minor populations of recipient-derived DC, which could be enough to induce an immune response. Finally, the presence of pro-inflammatory cytokines and the availability of TLR-ligands may influence the activation status of the DC-subsets.[26]

Six patients developed cytopenia after DLI. Five of these six patients received DLI in the accelerated phase of their disease. Keil et al.[24] showed that a level of about 5% donor cells was enough to protect against critical aplasia after DLI. We did not study chimerism in CD34+ cells, however, four patients with aplasia had > 94% recipient derived myeloid cells in peripheral blood at the time of DLI (data not shown). Two patients had about 60% recipient-derived myeloid cells, in one patient the duration of aplasia was 3 days during an episode with fever and one patient was pre-treated with hydroxyurea.

We showed that part of the recipient-derived MDC and PDC originate from the malignant CML progenitor cells. This situation is specific for CML and is not applicable for relapse of lymphoid malignancies or acute myeloid leukemias. Relapse of lymphoid malignancies or acute leukemias after allogeneic SCT probably show other dynamics of return of recipient cells. As the malignant clone in other malignancies does not produce MDC or PDC, return of recipient-derived DC subsets is less likely.

We performed this study to elucidate the mechanisms that determine the effectiveness of DLI in patients with relapsed CML in relation with DC-chimerism. Absolute numbers of MDC and PDC did not correlate with induction of alloreactivity, but median number of MDC was higher in the group of patients with an early relapse. The combination of donor chimerism in T cells and the presence of recipient DC subsets induces efficient GVL reactivity (all patients enter CMolR) in association with GVHD in 66% of the patients. However, in patients with a molecular relapse, low-dose DLI can induce mild alloreactivity, which is enough to reach CMolR without significant GVHD. This study confirms the importance of regular BCR-ABL measurements, for early identification of a molecular relapse. Further studies are needed for a better understanding of factors that influence the effectiveness of DLI and for the development of strategies to enhance the effectivity of DLI in patients with other malignancies than CML.

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Chapter 3

Multiple myeloma patients receiving pre-emptive donor lymphocyte infusion after partial T cell-depleted allogeneic stem cell transplantation show a long progression-free survival

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Abstract

The purpose of this study was to determine the role of pre-emptive donor lymphocyte infusion (pDLI) after partial T cell-depleted allogeneic stem cell transplantation (SCT) in patients with multiple myeloma (MM).

A cohort of 24 MM patients was treated with partial T cell-depleted myeloablative SCT between December 1997 and April 2002. These patients were intended to receive pDLI after SCT. The overall response rate after SCT was 83% (20 of 24 patients) with 10 patients (42%) in complete remission (CR). Transplant-related mortality within one year after SCT was 29%. Thirteen patients (54%) received pDLI and four patients in partial remission reached CR.

GVHD > grade I after pDLI developed in 4 out of 13 patients (30%). Four patients received therapeutic DLI, without preceding pDLI. Eleven patients (46%) are alive, with a median follow-up of 67 months (range, 48-100 months). Seven of these patients (29%) are in continuous CR (CCR), which was confirmed by a negative patient-specific IgH PCR in four patients. All seven patients in CCR received pDLI.

Although myeloablative SCT in MM induces high toxicity, we show that the concept of T cell depletion followed by pDLI is promising and needs to be investigated in a reduced intensity conditioning setting.

Introduction

Graft-versus-myeloma reactivity of donor lymphocyte infusion (DLI) for relapsed multiple myeloma (MM) after allogeneic stem cell transplantation (SCT) is well established. Response rates of 30% to 50% in relapsed MM patients have been reported.[1;2] DLI can be given as treatment for clinical relapse or as pre-emptive therapy after T cell-depleted allogeneic SCT.[3] In our center, all 24 patients transplanted upfront for MM in the period from December 1997 to April 2002 intended to receive pre-emptive DLI (pDLI) after discontinuation of immunosuppression and in the absence of GVHD. We analyzed response rates and toxicity in this cohort.

Patients and methods

Patients and treatment

Twenty-four patients were treated with an allogeneic SCT for MM between December 1997 and April 2002 at the Radboud University Nijmegen Medical Centre, The Netherlands. All patients had MM stage II or III. All patients younger than 60 years and with an HLA-identical sibling were offered an allogeneic SCT, according to the HOVON 24 study. Eight patients were actually included in the HOVON 24 study, which is published, although only the Nijmegen patients received pDLI.[4] Induction therapy included 2 to 6 VAD (vincristine, adriamycin and dexamethasone) courses. Four patients received an additional course of cyclophosphamide (used for stem cell collection in the HOVON 24 protocol) and 16 patients were treated with intermediate-dose melphalan (two times 70 mg/m^2) before the transplantation. One patient received standard-dose melphalan-prednisone. Patient characteristics are shown in *Table 1*. At the time this study was performed, the prognostic factors such as beta-2 microglobulin and cytogenetics were not routinely tested. In 14 of the 24 patients, conventional cytogenetic analysis of bone marrow was performed before SCT: two patients had multiple structural and numerical abnormalities and one patient showed loss of chromosome 22. The conditioning regimen consisted of cyclophosphamide (total dose 120 mg/kg) and total body irradiation (9 Gy) in 23 patients, and in 5 cases conditioning was intensified by the addition of idarubicin (total dose 42 mg/m^2). One patient with an unrelated donor received cyclophosphamide (120 mg/kg), TBI (9 Gy) and anti-thymocyte globulin (thymoglobulin, 8 mg/kg). Another patient was conditioned with cyclophosphamide (120 mg/kg) and busulphan (16 mg/kg) orally. The grafts were partially depleted of T cells by counterflow centrifugation

as described before.[5] The grafts for two patients were prepared by CD2 and CD19 depletion (Isolex, Nexell-Baxter, Irvine, CA, USA). The median numbers of CD3+ cells and CD34+ cells in the grafts were 0.7 x 10^6 (range, 0.3 x 10^6 - 1.03 x 10^6) and 2.4 x 10^6 (range, 1.2 x 10^6 – 5.64 x 10^6) per kg body weight of the recipient, respectively. All patients received cyclosporine A as GVHD prophylaxis.

Table1: Patient characteristics

Characteristic	Number of patients (N=24)
Sex, no of male/female	14/10
Median age, years (range)	50 (33-58)
Median time diagnosis-SCT, months (range)	8 (5-14)
WHO performancestatus	
0 -1	19
2 -3	5
Beta-2-microglobulin (mg/l)	
0 -3	8
>3	6
Unknown	10
Hemoglobin (g/l)	
≤ 10	9
> 10	14
Unknown	1
Lactate dehydrogenase	
Normal	16
Elevated	2
Unknown	6
M-protein heavy chain	
IgA	2
IgG	17
IgD	1
Light -chain Normal	3
Non secreting	1
Bone marrow plasma cells (%)	
≤ 50	19
> 50	4
Unknown	1

Abbreviations: Ig = immunoglobulin; WHO = World Health Organization;

Infusion of donor lymphocytes

pDLI was given on an intention-to-treat basis and was withheld in cases of preceding GVHD > grade I, active chronic GVHD (cGVHD) and in patients who were still on immunosuppressive therapy. DLI was performed as described before.[6] Briefly, lymphocytes were obtained from the original stem cell donor by leukapheresis using the blood cell separator Baxter CS 3000 (Baxter, Deerfield, IL, USA) or the Fresenius AS 104 (Fresenius, Oberursel, Germany). Lymphocytes were administered to the patient within 3 hours of completion of the leukapheresis procedure. No GVHD prophylaxis was administered after the infusion of the donor lymphocytes. The first nine patients received 0.1 x 10^8 CD3+ cells/kg, but because of GVHD, the dose of lymphocytes was reduced to 0.05 x 10^8 CD3+ cells/kg in the next four patients. Therapeutic DLI (tDLI) was given for progression or relapse of MM and T cell dose varied from 0.05 x 10^8 to 0.7 x 10^8 CD3+cells/kg.

Response Criteria

Complete remission (CR) was defined as complete disappearance of M-protein from blood and/or urine by immunofixation and normalization of the bone marrow.[7] Partial remission (PR) was defined as a decrease of more than 50% of the M-protein in the peripheral blood and a decrease in urinary light-chain excretion of more than 90% or to less than 0.2 g/24h. Minimal response (MR) was defined as 25% or more decrease of monoclonal proteins in the peripheral blood and/or more than 50% decrease in urinary light-chain excretion. Molecular remission was defined as a negative patient-specific polymerase chain reaction (PCR). The sensitivity of the PCR is $1x10^{-5}$.

End points and statistical analysis

The data were analyzed in April 2006. End points included response rate, overall survival (OS) and progression free survival (PFS).

PFS was determined from transplantation until progression, relapse or death, whichever came first. OS was calculated from transplantation until death. PFS and OS were estimated by the Kaplan-Meier method.

Multivariate analysis was performed using the Wilks Lambda test.

Results

Outcome and toxicity of SCT

Twenty of 24 patients (83%) were in remission before transplantation, including 4 patients in CR and 16 in PR. Three patients showed a minimal response and one was refractory to the chemotherapy before transplantation. Twenty-two patients were evaluable 3 to 6 months after transplantation. Two patients had died at this time, one from infection and one from progressive disease. The overall response rate (of all 24 patients) after allogeneic stem cell transplantation was 83% (20 of 24 patients) with 10 patients (42%) in CR and 10 in PR.

Acute GVHD (aGVHD) occurred in 12 patients (50%): seven patients (29%) with grade I, three patients (13%) with GVHD grade II and two patients (8%) with grade III. Twenty-three patients were evaluable for cGVHD. Seven patients (30%) developed chronic GVHD, which was limited in five patients and extensive in two patients.

Table 2: Disease status after SCT, response after DLI and at last follow-up in 13 patiens treated with pDLI.

After SCT	**After pDLI**	**Last follow-up**	**PCR**
5 patients in CR	5 remain in CR	3 CCR, 2 alive with relapse	2 CCR confirmed by a negative PCR
1 patient with VGPR	1 CCR	1 CCR	Confirmed by negative PCR
4 patients in PR	These 4 patients reach CR	3 CCR, 1 relapse	1 CCR is confirmed by a negative PCR
2 patients with no remission	PR in 1, PD in1	Both died (1 of GVHD and 1 of PD)	
1 patient is non-secreting	1 non-secreting	Died of relapse	

Abbreviations: CR = complete remission; CCR = continuous CR; DLI = donor lymphocyte infusion; PCR = polymerase chain reaction; PR = partial remission; PD = progressive disease; VGPR = very good partial response.

DLI after SCT

Thirteen of the 24 patients (54%) received pDLI after SCT. Median time from SCT to pDLI was 7.5 months (range, 3.5-13.3 months). Six patients (46%) developed GVHD after pDLI: grade I in two patients, grade II and III in one patient each and grade IV GVHD in two patients. Eleven of 24 patients did not receive pDLI, because of GVHD (n=5), rejection (n=1), rapid progressive disease (n=1), death (n=1), poor performance status (n=1) and donor related problems (n=2).

Four patients received tDLI for progressive disease, without previously having had pDLI. Median time to tDLI was 18.7 months (range, 13.6–47.2 months). Two patients developed GVHD after tDLI, and this was grade I and IV in each patient.

Outcome of DLI and overall survival

Disease status at the time of pDLI was CR in five patients, VGPR in one, PR in four, one patient was non-secreting and two patients were not in remission (*table 2*). Overall, 10 patients have reached a clinical CR after pDLI. Five patients were in CR before pDLI and the 5 patients with (VG)PR after SCT also reached a clinical CR after pDLI. Three patients relapsed later; two patients are alive and still receiving treatment for MM.

Nine out of 13 patients (69%) who received pDLI are alive at present and seven are in CCR. In four of the patients in CCR a patient-specific PCR for the immunoglobulin heavy-chain rearrangement was generated and all four patients are persistently PCR-negative.[8] In two patients the PCR was negative prior to pDLI, in 1 patient the PCR was weakly positive and became negative after pDLI. In another patient, the PCR was not tested between SCT and pDLI. Overall, of the 7 patients in CCR, three were in clinical CR before pDLI. Four patients in CCR reached CR only after pDLI.

Four of the 13 patients who were given pDLI have died: both patients who were not in remission after SCT died, one from progressive disease and one from GVHD, respectively. The third patient responded to DLI, which was confirmed by a negative patient-specific PCR, but died of a relapsed MM 3.5 years after pDLI. Finally, the patient who was nonsecreting died of progressive disease. In all patients reaching CCR the M-protein was < 10 g/l after SCT, indicating that tumor load at the time of DLI is related to the final outcome. Multivariate analysis was performed to study whether ß-2 microglobulin, M-protein level or percentage of plasma cells was correlated with CCR. None of these factors was significantly correlated with CCR, but patient numbers are too small to draw definite conclusions.

Of the four patients who received tDLI, one patient died owing to GHVD, another achieved a complete remission, but relapsed and died from progressive disease. Two patients entered a partial response and are alive at 64 and 58 months after SCT, respectively.

Figures 1 and *2* show the overall survival and progression-free survival. Eleven out of 24 patients (46%) are alive at present, with a median follow-up of 67 months (range, 48-100 months) from SCT. Seven of these patients are in CCR; four patients relapsed or showed progressive disease. Thirteen patients died: seven patients (29%) owing to transplant related mortality (TRM) within 1 year after SCT and five patients (20%) owing to progressive disease. One patient died 18 months after SCT from GVHD after tDLI for progression of MM.

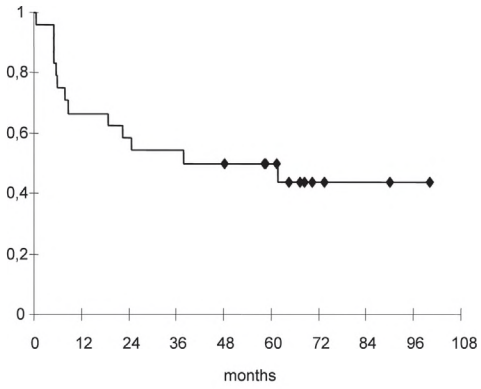

Figure 1: Overall survival from allogeneic stem cell transplantation

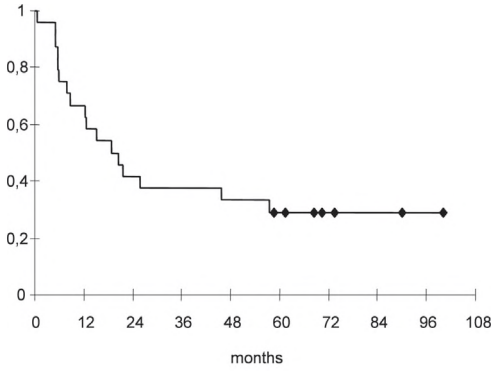

Figure 2: Progression-free survival from allogeneic stem cell transplantation

Discussion

Since partial T cell depletion of the graft is associated with a higher risk of relapse, a cohort of 24 patients (transplanted from 1997-2002) was planned for pDLI to boost graft-versus-tumor reactivity. Since only 13 patients received pDLI, our results have to be interpreted with caution. Nevertheless, in our cohort, 7 of 24 patients (29%) are in CCR with a median follow-up of 67 months and all 7 had received pDLI. Eight of the 24 patients were also included in the Dutch HOVON 24 trial, and described before.[4] However, pDLI was not given in the other centers participating in this study. In the HOVON 24 trial, only 3 of 53 patients (6%) were in CCR with a median follow-up of 20 months. This suggests that immunotherapy after T cell depleted SCT enhances the graft-versus-myeloma reactivity. In four patients with a clinical CCR, plasma cells were cryopreserved at diagnosis to set up a patient-specific IgH PCR and all four patients were in molecular remission. A recent study showed that molecular remission after SCT predicts a better relapse-free survival, although relapses after many years can occur.[9]

There is a strong association between graft-versus-myeloma reactivity and GVHD. The strongest predictors for response to DLI (therapeutic) in relapsed MM patients are the occurrence of aGVHD and cGVHD after DLI.[1;10] In reduced intensity SCT, patients with cGVHD have a significantly longer median event-free survival than patients without cGVHD.[11] In one multicentered study, patients with cGVHD had a more than two times less probability of relapse.[12] It is striking that in our study no CCRs were reached among patients with GVHD after SCT and who did not receive pDLI for this reason. The mechanism behind this observation is unknown; however, we made a similar observation in other hematological malignancies.[3]

The most serious toxicity of DLI is GVHD. Because two patients developed aGVHD grade IV after pDLI, the pre-emptive T cell dose was decreased from 0.1 x 10^8 CD3+ cells/kg to 0.05 x 10^8 CD3+ cells /kg. Four patients received the low dose pDLI, and none of these patients developed GVHD more than grade I. A comparable strategy is to start with low-dose DLI, followed by dose escalation if the patient does not reach CR. Peggs et al. studied the application of dose-escalated DLI following reduced intensity SCT in patients with lymphoid malignancies.[13] However, separation of graft-versus-malignancy from GVHD was achieved in only a minority of the patients. Another approach to reduce the incidence of GVHD after DLI is the transfer of CD8-depleted donor lymphocytes. In a recent study from Meyer et al, 11 patients with high-risk hematological malignancies received a total of 21 CD8-

depleted pDLIs. Two patients with HLA-C mismatched donors developed grade II and III aGVHD followed by limited cGVHD.[14] These prophylactic CD8 depleted DLIs accelerate immune reconstitution; however, the effect on disease relapse rate has to be studied. Combination therapy of low-dose thalidomide and DLI for MM is described by Kroger et al.[15] In this study, patients with progressive or residual disease after allogeneic SCT receive low dose DLI (0.05 x 10^8CD3+ cells/kg for patients with related donors) in combination with low dose thalidomide. Two patients developed aGVHD grade I of the skin and also two patients developed *de novo* cGVHD.

Only 54% of the patients were able to receive pDLI. This is comparable with the study of Alyea et al.[16] In their study of T cell depleted (CD6 depleted) allogeneic SCT followed by CD4+ DLI, 58% of the patients actually received DLI. The other patients did not receive DLI because of SCT-related complications or early relapse. In our study, patients actually receiving pDLI are the patients who had no complications from the SCT or rapidly progressive disease. Since pDLI was given relatively late (at a median of 7.5 months after SCT) the conclusion on the favorable effect of pDLI is skewed towards good prognosis patients. However, analysis of progression free survival and overall survival were made on intention-to-treat basis.

Despite the favorable outcome in patients receiving pDLI, TRM was high (29%). TRM in allogeneic SCT for MM prepared with myeloablative conditioning varies from 17% to 38%.[17;18] Nonmyeloablative SCT significantly reduced TRM, even in patients with advanced age and comorbidity.[19] However, in the setting of nonmyeloablative SCT with unmanipulated grafts, the incidence of cGVHD is high. The optimal procedure of performing allogeneic SCT in MM is still unknown.

An important question remains the place of upfront allogeneic SCT in patients with newly diagnosed MM, and which patients may benefit most. Results of a prospective multicenter trial comparing tandem autologous SCT and tandem autologous-allogeneic SCT were published recently.[20] Patients with newly diagnosed MM and with two adverse prognostic factors were included. After four courses of VAD, these patients received high dose melphalan followed by autologous SCT. Then, depending on the availability of an HLA-identical sibling, these patients received either a RIC-allogeneic SCT or a second autologous SCT. There was no significant difference in EFS and OS between these two strategies. On the other hand, a recent prospective study of Bruno at al. showed a superior outcome for the tandem autologous-allogeneic approach compared with tandem autologous transplant.[21]

On the basis of the data in our study and the reduction in TRM after reduced

conditioning, we are performing a pilot study in which patients receive an autologous SCT followed within 6 months by a partially T cell-depleted allogeneic SCT after reduced intensity conditioning with fludarabine (120 mg/m^2) and cyclophosphamide (4800 mg/m^2). From January 2006, 13 patients were transplanted according this regimen and all 13 patients engrafted. Currently, these patients receive low dose DLI after discontinuation of immunosuppressive therapy. Low dose DLI, with escalation, is given to patients not in CR (measured with PCR in the absence of M-protein) and in the absence of GVHD.

Further studies are needed for better immunomodulatory therapies after partially T cell-depleted allogeneic SCT for patients with MM. With reduced intensity conditioning regimens, the TRM after allogeneic SCT is significantly reduced; however, effectively boosting graft-versus-myeloma-reactivity without GVHD as side effect remains a dilemma in allogeneic SCT for MM.

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Chapter 4

Partial T cell-depleted allogeneic stem cell transplantation following reduced intensity conditioning creates a platform for immunotherapy with donor lymphocyte infusion and recipient dendritic cell vaccination in multiple myeloma

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Abstract

Allogeneic stem cell transplantation (SCT) in multiple myeloma (MM) may induce a curative graft-versus-myeloma (GVM) effect. Major drawback in unmanipulated reduced intensity conditioning (RIC) SCT is the risk of severe and longstanding graft-versus-host-disease (GVHD). This study demonstrates that transplantation with a partial T cell-depleted graft creates a platform for boosting GVM immunity by pre-emptive donor lymphocyte infusion (DLI) and recipient dendritic cell (DC) vaccination, with limited GVHD. All twenty MM patients engrafted successfully. Chimerism analysis in 19 patients evaluable at three months revealed that 7 patients were complete donor, while 12 patients were mixed chimeric. Grade II acute GVHD occurred in 7 patients (35%) and only 4 patients (21%) developed chronic GVHD. Fourteen patients received posttransplantation immunotherapy, 8 pre-emptive DLI, 5 patients both DLI and DC-vaccination and 1 patient DC-vaccination only. DC-vaccination was associated with limited toxicity and none of these patients developed GVHD. Importantly, overall treatment-related mortality at one year was low (10%). Moreover, the overall survival (OS) is 84% with median follow-up of 27 months, and none of the patients died from progressive disease. These findings illustrate that this novel approach is associated with limited GVHD and mortality, thus creating an ideal platform for adjuvant immunotherapy.

Introduction

Allogeneic stem cell transplantation (SCT) may cure patients with multiple myeloma (MM) because of a graft-versus-myeloma (GVM) effect. Myeloablative (MA) conditioning has been limited by a high treatment-related mortality (TRM), and at present, reduced-intensity conditioning SCT (RIC-SCT) following autologous SCT seems a promising approach. Importantly, TRM following RIC-SCT is reduced from 30 to 40% to 10 to 20%.[1] However, three prospective trials comparing autologous transplantation followed by RIC-SCT versus double autologous SCT showed contradictory results in clinical outcome.[2-4] The study by Bruno et al.[2] showed a superior overall survival (OS) for autologous SCT followed by allogeneic RIC-SCT. In line with this study, Rosiñol et al.[4] observed a trend towards a longer progression-free survival (PFS) for patients treated with auto/RIC-SCT, but no significant differences in event free survival (EFS) and OS. In contrast, the Intergroupe Francophone du Myelome (IFM) observed no differences in EFS and OS comparing double autologous SCT versus auto/RIC-SCT in high risk patients. Although these differences in outcome may be explained by different inclusion criteria and treatment schedules, they illustrate that improvement of the GVM effect, without the toxicity and morbidity of graft-versus-host disease (GVHD) after allogeneic RIC-SCT, is a prerequisite to further establish this therapeutic approach.

Previously, we showed that partial T cell-depleted allogeneic SCT followed by pre-emptive donor lymphocyte infusion (DLI) resulted in long-term complete remission (CR) in about one third of MM patients.[5] In this cohort of 24 patients, 1-year TRM after MA conditioning was 29%. But a continuous CR in seven MM patients after pre-emptive DLI with a median follow-up of 8.6 years encouraged us to investigate partial T cell-depleted allogeneic SCT in the RIC setting, combined with pre-emptive immunotherapy with DLI. The major advantage of T cell-depleted grafts is reduction of severe and prolonged GVHD, but effective posttransplantation immunotherapy is essential to overcome the higher rate of relapse. As a novel approach we incorporated recipient-derived dendritic cell (DC) vaccination in the posttransplantation strategy for patients with residual disease after two pre-emptive DLI dosages.

DC are the professional antigen-presenting cells (APCs) of the immune system, and are essential for the induction of antigen-specific T cell immunity. In the setting of allogeneic SCT and DLI, alloreactive T cell responses targeting minor histocompatibility antigens (MiHA) on malignant cells of the recipient can be induced directly by recipient-derived DC and indirectly by donor-derived DC because of

cross-presentation.[6] Boosting GVM immunity by vaccination with donor-derived DC loaded with hematopoiesis-restricted MiHA seems most ideal, but this approach is hampered by the limited number of known MM-expressed MiHA. Studies in mouse models demonstrated that recipient DC play a pivotal role in the initiation of alloreactive CD8+ T cell-mediated immunity against leukemia.[7,8] Moreover, the presence of recipient DC in the setting of mixed chimerism has a positive impact on the effectiveness of DLI.[9] Because recipient DC and myeloma tumor cells are both derived from the hematopoietic system, immune responses induced by recipient-derived DC may enhance GVM with limited GVHD in other tissues, like mucosa, liver, and skin.

Here, we show the results of partial T cell-depleted RIC-SCT after autologous transplant for MM, with limited GVHD and a low 1-year TRM of 10%. Furthermore, we investigated the feasibility of generating mature recipient-derived DC from cryopreserved apheresis products, the immunogenicity of the vaccine, and the toxicity of recipient-derived DC-vaccination. Our study indicates that partial T cell-depleted RIC-SCT is feasible, results in excellent engraftment, and offers opportunities for posttransplantation cellular immunotherapy with DLI in some patients combined with DC vaccination. Importantly, our approach keeps open the treatment with novel agents (bortezomib and lenalidomide) in case of progressive or relapsed disease even in combination with DLI.

Materials and Methods

Transplantation procedure

From January 2006 to May 2008, 20 patients have been included in a pilot study of partial T cell-depleted, allogeneic RIC-SCT for MM. All patients were pre-treated for symptomatic MM with induction chemotherapy and high-dose melphalan (HDM) followed by autologous SCT (conform HOVON-50 or HOVON-65 studies or standard induction scheme at that time).[10] Patients <65 years with an HLA-identical sibling donor were offered upfront allogeneic RIC-SCT within 6 months after autologous transplant, regardless of risk factors or disease status. Before RIC-SCT, autologous PBMC were collected by apheresis, washed to deplete platelets, and cryopreserved for posttransplant DC vaccination (*Figure 1*). The conditioning regimen consisted of cyclophosphamide (Cy) 1200 mg/m^2 intravenously in combination with fludarabine (Flu) 30 mg/m^2 on each of four consecutive days (days -5, -4, -3, -2 before SCT).

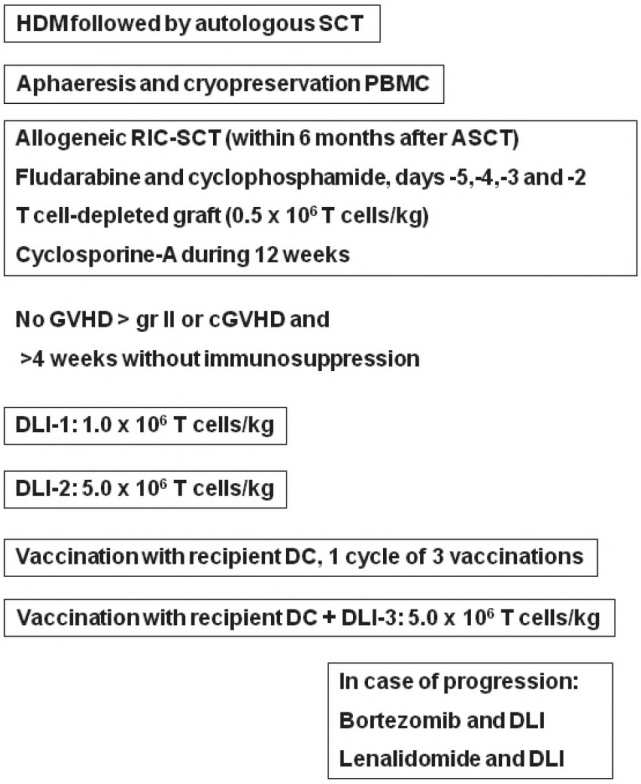


Figure 1: Flow-chart of the tandem autologous SCT followed by RIC-SCT and posttransplantation cellular immunotherapy.

Donor stem cell grafts were depleted from T and B cells by anti-CD3 and anti-CD19 immunomagnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany). Following depletion, CD3+ T cells were added back to generate a stem cell graft containing a fixed number of 0.5 x 10^6 T cells/kg body weight of recipient. GVHD prophylaxis consisted of Cyclosporine A (CsA) 3 mg/kg/day intravenously starting on day -1 until CsA could be taken orally. CsA was administered orally at a dose of 6 mg/kg/day until 8-10 weeks after SCT followed by a gradually tapering off in 4 weeks. Acute and chronic GVHD (aGVHD, cGVHD) were classified grade I-IV and limited or extensive, respectively, according to the criteria described by Glucksberg [11] and Shulman.[12]

Evaluation of response and chimerism analysis

Responses were evaluated according to the response criteria for MM described by Durie et al. in 2006.[13] Bone marrow (BM) aspirates during posttransplantation immunotherapy were performed in patients receiving DC vaccination. Lambda free light chains were measured using the serum free light chain (FLC) assay (Freelite, Birmingham, UK). For measuring kappa free light chains, we used the ELISA assay, as described by Lamers et al.[14] This ELISA was shown to parallel FLC kappa assay, with lower absolute values. To define CR, the FLC ratio was measured with the Freelite assay for both lambda and kappa free light chains. The data were analyzed in December 2008.

Molecular remission was defined as a negative patient-specific IgH-polymerase chain reaction (PCR).[15] The sensitivity of the PCR is $1x10^{-5}$. The patient-specific IgH-PCR did not play a role in the decision on the treatment schedule, because molecular analysis of disease load in BM was performed retrospectively.

Real-time quantitative PCR of single nucleotide polymorphisms (SNP) and/or the SMCY gene was used for chimerism analysis as described previously.[16-18] Briefly, recipient/donor pairs were screened for discriminating SNPs. Quantification was based on real-time PCR with allele-specific primers for DNA-sequences containing the discriminating SNP and target DNA-specific probes.

Posttransplantation immunotherapy: treatment schedule

Patients without aGVHD grade >II and without cGVHD after RIC-SCT, were candidates for pre-emptive DLI 4 weeks after discontinuation of immunosuppression. The first DLI-dose consisted of $1.0x10^6$ T cells/kg body weight and the second dose two months later of $5.0\ x10^6$ T cells/kg body weight (*Figure 1*). Patients with residual disease after two DLIs were eligible for recipient-derived DC vaccination. Exclusion criteria for vaccination were progressive disease (PD), extensive or uncontrolled GVHD, recent use of immunosuppressive drugs, and active infections. Vaccinations were administered three times at 2-week intervals. The DC dose was maximal 30 $x10^6$ cells intravenously (i.v.) as a bolus injection and $15x10^6$ DCs intradermally (i.d.) in the upper leg near the inguinal lymph node region. If the yield of mature DC was too low, then only i.v. vaccination was given. Blood samples were taken from these patients before vaccination, on day 14 (after first vaccination), on day 28 (after second vaccination), on day 42 (after third vaccination) and on day 56 (28 days after the third vaccination). If no GVHD was induced, residual disease persisted and

sufficient DC were cryopreserved, a second series of vaccinations was started using the combination of DC vaccination with DLI $5.0x10^6$ T cells/kg body weight.
The study was approved by the Local Ethics Committee of Radboud University Nijmegen Medical Centre.

Posttransplantation immunotherapy: generation of DC vaccine

DC vaccines were generated under good manufacturing practice conditions in a clean room facility. Before the conditioning for RIC-SCT, patient peripheral blood mononuclear cells (PBMCs) were collected by leukapheresis of 9 liters of blood using the Cobe Spectra aphaeresis system (Gambro BCT, Breda, The Netherlands). PBMC were washed with CliniMACS buffer containing 5% human serum albumin (HSA) to deplete from platelets, cryopreserved in HSA plus 10% DMSO and stored in liquid nitrogen until use. For culturing DC, PBMC were rapidly thawed at 37°C and resuspended in CliniMACS buffer containing 100 U/ml Pulmozyme (Roche, Woerden, The Netherlands), 3 mM MgCl2 and 5% HSA. After a 30-minute incubation, PBMC were centrifugated, washed, and resuspended in XVIVO-15 medium (Cambrex Bio Sciences, Verviers, Belgium) plus 2% heat-inactivated virus-free human serum (HS). Monocytes were isolated by plastic adherence and cultured in XVIVO-15/2% HS supplemented with 800 U/ml GM-CSF and 500 U/ml IL-4 (CellGenix, Freiburg, Germany). Cells were harvested at day 3, counted and cultured at $0.5x10^6$ cells/ml in 6-well plates in XVIVO-15/2% HS containing GM-CSF (800 U/ml), IL-4 (500 U/ml) and 50 μg/ml keyhole limpet hemocyanin (KLH) subunits (Biosyn Arzneimittel GmbH, Fellbach, Germany). Two days before harvesting, KLH-loaded DC were maturated in XVIVO-15/2% HS containing GM-CSF (800 U/ml), IL-4 (500 U/ml), IL-1β (5 ng/ml), IL-6 (15 ng/ml), TNF-α (20 ng/ml) (all CellGenix Freiburg, Germany) and prostaglandin E2 (PGE2; Pharmacia & Upjohn, Puurs, Belgium, 1 μg/ml). Mature DC were harvested at day 9 and tested for microbial and phenotypic analysis. One third of the cells were used for immediate injection and remaining cells were cryopreserved for subsequent vaccinations.

Immunologic monitoring

T cell responses against KLH were measured using the ^{3}H-thymidine incorporation assay with PBMC of the patient before and after vaccination. Briefly, PBMC were restimulated in vitro with 50 μg/ml KLH subunits or 1 μg/ml PHA plus 100 U/ml IL-2 (i.e. positive control). At day 7 of incubation, T cell proliferation was determined

by ^{3}H-thymidine incorporation. The stimulation index was calculated as the counts ratio between stimulated and nonstimulated PBMC. Antibodies against KLH were measured in the serum of vaccinated patients by ELISA as described by De Vries et al.[19] A positive signal at a 400x dilution of the patient's serum was considered positive. Alloreactive CD8+ T cell responses against recipient MiHA were determined by major histocompatibility complex (MHC) tetramer staining. Therefore, patients and donors were first genotyped for known MiHA-mismatches using allele-specific PCR assays as described previously.[20;21] In case of MiHA mismatches, PBMCs were incubated with the appropriate phycoerythrin (PE)-labeled MHC tetramer complex for 20 minutes at room temperature. After washing with PBS/0.5% BSA, cells were labeled with the appropriate concentration anti-CD8-FITC (ProImmune, Oxford, UK), anti-CD3-PECy7 and anti-CD45-ECD (Beckman Coulter, Fullerton, California) for 15 minutes at 4°C. After washing, cells were resuspended in PBS/0.5%BSA and 7-amino-actinomycin D (7AAD; Sigma, St Louis, MO, USA) was added. Cells were analyzed using the Coulter FC500 flow cytometer (Beckman Coulter).

Results

Patient characteristics

Twenty MM patients received upfront allogeneic RIC-SCT after autologous SCT between January 2006 and May 2008 (*Table 1*). Median age of these patients was 57 years (range: 39-64 years) at the time of RIC-SCT and of these 20 patients 11 were male and 9 were female. Disease status following autologous SCT was complete response (CR) in seven patients (35%), very good partial response (VGPR) in two (10%), partial response (PR) in eight (40%), stable disease (SD) in two (10%) and progressive disease in one patient (5%). These results are comparable to the outcome after autologous SCT in other studies.[4]

RIC-SCT with partial T cell-depleted grafts resulted in successful engraftment with limited GVHD

All 20 patients received PB stem cell (PBSC) grafts from HLA-identical sibling donors. Median number of infused CD34+ cells was 8.3 x10^{6} per kg body weight of the recipient (range: 4.3-12.8 x10^{6}). Median number of infused CD3+ T cells was 0.50 x 10^{6} per kg (range: 0.31-0.77x10^{6}). Furthermore, median number of infused B cells was 0.22x10^{6} per kg (range: 0.05-0.75x10^{6}) and median number of infused NK cells

was 34.5 x 10^6 per kg (range: 7.9-97.1x10^6). After RIC-SCT, median time to reach leukocyte counts >1.0x10^9/l and platelet counts >20x10^9/l was 13 days (range: 10-20 days) and 9 days (range: 0-11 days), respectively. In 3 patients platelet counts did not decline to below 20x10^9/l.

Table 1: Characteristics of MM patients

Characteristic	**number**
Number of patients	20
Median age at transplantation , years (range)	57 (39-64)
Sex, no (%)	
Male	11 (55%)
Female	9 (45%)
Immunoglobulin class (%)	
IgG	11 (55%)
IgA	3 (15%)
Light chain	6 (30%)
Cytogenetics	17
Karyotypic anal ysis	
Normal	11
Deletion of chromosome 13*	1
Hyperdiploid/complex	5
Interphase FISH	
Deletion of chromosome 13	7
Beta2-microglobulin	
<3.5 mg/L	5
>3.5 mg/L and <5.5 mg/L	3
≥5.5 mg/L	1
Not done	11
Induction chemotherapy	
VAD	16
TAD	2
PAD	2

VAD indicates vincristine, doxorubicin, dexamethasone; TAD, thalidomide, doxorubicin, dexamethasone; PAD, Bortezomib, doxorubicin, dexamethasone; FISH, Fluorescent in situ hybridization; MM, multiple myeloma.

*Deletion of chromosome 13 by metaphase cytogenetics

Nineteen patients could be evaluated for PB cell chimerism at 3 months after RIC-SCT, and one patient died 2.2 months after RIC-SCT. All 19 patients showed successful donor engraftment and there was no secondary graft failure. At three months, seven patients were complete donor chimeric and 12 patients were mixed chimeric. In these 12 mixed chimeric patients, the median value of autologous PB cells was 7% (range: 2-27%). Two patients converted to complete donor chimerism after discontinuation of CsA at 5 and 6 months after RIC-SCT, respectively. At the last follow-up, 16 of the 19 patients were complete donor chimeric, and 3 patients were still mixed chimeric. These 3 patients included one patient in which the donor was unavailable for donating lymphocytes and 2 patients treated with pre-emptive DLI shortly before or after the last follow-up.

Importantly, none of the patients developed grade III or IV aGVHD. Grade II aGVHD occurred in 7 out of 20 (35%) patients. Nineteen patients were evaluable for cGVHD, of whom only four (21%) developed cGVHD. The median duration of CsA treatment was 99 days (range: 58-230 days). In 9 patients, CsA could be discontinued within 100 days. Three patients with cGVHD received CsA for more than 5 months.

Clinical response after partial T cell-depleted RIC-SCT and pre-emptive DLI

Nineteen patients could be evaluated for clinical response at three months after RIC-SCT. Eight patients (42%) were in CR, one (5%) patient in VGPR, seven (37%) patients in PR and three patients had stable disease after the auto/RIC-SCT tandem-procedure.

Twelve of the 19 patients (63%) received pre-emptive DLI of 1.0 x 10^6 T cells/kg after RIC-SCT, and seven of these patients also received a second dose-escalated pre-emptive DLI of 5.0 x 10^6 T cells/kg. Details of these twelve patients are shown in *Table 2*. Only one of the 12 patients developed GVHD grade I after pre-emptive DLI.

Two patients (UPN2 and UPN17) reached CR after pre-emptive DLI in a dose of 5.0 x 10^6 T cells/kg (*Figure 2A* and *2B*). For patient UPN2, a patient-specific IgH-PCR was developed and this PCR became negative after pre-emptive DLI indicating molecular remission. Furthermore, three patients (UPN1, UPN3 and UPN7) converted to complete donor chimerism after posttransplantation immunotherapy with DLI, 6, 22 and 25 months after SCT, respectively.

Seven of the 19 patients did not receive pre-emptive DLI, because of GVHD (n=4), infections (n=2) and donor unavailability in 1 patient.

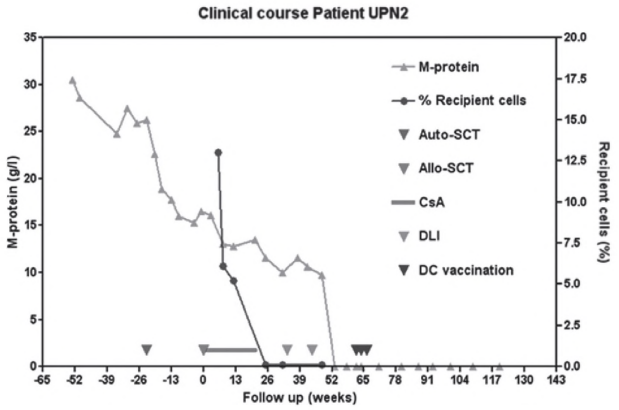
Table 2: Disease status and chimerism in patients receiving pre-emptive DLI after RIC-SCT

UPN	M-protein	Disease status 3 months after RIC-SCT*	Chimerism 3 months after RIC-SCT	Pre-emptive DLI: T cells/kg body weight	Outcome after pre-emptive DLI (after second DLI)
1.	IgG-κ	CR	2.1% recipient cells	1.0 x 10^6	CR, complete donor chimerism, Guillain-Barre syndrome, died 13 months after SCT from pneumonia
2.	IgG-κ	PR	5.2% recipient cells	1.0 x 10^6 and 5.0 x 10^6	CR, complete donor chimerism, DC-vaccination
3.	Light chain λ	PR	26.7% recipient cells	1.0 x 10^6 and 5.0 x 10^6	VGPR, 6.7% recipient cells, DC-vaccination
4.	IgG-κ	PR	Complete donor	1.0 x 10^6 and 5.0 x 10^6	PR, ongoing decline of M-protein
5.	IgG-κ	SD	Complete donor	1.0 x 10^6 and 5.0 x 10^6	SD, DC-vaccination
7.	Light chain λ	CR	14.0% recipient cells	1.0 x 10^6 and 5.0 x 10^6	CR, 12.8% recipient cells, DC-vaccination
8.	IgG-κ	CR	Complete donor	No pre-emptive, but therapeutic DLI	VGPR after therapeutic DLI for relapse, DC-vaccination
10.	IgA-κ	PR	8.6% recipient cells	No DLI, donor not available	Not applicable, DC-vaccination
12.	IgA-κ	PR	Complete donor	1.0 x 10^6 and 5.0 x 10^6	Relapse
13.	IgG- λ	PR	Complete donor	1.0 x 10^6 and 5.0 x 10^6	PR, DC vaccine did not fulfill quality criteria
14.	IgG-κ	SD	9.5% recipient cells	1.0 x 10^6	Relapse, 1.8% recipient cells
15.	Light chain λ	CR	2.2% recipient cells	1.0 x 10^6	Relapse, 1.1% recipient cells
17.	IgG- λ	PR	6.9% recipient cells	5.0 x 10^6	CR, 1.0% recipient cells
19.	Light chain κ	CR	5.5% recipient cells	1.0 x 10^6	CR, second pre-emptive DLI is planned

CR indicates complete remission; VGPR, very good partial response; PR, partial response; SD, stable disease; RIC-SCT, reduced-intensity conditioning-stem cell transplantation; DLI, donor lymphocyte infusion; DC, dendritic cell.

* Response 3 months after RIC-SCT.

a



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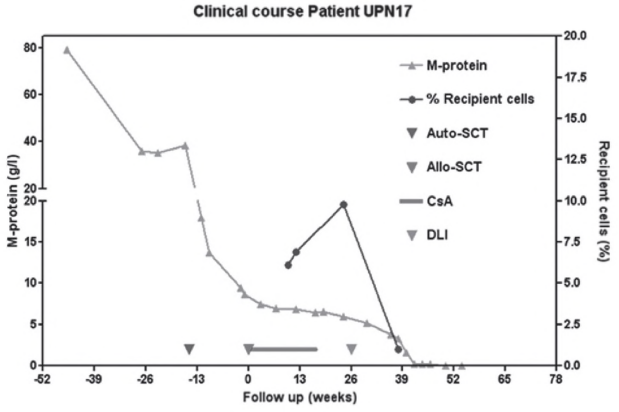

Figure 2: Clinical course of patient UPN2 (a) and patient UPN17 (b). The Y-axis on the left shows disease load as measured by serum free light chains or M-protein, and is shown with the green line. The Y-axis on the right shows the percentage recipient cells in peripheral blood and is shown with a purple line. Triangle in blue indicates autologous SCT, triangle in red indicates RIC-SCT, triangle in orange indicate DLI and triple-triangle in blue indicate 1 cycle of DC vaccinations.

Generation of recipient-derived DC vaccines

Following pre-emptive DLI, DC vaccines were generated for six patients as part of posttransplantation immunotherapy. Therefore, the cryopreserved apheresis product collected just prior RIC-SCT was thawed and used to generate mature monocyte-derived DC of recipient origin. Preclinical investigations showed that mature DC could be generated from cryopreserved apheresis products of MM patients that efficiently stimulated allogeneic T cell proliferation in vitro (*Figure 3*). The yield of

PBMC and CD14+ monocytes post-cryopreservation for the six patients was 45% to 90% and 25 to 57%, respectively (*Figure 4A*). DC culture from cryopreserved PBMC resulted in sufficient DC yield in four patients (i.e. 9-16% from CD14+ monocytes), but for two patients only a limited number of DC could be obtained (yield <5% from CD14+ monocytes). The final vaccine contained >95% viable DCs (*Figure 4A*). Furthermore, DC vaccines had a very mature phenotype with >85% expression of CD83 and the co-stimulatory molecules CD80, CD86 and CD40 (*Figure 4B*). Moreover, 58% to 95% of the DC in the vaccines expressed the lymph-node migration receptor CCR7. For 1 patient, we did not obtain good quality mature DC and this vaccine was not administered (data not shown). Because of the variability in the yield of DC from thawed PBMC, the number of administered DC varied. Four patients received at least one maximum dose, 1 patient received three vaccinations each with total 10 x 10^6 cells (i.v. and i.d.) and 1 patient received three i.v. vaccinations each with 4 x 10^6 cells (*Table 3*).

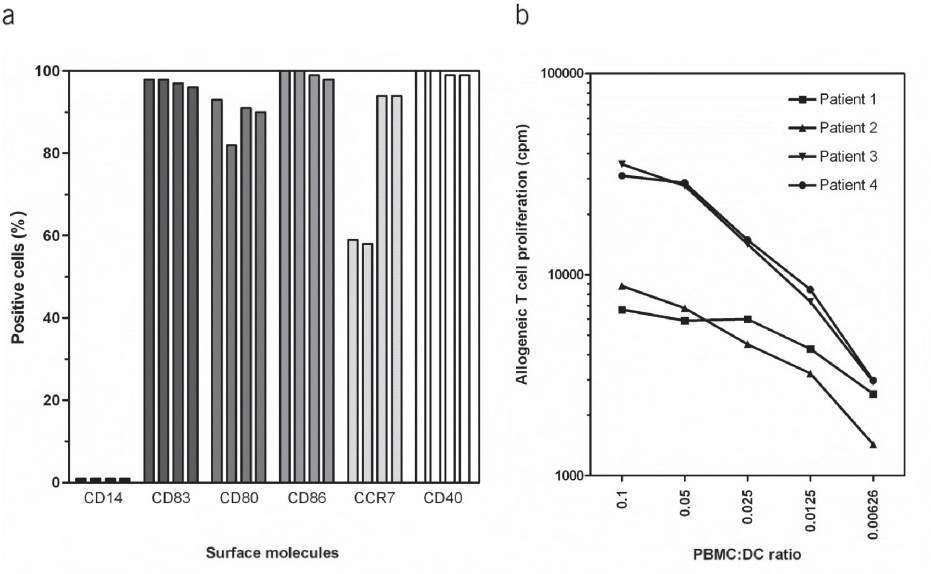

Figure 3: a. Preclinical study of characteristics of mature DC generated from cryopreserved apheresis products from 4 MM patients. Apheresis was performed after autologous SCT. Cultured DC had a mature phenotype with high expression of CD83, CD80 and CD86.

b. In vitro stimulation capacity of mature DC generated from cryopreserved apheresis products. DC from patient 1&2 and patient 3&4 were tested with responder cells from different healthy donors.

table 3: immune responses and toxicity after recipient DC vaccination

UPN	Number of vaccinated DC*			DLI†	Induration	Fever	GVHD	Anti-KLH response	
	Vac I	Vac II	Vac III					T cell§	Ab¶
3.	30/15	30/15	29/13	no	yes	yes	no	+++	-
	6.5/3.5	6.5/3.5	5/2.5	DLI (5.0×10^6 /kg)	no	no	no		
5.	4/0	4/0	3/0	no	na‡	no	no	+++	-
7.	30/15	33/15	34/17	no	yes	yes	no	+++	-
10.	28/14	27/13	27/13	no	yes	yes	no	+++	-
2.	7/3	7/3	6.5/3.5	no	yes	yes	no	++	-
8.	30/15	18/9	18/9	DLI (10.0×10^6 /kg)	no	no	no	+	-

DLI indicates donor lymphocyte infusion; MM, multiple myeloma; GVHD, graft-versus-host disease; DC, dendritic cell.

*Cell numbers are given in 10^6, intravenously/intradermally.

†Two patients received the combination of DLI and DC-vaccination. UPN3 received a second cycle of vaccinations in combination with DLI on the day of vaccination I. UPN 8 relapsed after RIC-SCT, and responded to therapeutic DLI. Because of relapsed MM, this patient received DC-vaccination in combination with DLI. DLI was co-infused with DC-vaccination II

‡ Local induration was not applicable to this patient.

§KLH-specific proliferation of PBMC after vaccination is depicted as stimulation index (SI): + SI >2<10; ++ SI≥10<100; +++ SI≥100

¶KLH-specific antibody titers in serum after vaccination: - no Ab or < 1:400; + Ab titer ≥ 1:400.

Vaccination with recipient-derived DC vaccines after RIC-SCT

Six MM patients were vaccinated with recipient-derived DC (*Table 2* and *3*). The median time from RIC-SCT to DC vaccination was 11.6 (range: 8.1-24.4) months. Median interval from last DLI to DC vaccination was 4.2 (range: 3.5-4.9) months. Four patients were vaccinated after 2 pre-emptive DLIs. In one patient, the donor

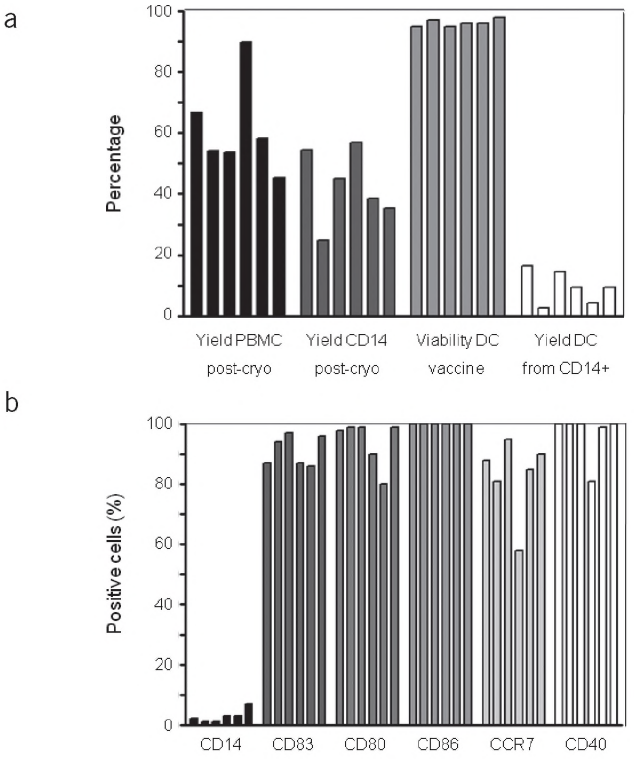

Figure 4: a. Characteristics of DC vaccine from thawed apheresis products. Post-cryopreservation, the yield of PBMC varied from 45% to 90% and yield of CD14+ monocytes varied from 25% to 57%. Yield of mature DC from CD14+ cells was sufficient in 4 patients (9%-16%), however less than 5% in two patients. Viability of the vaccination product was >90% for all administered vaccines.
b. DC vaccine phenotype. All administered DC vaccines had a mature phenotype with high expression of the cell surface antigens CD83, CD80, CD86, and CCR7.

was no longer available for donating lymphocytes and she was treated with DC vaccination only as posttransplantation immunotherapy. The sixth patient was treated with DC vaccination following two therapeutic DLIs. None of these patients showed clinically active disease at the time of vaccination.

DC were loaded with the antigenic protein KLH as an adjuvant to provide CD4+ T cell help and for boosting of alloreactive CD8+ T cell responses as well as to analyze the induction of a primary immune response posttransplantation. All patients showed a PB T cell proliferative response against KLH that could already be detected

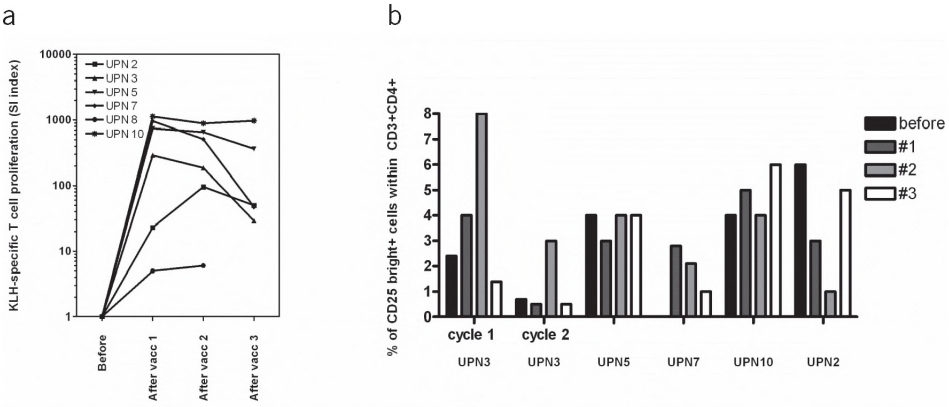

Figure 5: a. KLH specific T cell proliferation indicated as stimulation index.
b. Regulatory T cells (CD25 bright+ cells within the CD3+CD4+ population) before and after each DC vaccination in five patients treated with DC vaccination.

after one single DC-vaccination (*Table 3* and *Figure 5*). However, the anti-KLH T cell proliferative response in some patients decreased following subsequent DC-vaccinations (*Figure 5A*). Furthermore, antibody responses against KLH could not be detected (*Table 3*).

Five patients were evaluated for T cell recovery at the time of DC administration (*Table 4*). Median CD3+ T cell count was 0.7 $x10^9$/L, median CD4 T cell count 0.3 $x10^9$/L and median CD8+ T cell count was 0.4 $x10^9$/L indicating a not completely recovered immune system, especially from the CD4+ T cells at the time of vaccination. Natural Killer (NK) cells were recovered to normal in four of the five patients with a median of 0.2 $x10^9$/L CD3-CD16/56+ NK cells. CD4/CD8 ratios were still inversed in three of the five patients. Although CD4+ and CD8+ T cell counts were not completely recovered to normal levels, all five patients showed a strong *in vitro* polyclonal T cell proliferative response upon stimulation with PHA and IL-2 (*Table 4*).

Impact of DC-vaccination on regulatory T cells has been studied before and after each DC vaccination. Regulatory T cells were studied as the percentage of CD25 bright+ cells within the CD3+CD4+ population and are shown in *figure 5B*. There is no trend in increasing percentages of CD4+ CD25 bright+ cells in DC-vaccinated patients.

Table 4: T cell recovery at time of DC vaccination

UPN	Interval SCT-DC-vaccination (months)	CD3+ x10^9/L	CD3-CD16/56+ x10^9/L	CD19+ x10^9/L	CD4+ x10^9/L	CD8+ x10^9/L	PHA/IL2 response*
Normal (5-95 percentile)		1.2 (0.7-2.1)	0.3 (0.09-0.6)	0.2 (0.1-0.5)	0.7 (0.3-1.4)	0.4 (0.3-1.4)	
3.	11.8	0.7	0.23	0.2	0.4	0.3	+++
		1.5	0.64	0.07	1.6	0.7	
5.	11.3	0.8	0.2	0.0	0.2	0.5	++++
7.	11.3	0.8	0.2	0.05	0.4	0.4	++++
10.	8.1	0.3	0.12	0.08	0.2	0.1	++++
2.	14.4	0.7	0.06	0.22	0.2	0.6	++++

DC indicates dendritic cell; SCT, stem cell transplantation.

*PHA/IL2 induced proliferation of PBMC before vaccination is depicted as stimulation index (SI): + SI >2<10; ++ SI≥10<100; +++ SI≥100<500; ++++SI≥500

Characterization of T cell-responses

For all six patients who received DC vaccination, we performed genomic typing of twelve previously described MiHA. In two patients, a mismatch against a known MiHA was found with the immunogenic allele in the recipient. Patient UPN2 was mismatched for MiHA HY and patient UPN5 for HA-8. PB samples (before and after DLI as well as before and after DC vaccination) of patient UPN2 were analyzed with tetramers against HLA-A2 and HLA-B7 restricted epitopes in the male-specific SMCY protein. However, in these samples no SMCY-tetramer positive cells could be detected (data not shown). Samples of patients UPN5 (before DC-vaccination andafter each DC-vaccination) were analyzed with tetramers against the HLA-A2 restricted HA-8 antigen. Also in this patient we found no HA-8 tetramer positive cells in peripheral blood samples (data not shown). Although we could not detect tetramer-positive T cells against known MiHA in DLI and DC-treated patients, this does not exclude the presence of MiHA-specific or tumor antigen-specific T cell responses in these patients.

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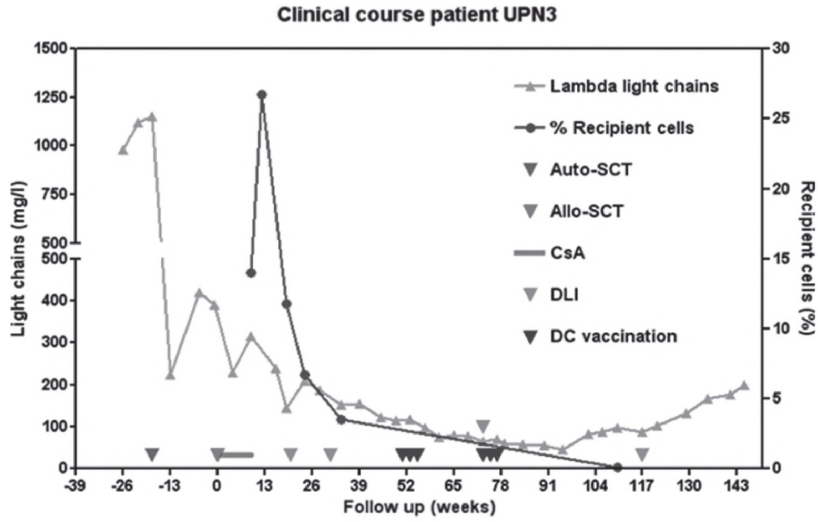

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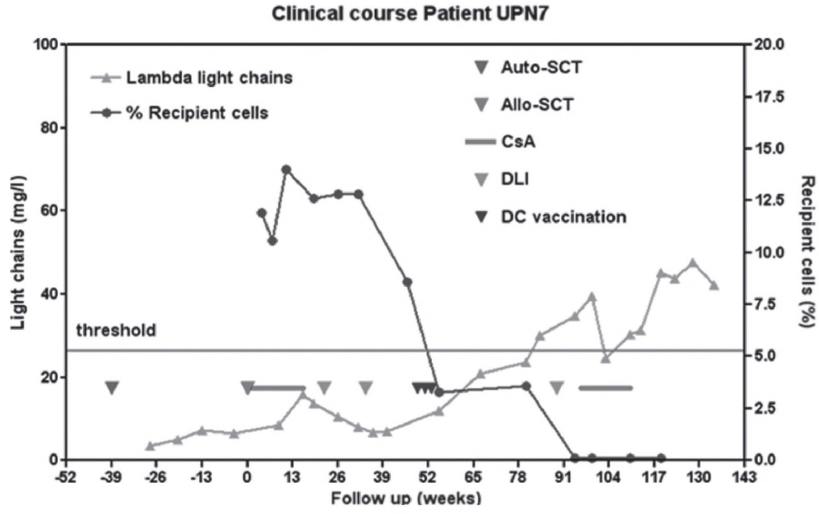


Figure 6: Clinical course of patient UPN3 (a) and patient UPN7 (b). The Y-axis on the left shows disease load as measured by serum free light chains or M-protein, and is shown with the green line. The Y-axis on the right shows the percentage recipient cells in peripheral blood and is shown with a purple line. Triangle in blue indicates autologous SCT, triangle in red indicates RIC-SCT, triangle in orange indicate DLI and triple-triangle in blue indicate 1 cycle of DC vaccinations.

Recipient DC-vaccination did not induce severe toxicity and GVHD

Toxicity of recipient DC-vaccination was limited to flu-like symptoms with mild fever and local induration at the injection site (*Table 3*). Importantly, none of the six patients developed clinical signs of GVHD, but two patients developed discrete dermal changes with folliculitis and eosinophilia in PB.

At the time of the first DC-vaccination, three patients were complete donor chimeric and three patients had still mixed chimerism with 6.7%, 12.8% and 8.6% recipient cells respectively (*Table 2*). DC vaccination alone did not induce conversion to complete donor chimerism. Patient UPN3 converted to complete donor chimerism after a second cycle of vaccinations in combination with DLI (*Figure 6A*). Patient UPN7 received a therapeutic DLI of 0.5 x 10^8 T cells/kg for increase of free light lambda chains, resulting in complete donor chimerism and GHVD gr II (*Figure 6B*).

Clinical outcome of DC-vaccination after RIC-SCT

Fourteen out of 20 patients were treated with posttransplantation immunotherapy (13 pre-emptive and 1 for relapse), including 8 patients with pre-emptive DLI alone, 5 patients received both DLI and DC vaccination and 1 patient DC vaccination only (*Table 2*).

DC vaccination in six patients did not result in induction of responses by itself. However, patient UPN3 showed a gradual decline of light chains after RIC-SCT during posttransplantation immunotherapy with DLI and DC-vaccinations (*Figure 6A*). Two years after RIC-SCT, light chains started rising again without clinical symptoms. An escalating dose of DLI was administered, but the serum free light chains continued to rise. Presently, this patient is treated with the combination of lenalidomide and DLI.

Patient UPN7 showed a rise in FLC lambda eight months after completion of DC vaccination (*Figure 6B*). Although this rise did not fulfill the criteria for PD, immunotherapy was continued because in our experience rise of FLCs predicts clinical relapse. He was treated with therapeutic DLI (0.5 x 10^8 T cells/kg) and developed GVHD grade II in combination with stabilization of free light lambda chains. Patient UPN5 and UPN10 did not respond to DC-vaccination.

Patient UPN8 reached a VGPR after two therapeutic DLIs of 5.0 and 10.0 x 10^6 T cells/kg for relapsed MM (*Figure 7*). Because of the relapse, DC-vaccination was combined with DLI in a dose of 10.0 x 10^6 T cells/kg. At the last follow-up immunofixation remains positive but the M-protein cannot be quantified.

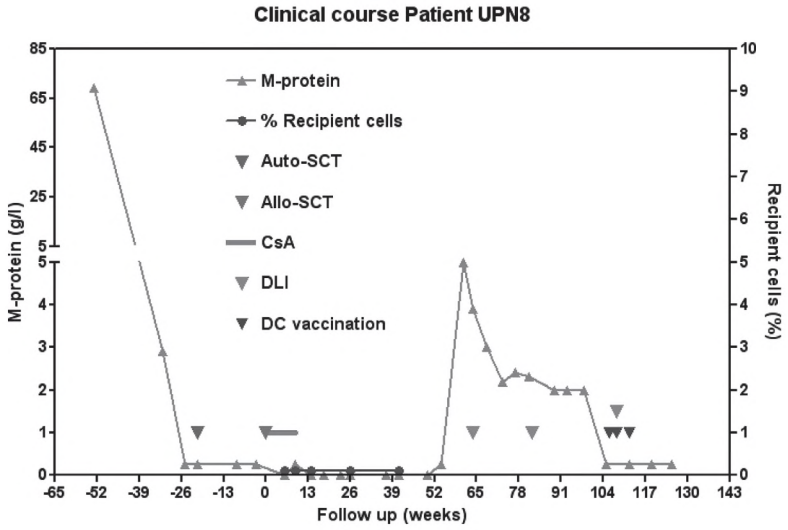


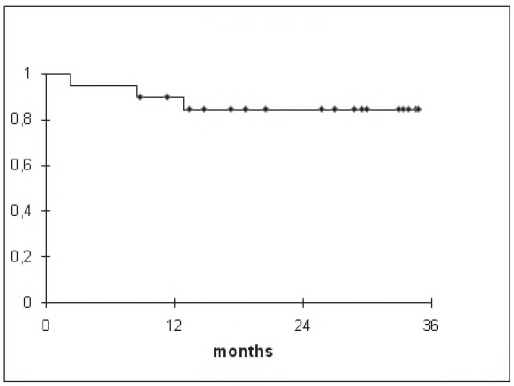
Figure 7: Clinical course of patient UPN8. The Y-axis on the left shows disease load as measured by serum free light chains or M-protein, and is shown with the green line. The Y-axis on the right shows the percentage recipient cells in peripheral blood and is shown with a purple line. Triangle in blue indicates autologous SCT, triangle in red indicates RIC-SCT, triangle in orange indicate DLI and triple-triangle in blue indicate 1 cycle of DC vaccinations.

Overall and progression free survival after RIC-SCT

With a median follow-up for surviving patients of 27 months (range: 8.9-34.9 months) the overall survival is 84% (*Figure 8A*). TRM was 5% at 100 days, and 10% at 1 year. One patient died from sepsis and cardiac failure 2.2 months after RIC-SCT and one patient from pulmonary cGVHD 9 months after RIC-SCT. One additional patient died in CR 13 months after RIC-SCT from the complications of pneumonia during recovery from a Guillain-Barré syndrome. None of the patients died from relapsed or progressive multiple myeloma.

At the last follow-up in December 2008, six patients were in CR, one patient reached a VGPR and two patients with PR were still receiving immunotherapy. Eight patients had started with systemic therapy (bortezomib, thalidomide, or lenalidomide) for PD after RIC-SCT (3 from stable disease, 2 from PR and 1 from VGPR) or relapse from CR (2 patients). The current PFS is shown in *Figure 8B*.

a



b

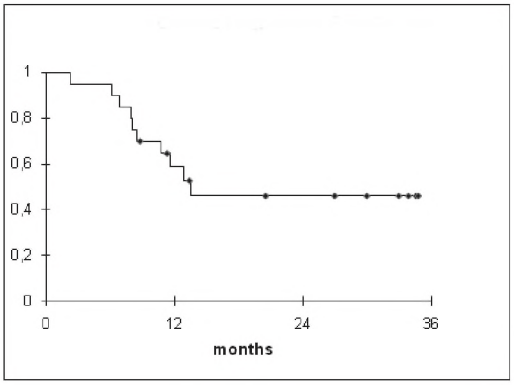


Figure 8. a. Overall survival of 20 patients after RIC-SCT with a median follow-up of 27 months.
b. Current progression-free survival of 20 patients after RIC-SCT. Progression was noted when systemic therapy was started.

Discussion

Here, we report on the feasibility of fludarabine-cyclophosphamide RIC-SCT in combination with a partial T cell-depleted graft for MM patients following induction chemotherapy and autologous transplant with HDM. One-year TRM was reduced to 10% in a cohort of 20 patients with a median follow-up of surviving patients of 27 months. This TRM is in line with other studies using RIC-SCT for MM.[1;4] OS rate is comparable with other studies in patients undergoing autologous SCT followed by RIC-SCT. In our study OS was 84% at 2 years. In the ECOG-study the actuarial

survival rate at 2 years was 78% and OS was 78% at 20 months in the study from Maloney et al.[1;22] All patients showed donor engraftment and no late graft failures occurred. Importantly, aGVHD was limited to grade I and II, and could be managed with CsA and corticosteroids. The prevalence of cGVHD was 21%, including 1 patient suffering from pulmonary disease, probably related to GVHD. The duration of immunosuppressive therapy with CsA was relatively short for a RIC-regimen with a median of 99 days, 3 patients were treated with CsA for more than 5 months due to cGVHD.

Pre-emptive DLI resulted in conversion from PR to CR in two patients. At the last follow-up, six of the seventeen evaluable patients were in CR and three of them have received pDLI. However, durability of these responses with a median follow-up of 27 months has to be shown by a longer follow-up. DC-vaccination alone did not induce GVM-responses after RIC-SCT. However, this study was designed to analyze the feasibility, immunogenicity, and toxicity of recipient-derived DC-vaccination. Important questions concerning the optimal dose of DC, route of administration, and combination with DLI have not been studied yet in the setting of allogeneic SCT. Our data indicate that partial T cell-depleted RIC-SCT creates a platform for posttransplantation cellular immunotherapy with pre-emptive DLI and DC-vaccination, given the low incidence and severity of GVHD and the short duration of immunosuppressive therapy. DLI has proved to be effective in MM as pre-emptive immunotherapy, however the optimal dose and timing is not known.[23] In this study, pre-emptive DLI started with a low dose of 1.0 x 10^6 T cell/kg four weeks after discontinuation of CsA followed by a second dose of 5.0 x 10^6 T cells/kg two months later. The lowest dose of 1.0 x 10^6 T cell/kg did not result in GVM reactivity, or in GVHD. Conversion of chimerism was observed in only 1 patient after this low-dose DLI. The second dose of 5.0x 10^6 T cells/kg resulted in CR in two patients without GVHD. Therefore, we apply a starting DLI dose of 5.0 x 10^6 T cells/kg in the current protocols.

Repeated DLIs were only given if a patient did not reach CR. Importantly, most responses to DLI were seen after the first or second DLI. Only one patient developed GVHD and decreasing FLCs after the third DLI. Repeated DLIs were given to patients with relapsed MM after RIC-SCT who responded to systemic therapy with Bortezomib or Lenalidomide (data not shown). These repeated DLIs did not result in long term remissions. We consider most patients not responding to the first or second DLI resistant to DLI. Prerequisites for the induction of a GVM effect by DLI

are effective antigen presentation and co-stimulation in conjunction with sufficient inflammation. In this study, we have been focusing on the antigen presentation, and introduced DC vaccination as posttransplantation immunotherapy. Another strategy to further boost GVM immunity after DLI could be blockade of negative regulatory mechanisms. For example, strategies aimed at reducing regulatory T cells or blocking of the T cell inhibitory PD-1/PD-L1 pathway.

Although pre-emptive DLI is effective, other therapies are needed to further improve the GVM effect of RIC-SCT. Both regulatory T cells and host APCs have been implicated in GVHD and graft-versus-leukemia (GVL) reactivity after DLI. The requirement of recipient APC for the induction of GVL has been clearly demonstrated by Mapara et al.[9] Moreover, Xia et al.[24] have shown that in long-term complete chimeras loss of DLI-induced GVL can be restored by infusion of host DC. In this study, we analyzed the feasibility of generating recipient-derived mature DC and the toxicity of vaccination with these DC.

To generate recipient-derived mature DC several months after allogeneic SCT, we used cryopreserved patient apheresis products obtained after autologous SCT and shortly before RIC-SCT. DC with a mature phenotype and sufficient CD80, CD83, CD86 and CCR7 expression could be generated from the cryopreserved PBMC from 6 out of 7 patients. The administered vaccine products all fulfilled the quality-criteria as described by Figdor et al.[25] The generated DC of one patient did not have a mature phenotype. Comparative studies with immature and mature DC have demonstrated that only mature DC stimulate T cell in vivo and it has been shown that immature DC can silence immune responses.[19] Therefore this not fully mature DC vaccine was not administered.

Induction of a primary immune response was measured by T cell responses against KLH. We showed that recipient-derived mature DCs loaded with KLH induce a potent primary T cell response after the first vaccination. However, the peripheral blood T cell proliferative response against KLH after the second and third DC injection decreased in most patients. DCs were not extra loaded with antigens because recipient-derived DCs are able to directly present recipient-specific MiHA to donor T cells. In this setting of using unloaded recipient-derived DC, we aimed at the induction of MiHA-specific donor T cell responses against known and unknown MiHA. Genotyping for known MiHA in the vaccinated recipients and their donors did identify MiHA-mismatches in some patients that could be involved in GVM and GVHD (data not shown). However, we were unable to detect antigen-specific T cells

against known MiHA using tetramer staining. Currently, we are analyzing whether recipient-derived DC did boost or induce T cell responses against unknown MiHA by functional characterization of alloreactive T cell lines generated from vaccinated patients.

Toxicity of recipient-derived DC was limited to fever the evening after the second and third vaccination and local induration at the injection site. Such toxicity is known from other vaccination studies and is probably due to immune responses against KLH.[26] None of the six patients developed GVHD after recipient-derived DC-vaccination, although two patients reported discrete skin changes.

This is the first study that applied recipient-derived DC vaccines after allogeneic SCT. One major limitation of recipient-derived DC vaccines after allogeneic SCT is the requisite to collect and to cryopreserve PBMC before SCT for generation of mature monocyte-derived DC several months later. Alternatively, donor DC loaded with recipient-specific MiHA may also induce alloreactive T cell responses after allogeneic SCT and the use of donor-derived DC for vaccination circumvents the obstacle of cryopreservation. To explore donor-derived DC vaccines for the induction of MiHA-specific immune responses after SCT, a set of hematopoietic-restricted MiHA with expression on tumor cells must be available for loading of donor-derived DC. Until now, the number of identified hematopoietic-restricted MiHA with expression on MM tumor cells was limited, and therefore hampered the application of this strategy. However, the proof of principle may be explored clinically by using MiHA HA-1 and LRH-1, which have been shown to be functionally expressed by MM tumor cells.[27;28]

Vaccination with donor-derived DC, loaded with tumor lysate or tumor-associated antigens, after SCT has been published before in 3 reports. In the first report, DC were cultured from granulocyte colony-stimulating factor (G-CSF) mobilized PB stem cells from the donor.[29] Donor-derived DC pulsed with irradiated tumor cells and primed T cells were injected in 4 patients with relapse after SCT. In the second report, DC cultured from PB cells from the donor and pulsed with tumor lysate were given to a patient transplanted for metastatic renal cell carcinoma.[30] Vaccination with donor-derived DC appeared to be safe in this single patient but did not induce graft-versus-tumor reactivity. In the third report, a patient with relapsed acute myeloid leukemia (AML) after SCT was vaccinated with WT1 peptide and KLH-pulsed donor-derived DC.[31] Immune responses were induced to the immunogenic antigen KLH, however T cell responses against WT1 were not detected and the relapsed leukemia did not respond.

New options to salvage patients with relapse or PD after allogeneic SCT are thalidomide, bortezomib and lenalidomide. El-Cheikh et al.[32] reported 37 patients treated with bortezomib as salvage treatment for relapse or progression following RIC-SCT. An objective disease response (including CR, VGPR and PR) was achieved in 27 patients (73%). Lenalidomide treatment for relapse MM was reported by Minnema et al.[33] Lenalidomide alone or in combination with dexamethasone resulted in a response rate of 87.5%. Importantly, some patients developed acute GVHD when lenalidomide was given as monotherapy within months after SCT or DLI. These studies are performed in patients with relapsed or PD; however the role of new agents in combination with cell therapies for residual disease after RIC-SCT has not been explored yet.

In conclusion, partial T cell-depleted RIC-SCT has the advantage of a low one-year TRM of 10%, limited severe GVHD, and sustained GVM reactivity. This strategy opens the possibility for posttransplantation immunotherapy, alone or in combination with new agents. Due to the low incidence of acute and chronic GVHD, the quality of life in these patients remains good and consequently pre-emptive immunotherapy can be performed in a majority of patients.
Therefore, in the era of RIC-SCT for MM posttransplantation strategies have become more important than before. We show that vaccination with recipient-derived DC is feasible, safe, immunogenic and most importantly, does not induce GVHD. The potential of this approach to induce GVM reactivity is not yet fully exploited. Further studies on the coinfusion of donor lymphocytes with recipient-derived DC or MiHA-loaded donor-derived DC are needed.

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Chapter 5

Reduced intensity conditioning followed by partial T cell-depleted allogeneic SCT for relapsed or progressive transformed non-Hodgkin's lymphoma, follicular lymphoma and CLL

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Abstract

This study analyzed the feasibility of partial T cell-depleted reduced intensity conditioning allogeneic stem cell transplantation (RIC-SCT) combined with pre-emptive donor lymphocyte infusion (pDLI) for relapsed transformed non-Hodgkin's lymphoma, follicular lymphoma, mantle cell lymphoma and chronic lymphocytic leukemia. In most patients, we applied a two-step approach with pretransplant host T cell-depletion followed by conditioning within three weeks. Twenty-nine patients were enrolled in this study and 27 received the scheduled RIC-SCT. Chimerism-analysis in 26 patients at three months revealed that thirteen patients were complete donor, while eleven were mixed chimeric. Two patients had complete autologous haematopoietic recovery; both received an unrelated donor transplant without additional host T cell-depletion before conditioning. Acute GVHD ≥ II occurred in five patients (19%) and fifteen (64%) developed chronic GVHD which was extensive in three. Five patients received pDLI. Most complications were viral infections or EBV-associated lymphoproliferative disease. The non-relapse mortality at one-year and relapse rate are low (both 14%). With a median follow-up of 19 months, 2-years estimated overall survival and current lymphoma-free-survival are 83% and 74% respectively. This study indicates that partial T cell-depleted RIC-SCT combined with host T cell-depletion before start of conditioning results in good engraftment and high lymphoma-free-survival without invalidating GVHD.

Introduction

Reduced intensity conditioning allogeneic stem cell transplantation (RIC-SCT) offers the opportunity for SCT in patients with comorbidities and in older patients. RIC-SCT induces durable engraftment with reduced toxicity, but morbidity and late mortality related to graft-versus-host-disease (GVHD) is still considerable when undepleted grafts are utilized.[1] In the myeloablative setting, the incidence of severe and longstanding GVHD can be reduced by removal of the T cells from the graft. However, T cell-depletion is associated with increased incidence of tumor relapse, post-transplant opportunistic infections and graft failure.

To reduce the incidence of tumor relapse after T cell-depleted SCT, the strategy of pre-emptive donor lymphocyte infusion (pDLI) for high-risk patients can be used.[2;3] Pre-emptive DLI in patients who had no significant GVHD after myelo-ablative partial T cell-depleted SCT has resulted in low relapse rates in patients at high risk for relapse.[4] Also in animal models, it has been demonstrated that delayed T cell infusions after allogeneic SCT may maintain the antitumor efficacy of engrafted T cells with limited nonspecific alloreactivity.[5]

We applied the strategy of partial T cell-depleted SCT followed by pre-emptive DLI in the reduced intensity setting for patients with relapsed or progressive lymphoproliferative diseases. RIC-SCT often results in mixed chimerism and incidence of graft rejection is increased, particularly in patients who receive T cell-depleted allografts. Therefore we used a clinical protocol with an induction course to deplete host T cells prior to the administration of RIC as described by Bishop et al.[6] Here, we report the results of partial T cell-depleted RIC-SCT followed by pre-emptive DLI in patients with relapsed or progressive transformed non-Hodgkin's lymphoma (NHL), indolent NHL and chronic lymphocytic leukemia (CLL). This study shows that partial T cell-depleted RIC-SCT is feasible, results in stable engraftment if host T cell-depletion is applied before the start of the conditioning regimen and offers opportunities for post-transplantation cellular immunotherapy with DLI.

Methods

Patients

Between January 2003 and October 2008, 29 patients were enrolled in a study on partial T cell-depleted, allogeneic RIC-SCT followed by pre-emptive DLI for NHL and CLL. From January 2003 until January 2007, only patients with relapsed or

progressive aggressive NHL (transformed or follicular NHL grade 3) and availability of an HLA-identical sibling donor were eligible for inclusion. From January 2007, inclusion criteria were extended to relapsed or progressive indolent lymphomas (follicular lymphoma grade 1 or 2 and lymphoplasmocytic lymphoma), relapsed mantle cell lymphoma after autologous transplant and progressive CLL/small lymphocytic lymphoma (SLL) and availability of an HLA-identical sibling or an HLA-compatible unrelated donor. All patients were required to have chemotherapy-sensitive disease. Before SCT, most patients were treated with fludarabine and cyclophosphamide to attain minimal residual disease. Further eligibility criteria included a WHO-performance score of 0-1, age 18-65 years and normal organ functions. Written informed consent was obtained from all patients. The study was reviewed and approved by the Local Ethics Committee of Radboud University Nijmegen Medical Center.

Conditioning regimen and GVHD-prophylaxis

All patients received partial T cell-depleted allogeneic RIC-SCT, with some modifications of the RIC-regimen. From January 2003, RIC consisted of total lymphoid irradiation (TLI) on each of three consecutive days followed by cyclophosphamide 50 mg/kg body weight intravenously on each of four consecutive days (total dose 200 mg/kg bodyweight). TLI incorporated mantle field, inverted Y-fields including the spleen, the inguinal and femoral lymph node regions. A total dose of 12 Gy was delivered in 2 Gy fractions, twice a day, in an overall treatment time of three days. Four patients with NHL (two patients with transformed NHL and two patients with relapsed follicular NHL grade 3) received this conditioning regimen.

From January 2006, RIC for patients with an HLA-identical sibling donor consisted of induction chemotherapy with fludarabine and cyclophosphamide to deplete circulating host T-cells as described before by Bishop et al.[6] If CD4+ count on day 17 was $<0.05 \times 10^9$/L, the conditioning regimen was started within three weeks (after day 17). The conditioning consisted of cyclophosphamide 1200 mg/ m^2 intravenously in combination with fludarabine 30 mg/m^2 on each of four consecutive days (days -5, -4, -3,-2 before SCT).

RIC for patients with an unrelated donor consisted of the same conditioning regimen with fludarabine, cyclophosphamide (days -8,-7,-6,-5) in combination with anti-thymocyte globulin (ATG, Thymoglobulin, Genzyme, Europe) 2.0 mg/kg/day for 4 consecutive days (days -4,-3,-2,-1) to reduce the risk of rejection. Our hypothesis was

that in-vivo T cell-depletion by ATG would induce enough host T cell-depletion for engraftment. Because two patients with unrelated donors had a primary take failure with autologous repopulation, induction chemotherapy for host T cell-depletion was also incorporated in the conditioning strategy for patients with an unrelated donor.
All allografts were depleted of T and B cells by anti-CD3 and anti-CD19 immunomagnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany) or by CD34 enrichment. After depletion or enrichment, T cells were added back to generate a graft with a fixed number of 0.5 x 10^6 T cells/kg body weight of recipient. For all patients GVHD-prophylaxis consisted of Cyclosporine a (CsA) 3 mg/kg/day intravenously starting on day -1 until CsA could be taken orally. CsA was administered orally at a dose of 6 mg/kg/day until 8-10 weeks after RIC-SCT followed by a gradually tapering off.

Pre-emptive DLI: treatment schedule

Patients without aGVHD grade >II and without cGVHD after RIC-SCT, intended to receive pre-emptive DLI four weeks after discontinuation of immunosuppression. The first DLI-dose consisted of 1.0 x 10^6 T cells/kg body weight. An escalated dose of 5.0 x 10^6 T cells/kg body weight was given two months later if there was persistent disease and no GVHD. Patients with a decrease of donor cells were treated with DLI with the goal to reach complete donor chimerism. Patients with relapse were eligible for therapeutic DLI. The lymphocyte dose of therapeutic DLI depended on previous lymphocyte doses of DLI and previous GVHD, but in principle a lymphocyte dosage of 10 x 10^6/kg body weight was administered.

Evaluation of response, GVHD and chimerism

Responses were assessed using standard disease-specific criteria.[7;8] Acute and chronic GVHD were classified grade I –IV and limited or extensive, respectively, according to the criteria described by Glucksberg et al [9] and by Shulman et al.[10]
Chimerism analysis was performed on whole blood by real-time quantitative polymerase chain reaction (PCR) of polymorphisms as described previously.[11-14] Briefly: recipient/donor pairs were screened for discriminating polymorphisms. Quantification of donor and recipient cells is based on real-time PCR with allele-specific primers for DNA-sequences containing the discriminating polymorphism and target DNA-specific probes.

Supportive care and definition of infectious complications

Supportive care measures were the same in all patients. Anti-microbial prophylaxis consisted of 500 mg ciprofloxacin given twice daily, and 500 mg valaciclovir given three times daily for two months. Fluconazole was given at a dose 200 mg/day orally only to those who were considered colonized by Candida albicans. No mould active antifungal prophylaxis was used. Patients received trimethoprim-sulphamethoxazole 480 mg/day orally for three months, as prophylaxis against infections due to Pneumocystis jiroveci (PCP). In case of GVHD requiring immunosuppressive therapy, PCP prophylaxis was continued or restarted.

Early bloodstream infection (BSI) was defined as a BSI occurring in the first 30 days post SCT. A blood culture was considered positive if a microorganism was recovered from one or more bottles, with the exception of coagulase-negative staphylococci, for which two separate positive blood cultures with the same strain were required.[15]

Invasive fungal disease (IFD) was defined according to the EORTC/MSG consensus statement.[16;17]

Monitoring for Epstein-Barr virus (EBV) and Cytomegalovirus (CMV) load was performed by quantitative PCR twice weekly during hospitalization and weekly thereafter. CMV-infection (CMV-I) and CMV-disease (CMV-D) were defined according to current consensus.[18] Patients with CMV-I received pre-emptive valganciclovir 900 mg bid. EBV-related lymphoproliferative disorder was defined according the WHO classification.[19] The cut-off value of the DNA load defining EBV-reactivation was 1000 copies/mL.

Statistical Methods and definitions

Data were analyzed as of June 2009. The Kaplan-Meier method was performed to assess overall survival (OS) and current lymphoma-free-survival. OS was calculated from the date of stem cell infusion until death from any cause. One patient did not proceed after induction chemotherapy, the scheduled date of stem cell infusion was chosen for calculation of survival. Lymphoma free survival was calculated from transplantation until progression or death from any cause. Patients who achieved complete remission after therapeutic DLI for progression were included in current lymphoma free survival. All patients who started with chemotherapy for lymphocyte depletion were included in the analysis of overall survival and current lymphoma-free-survival. Engraftment, chimerism and GVHD were analyzed in 27 patients that actual received RIC-SCT.

The risk score was calculated according the definitions of the European Group for Blood and Marrow Transplantation.[20;21]

Table 1: Patient characteristics

Characteristic	
No of patients	29
Median age, y (range)	50 (37-65)
Sex, no (%)	
Male	19 (66)
Female	10 (34)
Histology	
Aggressive lymphoma:	
Transformed NHL	9
Follicular lymphoma grade 3	5
Mantle cell lymphoma	1
Indolent lymphoma:	
Follicular lymphoma grade 1 and 2	5
Lymphoplasmocytic lymphoma	1
CLL/SLL	8
Prior no of chemotherapy regimens , median (range)	2 (1-6)
Prior Rituximab-chemotherapy combinations	22
Prior ProMaceCytaBom	8
Prior fludarabine/cyclophosphamide	16
Prior autologous transplant	2
Disease state at start of induction chemotherapy	
Complete remission	9
Partial remission	18
Progressive disease/not evaluated	1/1
Donor characteristics, stem cell source and RIC -regimen (n=27)	
Sibling, HLA-identical (PBSC/marrow)	20 (18/2)
TLI/Cyclophosphamide	4
Induction and conditioning with Flu/Cy	16
Unrelated (PBSC/marrow)	7 (6/1)
Flu/Cy/ATG	5
Induction Flu/Cy and conditioning with Flu/Cy/ATG	2

NHL indicates non-Hodgkin's lymphoma; CLL/SLL indicates chronic lymphocytic leukaemia/small lymphocytic lymphoma; ProMaceCytaBom indicates combination chemotherapy with cyclophosphamide, adriamycin, etoposide, prednisone, cytarabine, vincristine, bleomycin and methotrexate; PBSC indicates peripheral blood stem cells; TLI indicates total lymph node irradiation; Flu indicates fludarabine; Cy indicates cyclophosphamide and ATG indicates anti thymocyte globulin.

Results

Patient characteristics

Between January 2003 and October 2008, twenty-nine patients were enrolled in this study. Median age of these patients was 50 years (range, 37-65 years) at the time of SCT. Six patients were treated for relapsed indolent lymphoma (including follicular lymphoma grade 1 and 2, and lymphoplasmocytic lymphoma), eight patients were transplanted for progressive CLL / SLL and 15 patients for aggressive lymphoma (including follicular lymphoma grade 3 (n=5), transformed lymphoma (n=9) and relapsed mantle cell lymphoma (n=1)). Transformation from indolent lymphoma (follicular or marginal zone) to diffuse large B cell lymphoma had occurred in 8 patients and from follicular to blastoid lymphoma in 1 patient. Details on patients and disease characteristics are summarized in *table 1*.

The EBMT risk score was calculated for each patient and is shown in *table 2*.

Table 2: EBMT risk score

Risk Category	
Age classes, y, no (%)	
<20	0
20 -40	4 (14%)
>40	25 (86%)
Disease stage, no (%)	
Early	0
Intermediate	8 (28%)
Advanced	21 (72%)
Time interval, months, no. (%)	
<12	0
>12	29 (100%)
Histocompatibility, no. (%)	
HLA -identical sibling	22 (76%)
Unrelated donor	7 (24%)
Sex combination, no. (%)	
Other	22 (76%)
Recipient male, donor female	7 (24%)
Risk score, no. (%)	
0-3	0
4	9 (31%)
5	11 (38%)
6	7 (24%)
7	2 (7%)

Pretransplant lymphocyte depletion by fludarabine and cyclophosphamide

Twenty patients received induction chemotherapy with the intention to deplete CD4+ counts to a level of <0.05 x 10^9/L (*Figure 1*). In seventeen patients the target level of CD4+ depletion was reached after one cycle of chemotherapy. Three patients did not reach the target level of CD4+ cells. Two patients received a second cycle of induction chemotherapy and proceeded to RIC-SCT. The third patient who did not reach the target level of CD4 developed an EBV-driven Richter's transformation of his B-CLL after the first course of induction chemotherapy. Treatment with Rituximab induced a complete remission and the patient received a conventional myeloablative allogeneic SCT (MAC-SCT).

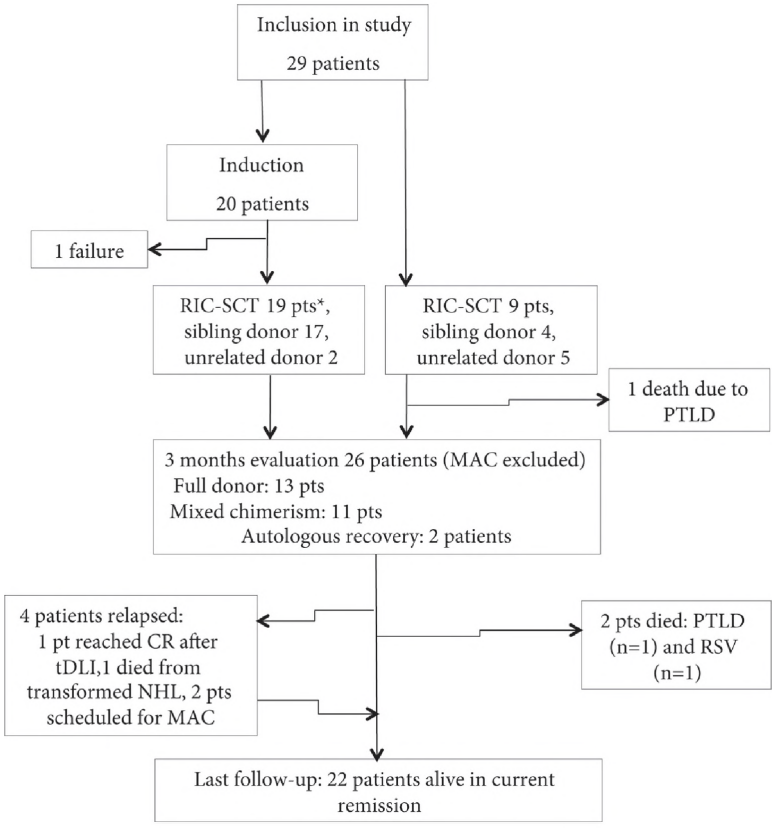

Figure 1: Flow-diagram showing the conditioning regimen, chimerism evaluation and outcome of all 29 patients enrolled in this study, one patient recieved myeloablative SCT (MAC-SCT) because of progression.

Median time between start of the induction chemotherapy and infusion of allogeneic stem cells was 33 days (range, 16-39 days) for patients receiving one induction course. For two patients receiving two induction courses, time between first induction course and infusion of the stem cells was 68 and 75 days, respectively.

Nine patients did not receive pretransplant lymphocyte depletion by induction-chemotherapy; including five patients with an unrelated donor receiving ATG and four patients treated with TLI and cyclophosphamide as conditioning regimen.

Median numbers of CD3+, CD4+ and CD8+ cells before start of the conditioning regimen in both groups, with and without pretransplant lymphocyte depletion, are shown in *table 3.*

Table 3: Median host T cell counts before start of conditioning regimen

	CD3+ x109/L	CD4+ x109/L	CD8+ x109/L	CD4+/ CD8+ ratio
Normal (5 - 95 percentile)	1.2 (0.7-2.1)	0.7 (0.3-1.4)	0.4 (0.3-1.4)	
After treatment with induction chemotherapy to deplete host T cells (n=20)	0.06 (range, 0.00-2.12)	0.02 (range, 0.00-0.34)	0.04 (range, 0.00-1.68)	0.5
Without induction chemotherapy, in heavily pre-treated patients with NHL or CLL (n=8)	0.64 (range, 0.11-1.73)	0.27 (range, 0.04-0.53)	0.27 (range, 0.06-0.53)	1.04

Donors and graft composition

Twenty-seven patients received a partial T cell-depleted graft after RIC; one patient did not proceed after the induction regimen because of severe hypertension and one patient proceeded to MAC- SCT (*Figure 1*). Donors for twenty patients were HLA-identical siblings (18 grafts from peripheral blood stem cells (PBSC) and 2 grafts from bone marrow) and unrelated donors for seven patients (6 PBSC and 1 bone marrow). Of the seven unrelated donor transplants, two were mismatched for one HLA-class I molecule. Cytotoxic T lymphocyte precursor frenquency (CTLp) was $7/10^6$ for one patient and Helper T lymphocyte frequencies (HTLp) were $<1/10^6$ for both patients.

Median number of infused CD34-positive cells was 5.6 (range, 0.6-11.6) x 10^6 per kg

body weight of recipient. Median number of CD3-positive T cells infused was 0.55 (range, 0.06-1.06) x 10^6 per kg.

Engraftment and chimerism

Median time to reach leukocytes >1.0 x 10^9/L was 12 days (range, 7-24) and median time to reach a number of platelets > 20 x 10^9/L was 10 days (range 0-19). In five patients platelets did not decline to levels < 20 x 10^9/L. Hematopoietic recovery in patients with an unrelated donor was more rapid than in patients with a sibling donor. In seven patients with an unrelated donor, median time to reach leukocytes >1.0 x 10^9/L and platelets>20 x 10^9/L were 11 and 3 days, respectively.

Twenty-five of these 27 (93%) patients had successful donor-engraftment. Two patients, transplanted with a graft from an unrelated donor, had complete autologous hematopoietic recovery. In both patients the percentage of donor cells was <1% measured from two months after RIC-SCT at different time-points. In both cases this was considered to be a primary take failure. Both patients with take failure were not pre-treated with induction therapy to reduce host T cells and CD4 counts in these two patients before start of the conditioning regimen were 0.26 x 10^9/L and 0.04 x 10^9/L respectively. One patient with a take failure was transplanted with an allograft from a one-locus mismatched unrelated donor. Mismatch was both in graft-versus-host and host-versus-graft direction. The second patient was transplanted with bone marrow stem cells from HLA-identical unrelated donor.

Twenty-six of these 27 patients were evaluated for chimerism at three months after SCT (*Figure 1*). Thirteen patients were complete donor chimera (<1% recipient cells), eleven patients were mixed chimeric (>1% recipient cells), and two patients had complete autologous recovery. Median number of recipient cells in the eleven mixed chimeric patients was 10.4% (range 1%-68%). Median number of recipient cells was higher in patients transplanted with an allograft from an unrelated donor (26%) than in patients with a sibling donor (8%). *Figure 2* shows the time to reach donor chimerism (<5% recipient cells) in 25 patients with successful donor-engraftment. Nineteen of the twenty patients transplanted with a sibling-donor developed complete donor chimerism directly or after discontinuation of immunosuppression. Five engrafted patients transplanted with an unrelated donor remained mixed chimeric which converted to complete donor chimerism in two patients after pDLI. Three patients died before reaching complete donor chimerism and one patient is alive in mixed chimerism at last follow-up.

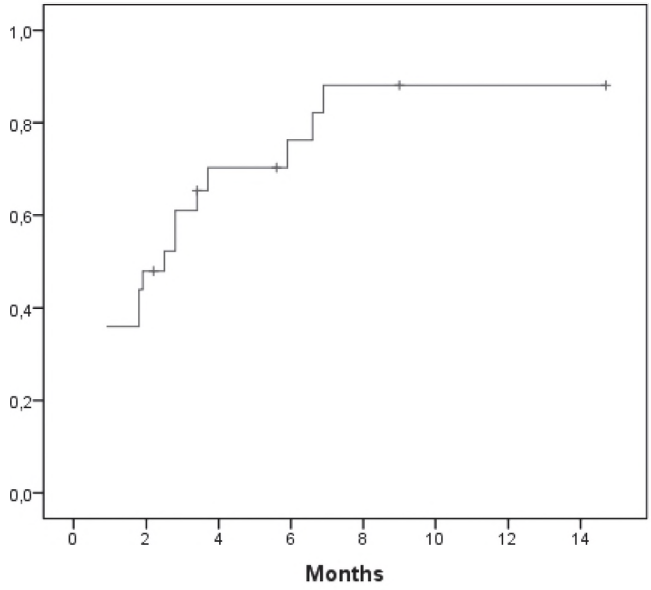

Figure 2: Time to complete donor chimerism in patients treated with RIC-SCT and with persisting donor engraftment (n=25).

GVHD and prophylaxis

Acute GVHD ≥ grade II occurred in five of 27 patients (19%): only one of these five patients suffered from aGVHD gr III. Median time from RIC-SCT to the onset of acute GVHD (including grade I) was 37 days. Twenty-five patients were evaluable for chronic GVHD. Sixteen (64%) patients developed chronic GVHD, which was extensive in three patients. Chronic GVHD was not preceded by acute GVHD in three patients. None of the seven patients with an unrelated donor who received ATG in the conditioning regimen, developed acute GVHD or chronic GVHD after RIC-SCT. GVHD was manageable with CsA, corticosteroids and UVB-therapy in all patients.

Median duration of GVHD-prophylaxis with CsA was 107 days (range, 55-245 days); only one patient was still using immunosuppression at last follow-up. Median duration of GVHD-prophylaxis was shorter for unrelated donor RIC-SCT than for patients transplanted with a sibling donor, 74 days and 138 days respectively.

Pre-emptive and therapeutic DLI after SCT

Five of 27 (19%) patients received pre-emptive DLI after RIC-SCT: two patients transplanted with a sibling donor and three patients with an unrelated donor. Only one patient received a second dose-escalated DLI of $5.0x10^6$ T cells/kg. Both patients receiving DLI from a matched sibling donor were complete donor chimeric at the time of lymphocyte infusion and none of them developed GVHD. All three patients treated with pDLI and transplanted with an allograft from an unrelated donor were mixed chimeric at the time of DLI and two of these three patients developed GVHD after pre-emptive DLI (dose of $1.0x10^6$ T cells/kg), grade I and III respectively. Both patients with GVHD converted from mixed chimerism to complete donor chimerism. Notably, twenty-two patients did not receive pre-emptive DLI, because of chronic GVHD (n=15), acute GVHD (n=2), infection (n=1), death (n=2), and primary take failure in two patients.

Four patients relapsed after RIC-SCT. Both patients with primary take failure relapsed, 13 and 14 months after RIC-SCT, and are scheduled to receive MAC-SCT. One patient died from rapidly progressive relapsed aggressive NHL after unrelated donor RIC-SCT before therapeutic DLI could be arranged. The fourth patient was transplanted for transformed NHL and relapsed 18 months after RIC-SCT. This patient was treated with two therapeutic DLIs, $10x10^6$ T cells/kg and $70x10^6$ T cell/kg respectively and responded favorably. Both DLIs resulted in graft-versus-lymphoma reactivity without GVHD. At the last follow-up, 79 months after RIC-SCT, this patient is still in remission.

Infectious complications of partial T cell-depleted RIC-SCT

As expected and documented before in RIC-SCT, the incidence of early bloodstream infections was low (10%) in our study. Furthermore, no cases of invasive fungal disease were diagnosed after RIC-SCT.

In contrast, viral infections were a major problem. Herpes zoster reactivations and infections occurred frequently (38%); with eight localized and three generalized infections. These infections occurred at a median of 195 days post-transplant (range 139-394), and all occurred after cessation of valaciclovir prophylaxis. Therefore we adjusted the protocol and valaciclovir prophylaxis is now given for one year. Five of the 29 (14%) patients had a CMV infection (CMV-I) and none developed CMV disease. CMV-I was diagnosed at a median of 44 days post-transplant (range, 33-188 days) relatively early in comparison to other reports.[18;22] CMV-I occurred

predominantly in patients who were CMV positive and received CMV negative grafts (data not shown). One of the patients with CMV-I suffered from GVHD after pDLI and received immunosuppression. Recurrent upper and lower respiratory tract infections, probably of viral origin, occurred in approximately 30%, although not all infections were microbiologically confirmed. One case of respiratory syncytial virus (RSV) was diagnosed.

Three patients (11%) developed an EBV-related lymphoproliferative disease (LPD); two after SCT (being true PTLD) and one patient developed both EBV related LPD during fludarabine/cyclophosphamide induction prior to the SCT and EBV associated Hodgkin's lymphoma after MAC-SCT.

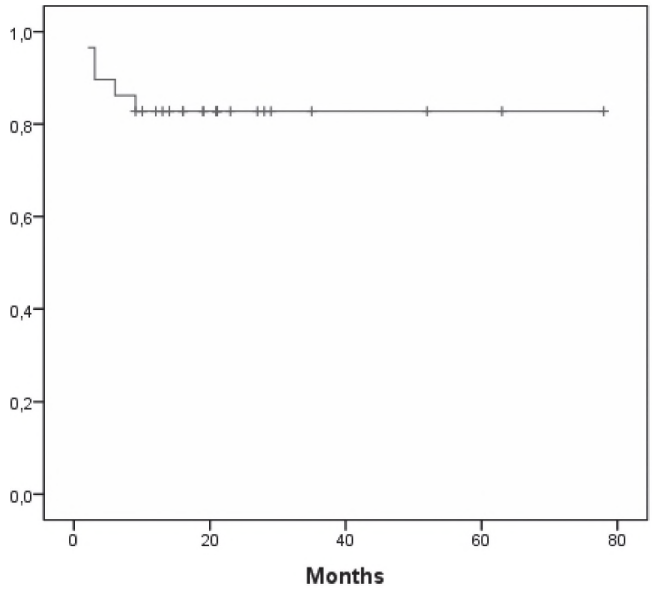

Figure 3: Estimated two-year OS of all 29 included patients was 83%.

Survival

The median follow-up for all 29 patients was 19 months (range, 3.4-79.2 months). Estimated two-year overall survival is 83% (95% confidence interval, 69%-97%) (*Figure 3*). Five patients have died after inclusion in this study (*Figure 1*). One patient

died <100 days after RIC-SCT due to PTLD. Three other patients died after RIC-SCT, one patient from relapse of blastoid transformed lymphoma, another patient

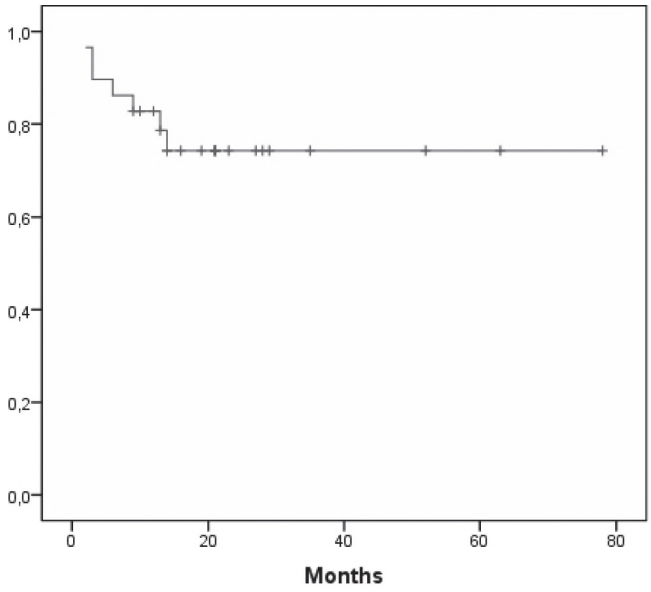

Figure 4: Estimated two-year lymphoma-free survival was 74% of all included patients.

from PTLD and one patient from infectious causes (pulmonary infection with RSV). One patient was treated with induction chemotherapy but did not proceed to RIC-SCT because of severe hypertension. This patient died 15 weeks after the induction course from infectious complications. Based on an intention-to-treat analysis, the non-relapse mortality at one year is 14%.

Twenty-four of 29 patients are alive at last evaluation in June 2009. Twenty-two patients are in complete remission at last follow-up. Both patients with a primary take failure relapsed and they are scheduled to receive MAC-SCT. Estimated two-year lymphoma-free-survival is 74% (95% confidence interval, 58%-91%) (*Figure 4*).

Discussion

This study was initiated to address the feasibility of partial T cell-depleted grafts in the setting of reduced intensity conditioning followed by pre-emptive post-transplantation immunotherapy with DLI for heavily pre-treated patients with aggressive lymphomas (transformed NHL and follicular lymphoma gr III) and indolent lymphoproliferative disorders (follicular NHL gr I/II and CLL). The application of RIC-SCT has resulted in reduced treatment-related mortality (TRM). Earlier studies using myeloablative conditioning for low-grade NHL were associated with a TRM of 26% to 40%.[23-25] TRM of myeloablative conditioning for aggressive lymphoma was in the same range of 25% to 44%.[26;27] Non-relapse mortality at one year in this study was 14%. Also, survival and current lymphoma-free-survival are favorable in our trial which were 83% and 74%, respectively. Other series reporting outcomes of RIC-SCT for lymphoma show variable results. The retrospective analysis from EBMT on RIC-SCT for lymphoma reported an OS of 62% at one year but TRM still exceeded 30%.[28] Three other studies on RIC-SCT for lymphoproliferative diseases, of which two used Alemtuzumab for in vivo T cell-depletion, showed a clear reduction of TRM of 11% to 16% with an OS of 68% to 88% at two-years.[29-31]

In this study partial T cell-depletion in vitro was performed to prevent severe acute and long-standing extensive chronic GVHD. The advantage from T cell depleted grafts consists of a better quality life due to less morbidity caused by GVHD. The incidence of acute GVHD ≥ gr II was 19%, including one patient with gr III and no patients with grade IV. Chronic GVHD developed in 64% of the patients which was extensive in only three patients. All patients with GVHD could be managed with CsA and corticosteroids and none of these patients died from GVHD. Hardy et al. analyzed donor T cell engraftment and GVHD after the same two step-RIC regimen in both T cell-depleted and T cell-replete recipients.[32] Interestingly, the overall incidence of acute GVHD was nearly identical (70%) in both groups. This can be explained by the rapid CsA tapering in their T cell-depleted group followed by early DLI-administration. In our study, the duration of CsA-treatment was longer and administration of DLI started if patients were 4 weeks without immunosuppression and without significant GVHD.

Pre-emptive DLI was part of our approach to boost GVL after partial T cell depleted RIC-SCT. Because 64% of the patients developed (predominantly limited) chronic GVHD, only 19% of the patients did receive pDLI. DLI was truly pre-emptive in two patients transplanted with a graft from a sibling donor. Three unrelated donor

transplant recipients were treated with pDLI to prevent relapse and for the correction of incomplete donor chimerism. This resulted in complete donor chimerism in two patients however in association with GVHD.

Although fifteen of the 29 patients in our study had a high grade lymphoma, relapse rate was low. The higher incidence of limited chronic GVHD in our study may explain the low relapse rate and is in line with the existence of a graft-versus-lymphoma (GVL) effect. Furthermore, GVL-reactivity may be more effective in transformed NHL than in other aggressive lymphomas, because of the underlying immune sensitive low-grade component.[33]

All patients had chemotherapy-sensitive disease and were at least in PR at the time of RIC-SCT. Previous analyses of nonmyeloablative and RIC-SCT in NHL-patients have shown that chemotherapy-resistance is an important prognostic factor on outcome. A recent analysis on the impact of the hematopoietic cell transplantation comorbidity index (HCT-CI) on outcomes of NHL patients treated with RIC-SCT underscores that response to chemotherapy pre-transplantation is an important predictor of overall transplantation outcome.[34] Also an EBMT analysis from the Lymphoma Working Party identified chemotherapy-resistant disease as a predictor of poor response to RIC-SCT.[28]

Recently, the EBMT risk score which was developed for patients with CML, was shown to be predictive for hematological diseases in general.[21] We calculated the EBMT risk score retrospectively to describe the pretransplant risk factors in our patient group, but did not use this tool for decision on treatment strategies. The majority of the patients (69%) in our study had a risk score of ≥5, indicating that this patient group was at high-risk for transplantation-related mortality and decreased survival.

Infectious complications were modest especially considering the highly immunosuppressive nature of our approach with the use of fludarabine induction, ex-vivo partial T cell-depletion and in vivo T cell-depletion with ATG in 26% of patients. Due to the absence of severe tissue damage in RIC-SCT the incidence of early bacterial infections was limited, with only three cases of early bloodstream infections (10%), similar to previous studies.[35] No cases of IFD were diagnosed after RIC-SCT despite the fact that no mould active prophylaxis was used. This is remarkable since incidences of IFD up to 10-15% have been documented before in RIC-SCT.[35;36] Although in our institution the overall incidence of IFD is low even in MAC, this can be largely explained by the low incidence of GVHD requiring long term immunosuppressive therapy. This probably also explains the low incidence of

CMV-D. The number of CMV infections was modest and most infections occurred predominantly in the first 100 days of SCT during CsA as GVHD-prophylaxis.
One concern is the occurrence of EBV related PTLD in three patients. The incidence of 10% (3/29) is high, but earlier reports have noticed an increase in PTLD after RIC-SCT up to 15%.[37-39] Although no firm conclusions can be drawn regarding the specific risk factors for PTLD in our cohort, other studies have suggested that the profound immunosuppression and incomplete depletion of recipient B-cells contributed to the higher incidence of PTLD in RIC-SCT, especially when ATG was used.[37]
Concerning the viral complications, strategies to support reconstitution of anti-viral immunity are important. Reconstitution of virus-specific T cells by using unmodified DLI will lead to GVHD in a significant number of patients. Two categories of new strategies have been developed to reconstitute virus-specific T cells post-transplant; (i) applying DLI which is depleted from alloreactive T cells or (ii) treatment with in vitro expanded virus-specific T cells.[40] Both strategies are attractive as prophylaxis or as treatment; however the generation of these products is time consuming and is confined to centers with specialized Good Manufacturing Practice (GMP) laboratories.
A variety of different regimens, usually containing fludarabine, are currently used as conditioning for RIC-SCT. Host T cell-depletion by one or two courses induction chemotherapy with fludarabine and cyclophosphamide was added to the conditioning regimen as described before by Bishop et al.[6] The extent of immunosuppression was assumed to be sufficient to facilitate donor-engraftment of a partial T cell-depleted graft. Two recipients of unrelated donors, who did not receive the induction course to deplete host T cells, developed a primary take failure. Therefore, in the setting of RIC-SCT with partial T cell-depleted grafts the use of an induction course for host T cell depletion appeared to be necessary. Furthermore, patients transplanted with stem cells from an unrelated donor remained mixed chimeric and converted to complete donor chimerism only after pDLI. In this setting the combination of both in vitro and in vivo T cell depletion by ATG induces deep T cell depletion which reduces the risk of GVHD however increases the risk of mixed chimerism and rejection.
Although our study shows favorable results regarding TRM, OS and relapse rate, there are limitations to this approach; (i) patient selection must be careful because the period of immunosuppression is long after the use of an induction course followed by the conditioning three weeks later in heavily pre-treated patients. Chemotherapy-responsiveness is therefore a prerequisite for this approach (ii) unrelated donors have

to be willing to donate lymphocytes some months after stem cell donation for pDLI and (iii) incorporation of strategies to reconstitute viral immunity are needed to make further advances in this approach.

In summary, these results demonstrate that partial T cell-depleted RIC-SCT with pre-transplant host T cell-depletion results in fast and stable engraftment. All patients transplanted with stem cells from an HLA-identical sibling donor develop complete donor chimerism during follow-up. The induction course to deplete host T cells is essential, because omitting this induction course resulted in a primary take failure in two patients. Partial T cell-depletion in vitro in combination with a relatively short course of CsA resulted in acute GVHD grade ≥II in 19% of the patients which is manageable with corticosteroids, CsA and UVB. No deaths were related to GVHD. Main complications were related to viral pathogens including varicella zoster infections and EBV-associated LPD. The rate of relapse was low, even in patients with high-grade NHL and despite the fact that only 19% of the patients received pDLI. Overall survival and current-lymphoma-free survival were favorable. Therefore, in the setting of partial T cell depleted RIC-SCT; additional improvement may be achieved by changing from unmodified pDLI for boosting GVL to transfer of lymphocytes for reconstitution of virus specific T cells.

Acknowledgements

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Chapter 6

Aberrant expression of the hematopoietic-restricted minor histocompatibility antigen LRH-1 on solid tumors results in efficient cytotoxic T cell-mediated lysis

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Abstract

CD8+ T cells recognizing minor histocompatibility antigens (MiHA) on solid tumor cells may mediate effective graft-versus-tumor (GVT) reactivity after allogeneic stem cell transplantation (SCT). Previously, we identified LRH-1 as a hematopoietic-restricted MiHA encoded by the *P2X5* gene. Here, we report that LRH-1 is aberrantly expressed on solid tumor cells. *P2X5* mRNA expression is demonstrated in a significant portion of solid tumor cell lines, including renal cell carcinoma (RCC), melanoma, colorectal carcinoma, brain cancer and breast cancer. Importantly, *P2X5* gene expression was also detected in a subset of primary solid tumor specimens derived from RCC, brain cancer and breast cancer patients. Furthermore, *P2X5* expressing solid tumor cells can be effectively targeted by LRH-1-specific cytotoxic T lymphocytes under inflammatory conditions. The expression of HLA-B7 and CD54 on tumor cells increases upon cytokine stimulation resulting in improved T cell activation as observed by higher levels of degranulation and enhanced tumor cell lysis. Overall, hematopoietic-restricted MiHA LRH-1 is aberrantly expressed on solid tumor cells and may be used as target in GVT-specific immunotherapy after SCT.

Introduction

Allogeneic hematopoietic stem cell transplantation (SCT) is an established curative treatment for patients with hematological malignancies and an experimental approach for solid tumors.[1;2] Initial evidence for a beneficial graft-versus-tumor (GVT) effect in solid tumors was demonstrated in patients with metastatic breast cancer.[3;4] Subsequent studies investigated the feasibility and safety of nonmyeloablative stem cell transplantation (NST) in order to reduce treatment-related toxicity.[5-8] In these nonmyeloablative regimens, immunosuppression allows donor engraftment but frequently results in mixed chimerism. Conversion to complete donor chimerism can be achieved by withdrawal of immunosuppression and adoptive immunotherapy by donor lymphocyte infusion (DLI).[9] Several studies have demonstrated that treatment with NST and DLI can induce tumor regressions in some types of metastatic solid tumors.[5-8] However, this GVT effect is often accompanied by severe graft-versus-host disease (GVHD), which results in considerable morbidity and mortality. Furthermore, most responses in patients with metastatic solid tumors are partial and the rate of complete remissions is limited. Therefore, further development of SCT-based allogeneic immunotherapy for solid tumors requires novel strategies to selectively boost GVT immunity in the absence of GVHD.

The GVT effect is predominantly mediated by donor-derived T cells recognizing minor histocompatibility antigens (MiHA) on malignant cells of the recipient. However, ubiquitously expressed MiHA often induce GVHD, resulting in T cell-mediated destruction of normal tissues such as skin, liver and gut. Therefore, it would be highly beneficial to direct donor T cell immunity towards MiHA that are selectively co-expressed on hematopoietic cells and solid tumor cells by vaccination or T cell therapy. Only a few hematopoietic-restricted MiHA have been described that are aberrantly expressed on solid tumors, including HA-1[10;11], ECGF-1[12] and BCL2A1.[13;14] Therefore, it is important to enlarge the panel of MiHA that can be used for immunotherapy of solid tumors.

Previously, we have identified a hematopoietic-restricted MiHA, designated LRH-1, which elicits an HLA-B7-restricted cytotoxic T cell (CTL) response in leukemia patients treated with DLI.[15] LRH-1 is encoded by the *P2X5* gene and results from a single nucleotide frameshift polymorphism between recipient and donor cells.[15] *P2X5* expression has been demonstrated in myeloid progenitor cells and peripheral blood T cells, B cells and NK cells as well as lymphoid organs. Furthermore, *P2X5* is expressed in a broad range of lymphoid malignancies resulting in effective LRH-

1-specific CTL-mediated lysis.[16] In contrast, *P2X5* mRNA was not detected in primary cell cultures of non-hematopoietic origin such as fibroblasts, keratinocytes, melanocytes and proximal tubule renal epithelial cells. Moreover, *P2X5* expression is not detected in prominent GVHD target tissues such as skin, liver, colon and small intestine.[15]

Here, we investigated whether the *P2X5*-encoded LRH-1 epitope is expressed on solid tumors in addition to hematopoietic cells. We revealed that *P2X5* mRNA is aberrantly expressed in several types of solid tumors, including renal cell carcinoma (RCC), melanoma, colorectal carcinoma, brain cancer and breast cancer. Furthermore, we demonstrated that *P2X5*-expressing solid tumor cell lines are efficiently recognized and lysed by LRH-1-specific CTL. These findings illustrate that LRH-1 can be used as target in the development of MiHA-specific immunotherapy after allogeneic SCT for metastatic solid tumors.

Materials and methods

Cell lines

The following solid tumor cell lines were used: RCC cell lines SKRC 1, 7, 10, 12, 17, 18, 24, 33, 35, 52, and 59 were kindly provided by Dr. E. Oosterwijk (Department of Urology, Radboud University Nijmegen Medical Centre (RUNMC), Nijmegen, The Netherlands); melanoma cell lines BLM, FM3, MEL397, and 518A2 were kindly provided by Dr. J. de Vries (Department of Tumor Immunology, RUNMC, Nijmegen, The Netherlands); colorectal carcinoma cell lines HT29, KATO3, HCT15, HCT116, RKO, SW480, and SW620, and brain cancer cell lines BE(-2)C, CHP212, DAOY, D283med, PFSK-1, SK-N-DZ, SK-N-SH, SW1088, TE671, and U-87MG, and breast cancer cell lines BT549, CAMA-1, MCF-7, MDA-MB-134VI, MDA-MB-157, MDA-MB-175VII, MDA-MB-361, MDA-MB-436, SK-BR-3, and T47D were kindly provided by Dr. M. Schutte (Department of Medical Oncology, Josephine Nefkens Institute, Erasmus University Medical Center, Rotterdam, The Netherlands). Skin-derived fibroblasts were kindly provided by S. van der Velde-Visser (Department of Human Genetics, RUNMC, Nijmegen, The Netherlands). All cell lines were cultured in Iscove's modified Dulbecco's medium (IMDM) (Invitrogen, Carlsbad, California) supplemented with 10% fetal calf serum (FCS). Prior to analysis solid tumor cell lines and fibroblasts and keratinocytes were cultured for 4 days in the absence or presence of inflammatory cytokines IFNγ (100 U/ml) and TNFα (10 ng/ml).

Primary tumor specimens

mRNA from 42 primary solid tumor specimens was used for gene expression analysis. mRNA from 15 RCC samples, containing ≥ 80% tumor cells, was kindly provided by Dr. E. Oosterwijk, (Department of Urology, RUNMC, Nijmegen, The Netherlands), mRNA from 13 brain tumors (4 medulla blastomas and 9 gliomas), containing ≥ 80% tumor cells [17], was kindly provided by H. Jacobs (Department of Pediatric Oncology, RUNMC, Nijmegen, The Netherlands), and mRNA from 14 breast cancer samples, containing 70-90% tumor cells [18], was kindly provided by Dr. P. Span (Department of Chemical Endocrinology, RUNMC, Nijmegen, The Netherlands).

CTL culture

CTL were cultured as previously described.[15;16] CTL RP1 recognizes the 9-mer epitope TPNQRQNVC in the context of HLA-B*0702. The HLA-B7-alloreactive CTL clone KOR18 was kindly provided by Prof. E. Goulmy (Department of Immunohematology, Leiden University Medical Center, The Netherlands).

Immunofluorescence analysis

Tumor cell lines were studied for surface expression of HLA-B7 and adhesion molecules by flow cytometry. Cells were labeled with anti-HLA-B7 (clone BB7.1-PE; Chemicon Millipore, Billerica, Massachusetts), anti-CD54 (clone 84H10; Immunotech Beckman Coulter, Fullerton, California), or anti-CD58 (clone AICD58; Immunotech) and analyzed using the Coulter FC500 flow cytometer (Beckman Coulter, Fullerton, California). The fold induction in surface expression was calculated by dividing the mean fluorescence intensity (MFI) of cytokine-stimulated cells by the MFI of unstimulated cells.

Real-time quantitative RT-PCR of the *P2X5* gene

P2X5 mRNA and *CD45* mRNA expression were determined as previously described.[15;16] The hydroxymethylbilane synthase (*HMBS*) housekeeping gene was used to normalize expression. The following gene-specific primers and Taqman probes were used: *P2X5*; *P2X5*-F 5'-TCCTGGCGTACCTGGTCGT-3', *P2X5*-R 5'-CTTCATTCTCAGCACAGACGTTC-3' and *P2X5*-probe 5'-(TET)-TGGGTGTTCCTGATAAAGAAGGGTTACCA-(TAMRA)-3', *CD45*; *CD45*-F 5'TTAGAAATGAGTCGCATAAGAATTGC-3', *CD45*-R 5'-CTCCAGGATAGTCTCCATTGTGAAA-3' and *CD45*-probe 5'-(TET)-

ATTTCCGTGTAAAAGATC-(TAMRA)-3', and *HMBS*; *HMBS*-F 5'-GGCAATGCGGCTGCAA-3', *HMBS*-R 5'-GGGTACCCACGCGAATCAC-3' and *HMBS*-probe 5'-(VIC)-CTCATCTTTGGGCTGTTTTCTTCCGCC-(TAMRA)-3'. *P2X5* mRNA expression was quantified by determining calibration functions using JVM-2 as reference cell line. The level of *P2X5* expression was calculated relative to the *P2X5* expression in the JVM-2 cell line, which is susceptible to lysis by LRH-1-specific CTL.

Table 1: Characteristics of the solid tumor cell lines used in flow cytometry-based cytotoxicity assays.

Cell line	Tumor type	*P2X5* genotype[A]	*P2X5* mRNA expression[B]		HLA-B7 expression[D]		
			Control	IFNγ + TNFα[C]	Type	Control	IFNγ + TNFα[C]
DAOY	Brain tumor	C/C	0.88	0.70	endogenous	3.4	10.4
BLM	Melanoma	C/C	1.54	1.58	endogenous	1.5	5.1
SKRC33	RCC	C/-	0.60	0.96	endogenous	3.9	15.1
SKRC18	RCC	C/-	0.50	0.42	ectopic	6.5	6.8
FM3	Melanoma	-/-	0.66	0.61	ectopic	N/D	N/D
LRH-1$^+$ LCL	N/A	C/-	2.8	N/D	endogenous	N/D	N/D
LRH-1$^-$ LCL	N/A	-/-	2.5	N/D	endogenous	N/D	N/D

N/A indicates not applicable

N/D indicates not determined

A *P2X5* genotyping was performed by PCR amplification of genomic DNA using allele-specific probes.

B Normalized *P2X5* mRNA expression was determined by real-time quantitative RT-PCR and expressed relative to the expression level in the B-cell prolymphocytic leukemia (B-PLL) JVM-2 cell line.

C Inflammatory conditions are mimicked by tumor cell culture in the presence of inflammatory cytokines IFNγ and TNFα for 4 days.

D HLA-B7-cell lines were transduced with HLA-B7. Expression of HLA-B7 was measured by flow cytometry and expressed as mean fluorescence intensity (MFI). The MFI of the isotype control varied between 0.24-0.34.

P2X5 genotyping

P2X5 genotyping was performed as previously described.[16] The following gene-specific primers and Taqman probes were used: *P2X5*; *P2X5*-EXON3-F 5'-CCAAATCAAACCTCAGCACAGAC-3', *P2X5*-EXON3-R

5'-CTCAGTGCCTCTCTGGTTCCTTA-3', *P2X5* 5C allele-specific probe 5'-(FAM)-ATTGTGACCCCCAACCA-(MGB)-3' and *P2X5* 4C allele-specific probe 5'-(VIC)-TGTGACCCCAACCAG-(MGB)-3'.

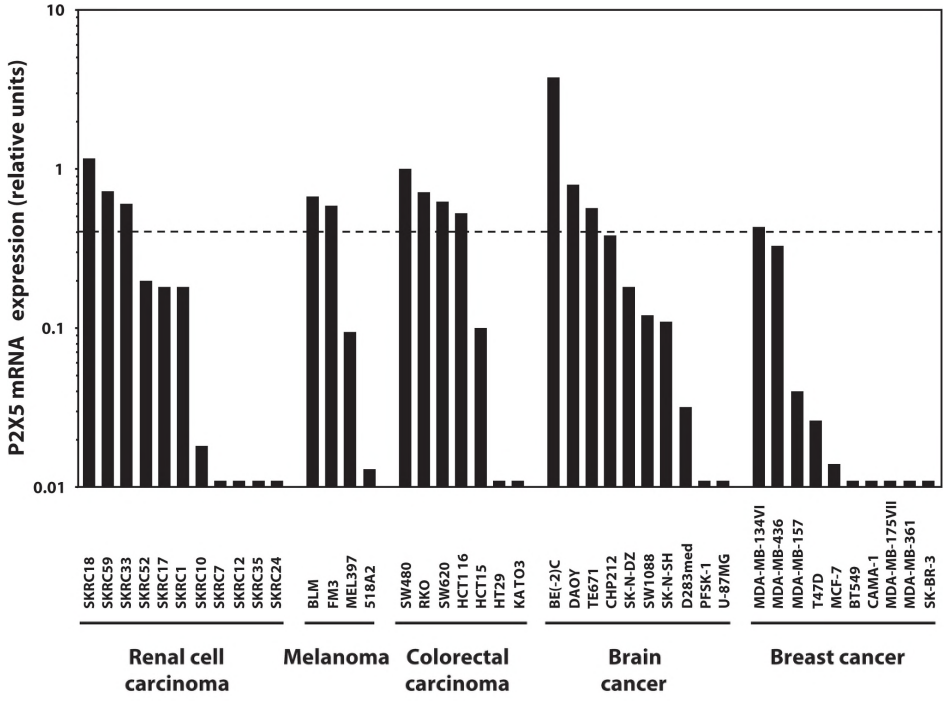

Figure 1: *P2X5* gene expression in solid tumor cells. *P2X5* mRNA expression was determined by real-time quantitative RT-PCR in 42 solid tumor cell lines. Cell lines from the following tumor types were analyzed: renal cell carcinoma (RCC; n=11), melanoma (n=4), colorectal carcinoma (n=7), brain cancer (n=10), and breast cancer (n=10). Expression is shown relative to the *P2X5* expression measured in reference B-cell line JVM-2, which is susceptible to lysis by LRH-1-specific CTL. The housekeeping gene *HMBS* was used for normalization. Solid tumor cell lines with *P2X5* expression higher than 0.4 were considered positive and potential targets for LRH-1-specific CTL, based on previous expression and recognition studies. This arbitrary threshold is indicated with a dashed line.

Flow cytometry-based cytotoxicity studies

Flow cytometry-based cytotoxicity assays were performed as previously described with minor adaptations.[16;19] Characteristics of the solid tumor cell lines used as targets are described in *Table 1*. Endogenous HLA-B7$^+$ cell lines were labeled with 2.5 μM carboxyfluorescein diacetate succimidyl ester (CFSE; Molecular Probes Europe, Leiden, The Netherlands). Alternatively, endogenously HLA-B7$^-$ cell lines were retrovirally transduced with LZRS-HLA-B*0702-IRES-EGFP as previously described[15] and used as targets without CFSE-labeling. Target cells (1 x 10^4) were co-cultured with unlabelled effector cells (3 x 10^4) at an E:T ratio of 3:1 in a total volume of 200 μl IMDM/10% FCS containing 25 U/ml IL-2 in 96-wells flat-bottom plates. For measurement of T cell degranulation at day 1, anti-CD107a (BD Biosciences, Franklin Lakes, New Jersey) was added to the T cells prior to co-culture. After 1 to 3 days of co-culture, cells were harvested and 7-amino-actinomycin D (7AAD) was added. Numbers of viable target cells were quantified by flow cytometry.

Results

Expression of the *P2X5* gene in solid tumor cells

To define whether non-hematopoietic malignancies could be targets for LRH-1-specific CTL, we have analyzed *P2X5* mRNA expression in solid tumor cell lines and primary tumor specimens by real-time quantitative RT-PCR. We tested a panel of 42 cell lines of various histological origin including RCC, melanoma, colorectal carcinoma, brain cancer and breast cancer. In addition, we analyzed primary tumor specimens derived from 15 patients with RCC, 13 patients with brain cancer, and 14 patients with breast cancer. Previously, we found that EBV-transformed lymphoblastoid cell lines (EBV-LCL) that have a mean expression level of 2.7 compared with the reference B cell line JVM-2 are significantly recognized by LRH-1-specific CTL.[15] In contrast, monocytes and fibroblasts with a mean *P2X5* mRNA expression level of 0.28 and 0.10, respectively, are not susceptible to LRH-1 CTL-mediated lysis. Based on these observations, we used a cut-off *P2X5* mRNA level of 0.4 to distinguish *P2X5*-positive from *P2X5*-negative cell types.[15] Interestingly, significant *P2X5* mRNA expression was observed in 13 out of 42 (31%) solid tumor cell lines, covering all cancer types studied (*Figure 1*). We detected *P2X5* transcripts in 3 out of 11 RCC cell lines, in 2 out of 4 melanoma cell lines, in 4 out of 7 colorectal carcinoma cell lines, in 3 out of 10 brain cancer cell lines, and in 1 out of 10 breast

Table 2: *P2X5* and *CD45* gene expression in primary solid tumor cells.

Tumor type	Sample	*P2X5* expression	*CD45* expression	Ratio (*P2X5*/*CD45*)
Renal cell carcinoma	1	3.84	6.51	0.6
	2	3.03	1.32	2.3
	3	2.30	1.49	1.5
	4	1.50	4.59	0.3
	5	1.34	8.47	0.2
	6	1.26	1.29	1.0
	7	0.47	0.78	0.6
	8	0.40	2.22	0.2
	9	0.34	1.01	0.3
	10	0.33	2.01	0.2
	11	0.22	1.98	0.1
	12	0.17	1.17	0.1
	13	0.16	2.45	0.1
	14	0.11	1.92	0.1
	15	0.09	0.67	0.1
Brain cancer	1	1.11	1.56	0.7
	2	1.06	0.36	2.9
	3	0.70	0.24	2.9
	4	0.35	1.57	0.2
	5	0.23	0.90	0.3
	6	0.19	0.07	2.7
	7	0.13	0.07	1.7
	8	0.10	1.95	0.1
	9	0.08	1.09	0.1
	10	0.02	0.16	0.1
	11	0.02	0.79	<0.1
	12	0.02	1.34	<0.1
	13	0.01	0.09	0.2
Breast cancer	1	1.48	0.74	2.0
	2	0.54	0.40	1.4
	3	0.52	1.32	0.4
	4	0.38	0.49	0.8
	5	0.13	0.12	1.1
	6	0.08	0.69	0.1
	7	0.06	0.49	0.1
	8	0.06	0.45	0.1
	9	0.03	0.58	0.1
	10	0.03	0.36	0.1
	11	0.02	0.39	0.1
	12	0.02	0.40	<0.1
	13	0.01	0.17	0.1
	14	0.01	0.17	0.1

Normalized *P2X5* and *CD45* mRNA expression was determined by real-time quantitative RT-PCR and expressed relative to the expression level in the B-cell prolymphocytic leukemia (B-PLL) JVM-2 cell line.

cancer cell lines. In the primary tumor samples studied, we detected *P2X5* mRNA levels of ≥0.4 in 8 out of 15 RCC samples, 4 out of 13 brain cancer samples, and 4 out of 14 breast cancer samples (*Table 2*). To determine whether *P2X5* expression could be due to the presence of hematopoietic cells, we also analyzed expression of *CD45*. We calculated the ratio of *P2X5*/*CD45* and considered that *P2X5* expression in samples with a ratio <1.0 probably resulted from infiltrated hematopoietic cells. We observed that in at least 3 out of 15 RCC samples, 2 out of 13 brain cancer samples, and 2 out of 14 breast cancer samples *P2X5* mRNA levels of ≥0.4 with a *P2X5*/*CD45* ratio of >1.0 could be detected (*Table 2*). These findings corroborate with our results observed in solid tumor cell lines. Together, these data show that the LRH-1-encoding *P2X5* gene is significantly expressed in a subset of solid tumors.

P2X5-expressing solid tumor cells are efficiently lysed by LRH-1-specific CTL

Target cell recognition by LRH-1-specific CTL is controlled by a single cytosine deletion polymorphism (rs5818907) in exon 3 of the *P2X5* gene.[15] Therefore, *P2X5*-expressing tumor cell lines were genotyped at rs5818907 by allele-specific PCR. Based on this *P2X5* genotyping analysis, we have selected the homozygous (C/C genotype) or heterozygous (C/- genotype) LRH-1+ tumor cell lines DAOY (brain cancer), BLM (melanoma), SKRC33 and SKRC18 (both RCC) as targets for LRH-1-specific CTL in cytotoxicity assays (*Table 1*). For these experiments, we used a flow cytometry-based cytotoxicity assay, which facilitates determination of target cell proliferation and death by both rapid and more slowly T cell effector mechanisms.[19] Moreover, the addition of low-dose IL-2 to the co-cultures prolongs CTL survival, which allows continuous exposure and serial killing of tumor cells by the CTL.

Using this in vitro assay, we have demonstrated that LRH-1-specific CTL efficiently lysed LRH-1$^+$ EBV-LCL, whereas no cytotoxicity was observed against LRH-1$^-$ EBV-LCL (*Figure 2a*). Interestingly, regarding the solid tumor cell lines, we observed that the LRH-1$^+$ brain cancer cell line DAOY could also be efficiently targeted by LRH-1-specific CTL (*Figure 2b*). Microscopic analysis confirmed high susceptibility of DAOY for cytotoxicity by LRH-1-specific CTL (*Figure 2c*). LRH-1$^+$ melanoma cell line BLM, which has a relative high *P2X5* mRNA expression level of 1.5, was less efficiently lysed by LRH-1-specific CTL (*Figure 3a*).

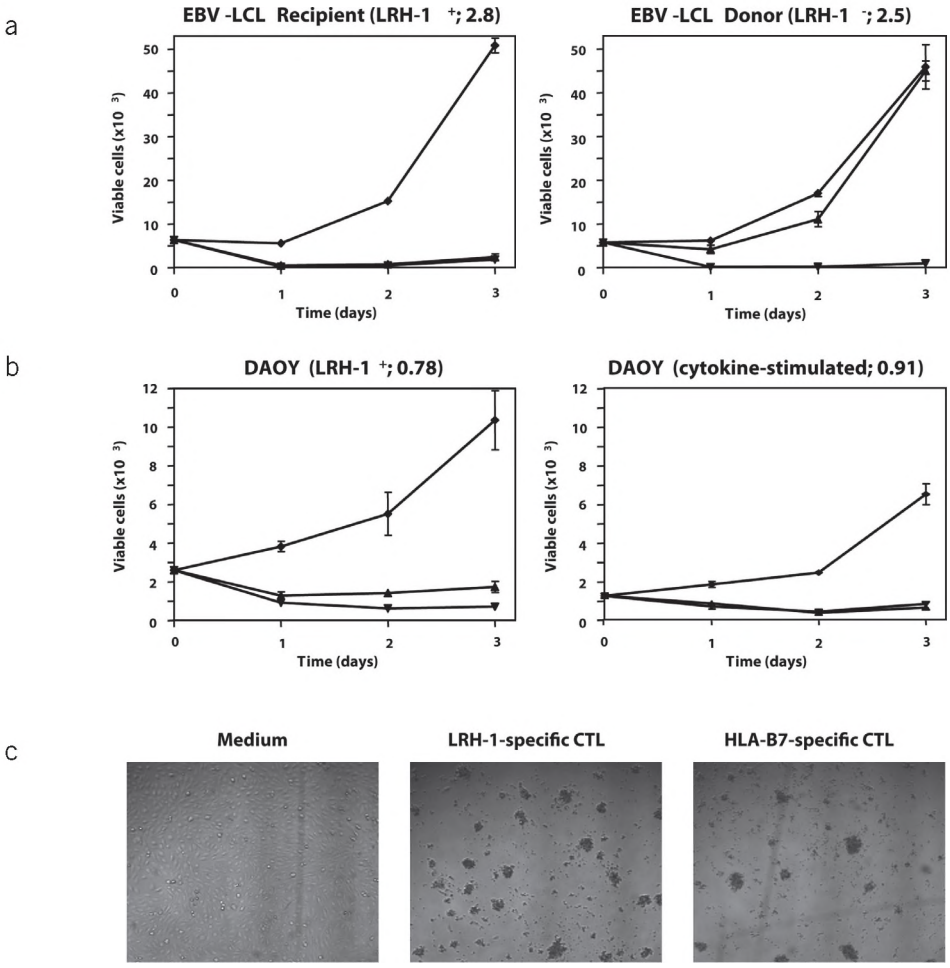

Figure 2: Cytotoxicity against solid tumor cell lines was determined after incubation with LRH-1-specific CTL (▲), HLA-B7-specific CTL (▼; positive control) or medium only (◆). Survival of unstimulated and cytokine-stimulated target cells in the absence or presence of CTL at an E:T ratio of 3:1 is shown of an LRH-1$^+$ and LRH-1$^-$ EBV-LCL (a) and of LRH-1$^+$ brain tumor cell line DAOY (b). *P2X5* mRNA expression is shown between parentheses. Data are depicted as mean ± SD of triplicate wells. (c) Microscopic analysis of cytokine-stimulated DAOY cells at 40 hours of co-culture with CTL or medium.

However, stimulation of BLM with inflammatory cytokines IFNγ and TNFα strongly enhanced susceptibility to lysis by LRH-1-specific CTL. Similar results were obtained with LRH-1$^+$ RCC cell lines. Lysis of RCC cell line SKRC33 was significantly enhanced

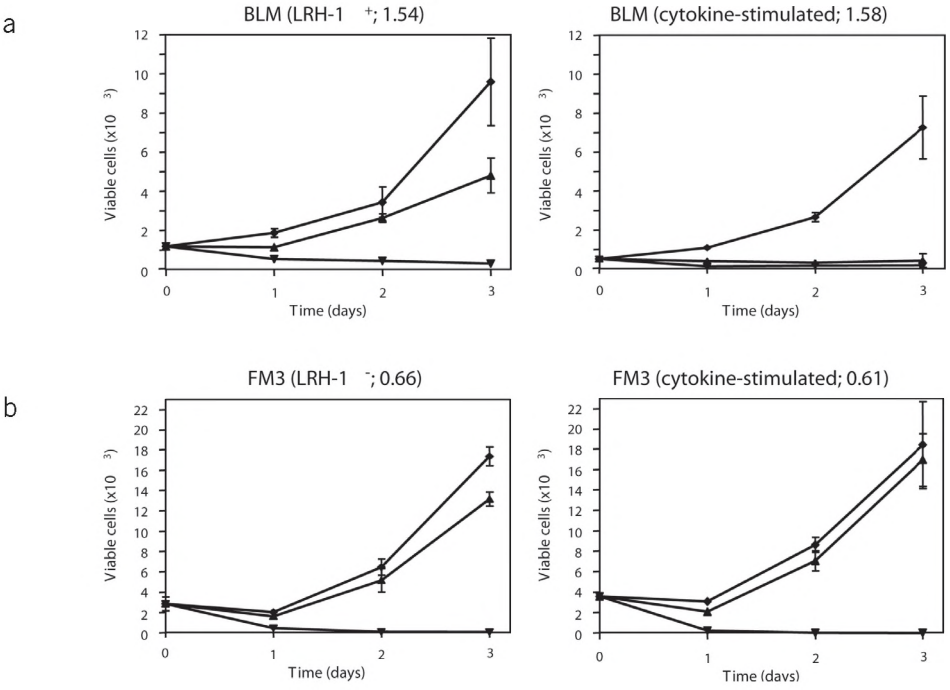

Figure 3: Cytotoxicity against LRH-1$^+$ melanoma cell line BLM (a) and LRH-1$^-$ melanoma cell line FM3 (b) was determined after incubation with LRH-1-specific CTL (▲), HLA-B7-specific CTL (▼; positive control) or medium only (◆). Survival of unstimulated and cytokine-stimulated target cells in the absence or presence of CTL at an E:T ratio of 3:1 is shown. *P2X5* mRNA expression is shown between parentheses. Data are depicted as mean ± SD of triplicate wells.

upon pre-incubation with IFNγ and TNFα (*Figure 4a*). In contrast, LRH-1 CTL-mediated lysis of SKRC18 could not be enhanced by inflammatory cytokines, which might be associated with the relative low *P2X5* mRNA expression of 0.42 (*Figure 4b*). In addition, SKRC 18 did not endogenously express HLA-B7 and HLA-B7 expression could not be upregulated by cytokine pre-incubation (*Figure* 5a). The observation that exogenous loading of SKRC18 with LRH-1 peptide resulted in complete lysis indicated that the low responsiveness is most likely caused by low epitope density at the cell surface. No cytotoxicity was observed against HLA-B7-transduced LRH-1$^-$ FM3 melanoma cells either unstimulated or pre-stimulated with inflammatory

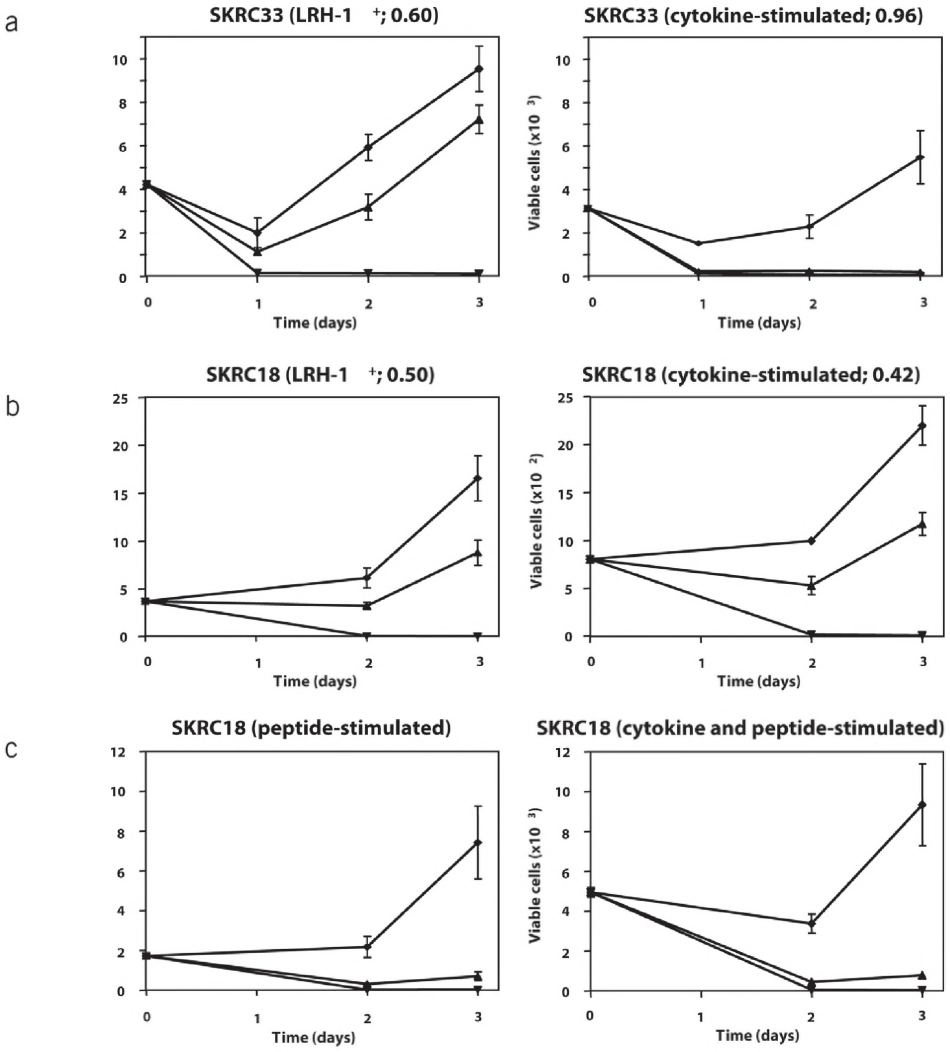

Figure 4: Cytotoxicity against LRH-1$^+$ RCC cell lines SKRC33 (a) and SKRC18 (b) was determined after incubation with LRH-1-specific CTL (▲), HLA-B7-specific CTL (▼; positive control) or medium only (◆). Survival of unstimulated and cytokine-stimulated target cells in the absence or presence of CTL at an E:T ratio of 3:1 is shown. *P2X5* mRNA expression is shown between parentheses. Data are depicted as mean ± SD of triplicate wells. (c) LRH-1-peptide loading resulted in improved lysis of SKRC18.

cytokines (*Figure 3b*). Allo-HLA-B7-specific CTL lysed both LRH-1$^+$ and LRH-1$^-$ solid tumor cell lines (*Figure 2, 3* and *4*). Taken together, these data demonstrate that

LRH-1$^+$ solid tumor cell lines which have sufficient *P2X5* expression levels can be effectively targeted by LRH-1 CTL, especially under inflammatory conditions.

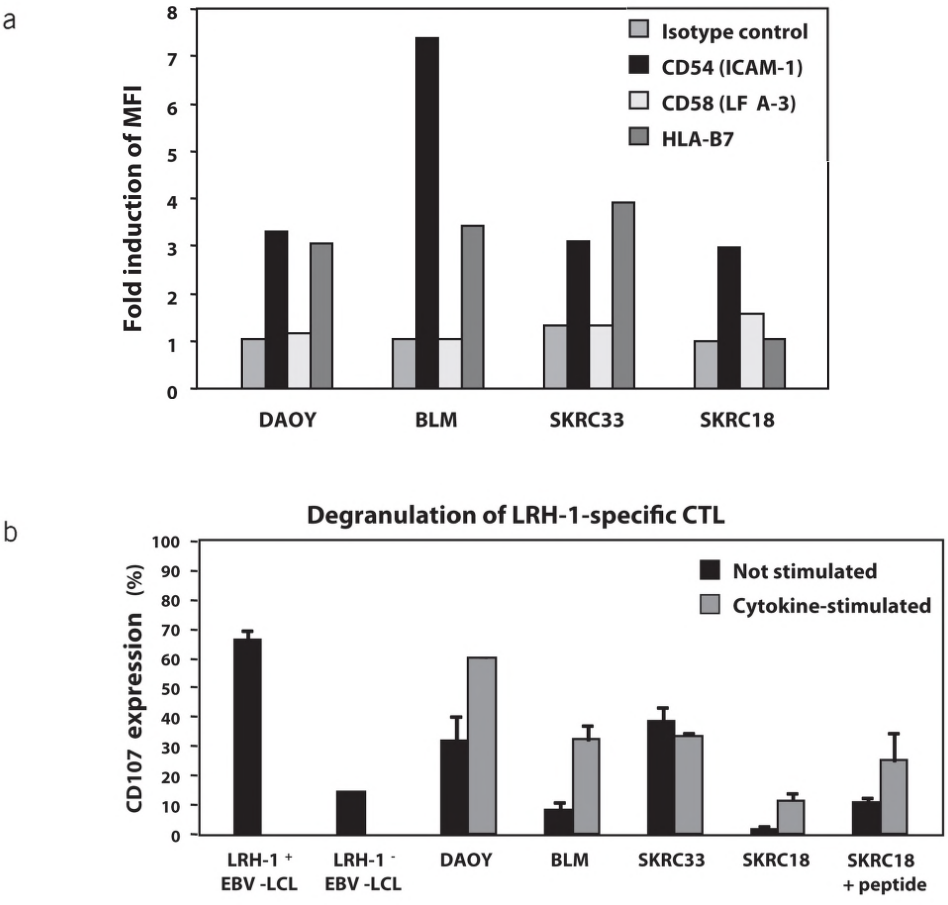

Figure 5: (a) The expression of adhesion and MHC molecules in epithelial cancer cell lines. Surface expression of HLA-B7, CD54 (ICAM-1) and CD58 (LFA-3) was determined by flow cytometry. Prior to analysis cells were cultured for 4 days in the absence or presence of inflammatory cytokines IFNγ and TNFα. Data are depicted as fold induction of the mean fluorescence intensity (MFI) upon cytokine stimulation. (b) Cytokine stimulation of cancer cells results in improved degranulation of CTL. Anti-CD107-antibody was added to the co-cultures of flow cytometry-based cytotoxicity assays. Degranulation of LRH-1-specific CTL was measured after 1 day of co-culture with solid tumor cell lines and control EBV-LCL. Data are depicted as mean ± SD of triplicate measurements.

Table 3: *P2X5* gene expression in keratinocytes and fibroblasts.

Tissue type	Sample	*P2X5* mRNA expression[A]	
		Control	IFNγ + TNFα[B]
Keratinocytes	1	0.01	0.01
	2	0.01	0.01
	3	0.01	0.01
Fibroblasts	1	0.04	0.06
	2	0.14	0.33
	3	0.19	0.08

A Normalized *P2X5* mRNA expression was determined by real-time quantitative RT-PCR and expressed relative to the expression level in the B-cell prolymphocytic leukemia (B-PLL) JVM-2 cell line

B Inflammatory conditions are mimicked by cell culture in the presence of inflammatory cytokines IFNγ and TNFα for 4 days prior to RNA isolation

Enhanced tumor cell lysis under inflammatory conditions is associated with higher levels of degranulation by LRH-1-specific CTL

To determine whether up-regulation of *P2X5* mRNA could explain the increased tumor cell lysis upon stimulation with inflammatory cytokines, we measured *P2X5* mRNA levels of untreated and cytokine-treated solid tumor cell lines by real-time quantitative RT-PCR. However, no significant change in *P2X5* mRNA expression was observed in the presence of cytokines (*Table 1*). In addition, we observed that *P2X5* expression remains undetectable in keratinocytes and very low in skin-derived fibroblasts in the presence of inflammatory cytokines (*Table 3*). On the other hand, flowcytometric analysis showed that expression of adhesion molecule CD54 (ICAM-1) increased 3.0 to 7.4-fold in solid tumor cell lines upon cytokine stimulation, whereas no significant changes were observed in expression of CD58 (LFA-3) (*Figure 5A*). Furthermore, cytokine stimulation resulted in more than 3-fold increase of HLA-B7 expression in cell lines which are endogenously HLA-B7$^+$ (*Figure 5A*).

Next, we determined whether up-regulation of HLA-B7 and CD54 expression on solid tumor cells resulted in increased activation of LRH-1-specific CTL. Therefore, we measured CTL degranulation by expression of CD107a on the cell surface after 1 day

of co-incubation with solid tumor cell lines and control EBV-LCL. The proportion of LRH-1-specific CTL expressing CD107a following co-culture with LRH-1-positive and -negative EBV-LCL was 66% and 14%, respectively (*Figure 5B*). Consistent with the cytotoxicity data observed in the flow cytometry-based cytotoxicity assays, the percentage CD107a$^+$ CTL was high after co-culture with LRH-1$^+$ solid tumor cell lines DAOY (32%) and SKRC33 (39%), but relatively low for BLM (8%) and SKRC18 (2%). In addition, when co-cultured with tumor cells pre-treated with inflammatory cytokines the proportion of CD107a$^+$ LRH-1-specific CTL significantly increased to 60% for DAOY and 32% for BLM. SKRC18 tumor cells stimulated 25% of LRH-1-specific CTL to degranulate only after pre-treatment with cytokines and loading with LRH-1 peptide. Elevated levels of CTL degranulation correlated with better lysis of tumor cells in cytotoxicity assays.

Together, these results suggest that inflammatory cytokines improve the formation of an immunological synapse by up-regulation of MHC class I and CD54, resulting in higher levels of granule exocytosis and enhanced CTL reactivity. The increased activation of CTL upon cytokine stimulation results in improved tumor cell lysis as observed in cytotoxicity assays.

Discussion

MiHA are considered to play an important role in GVT reactivity after allogeneic SCT for solid tumors.[13;14;20] Especially MiHA with tumor-restricted tissue distribution are promising targets to boost GVT reactivity selectively without enhancing GVHD. For further development of tumor-specific immunotherapy it is important to enlarge the spectrum of molecularly identified MiHA which are selectively expressed in solid tumors and possibly hematopoietic cells. Here, we analyzed the expression of the LRH-1-encoding *P2X5* gene in a large panel of solid tumor cell lines and primary tumor specimens using real-time quantitative RT-PCR. Significant *P2X5* mRNA levels were detected in 31% of the solid tumor cell lines tested, including RCC, melanoma, colorectal carcinoma, brain cancer and breast cancer. Primary samples could be tested for RCC, brain cancer and breast cancer and showed similar numbers of *P2X5* positive samples per tumor type. Therefore, LRH-1 belongs to the few hematopoietic-restricted MiHA that additionally show an aberrant expression on solid tumor cells. Expression on solid tumor cells was previously demonstrated for MiHA HA-1[10;11], ECGF-1[12], and BCL2A1.[13;14] Enlargement of the panel of MiHA

is essential for development of effective posttransplantation immunotherapy. We and others observed that only a part of the tested cell lines and patient samples are positive for MiHA expressed on solid tumors. In addition, the applicability of MiHA is dependent on the frequencies of the MiHA and presenting-HLA phenotypes. Therefore, identification of additional MiHA is essential for further improvement of tumor-specific immunotherapy. All currently molecularly described MiHA are identified from patients with hematological malignancies or aplastic anemia. Molecular identification of MiHA from patients transplanted for a metastatic solid tumor has not yet been reported. However, Tykodi et al.[20] have described $CD8^+$ CTL clones recognizing MiHA on RCC tumor cells that have been isolated from patients transplanted for metastatic RCC. They demonstrated that CTL clones from two different patients recognize the same MiHA. Though the gene encoding this MiHA was linked to chromosome 19q, they did not describe the molecular characterization of this MiHA yet. Dorrschuck et al.[21] obtained RCC-reactive CTL from co-cultures of mixed lymphocytes with tumor cells. Using microcapillary liquid chromatography and MALDI-TOF spectrometry they identified an HLA-A*0301-associated non-polymorphic peptide, encoded by the ubiquitously expressed *Eps-15 homology domain-containing 2* (*EHD2*) gene. Since *EHD2* is broadly expressed in malignant and normal tissues, antigens encoded by this gene are no candidates for tumor-specific immunotherapy.

Recently, the first RCC-associated antigen, CT-RCC1, was identified using allogeneic T cells isolated from a patient treated with allogeneic SCT for metastatic RCC.[22] The antigenic CT-RCC region is a part of the human endogenous retrovirus (HERV) type E locus. Interestingly, HERV-E is selectively expressed on RCC cells, but not on normal tissues including renal cells. However, CT-RCC is presented by HLA-A11 which is only present in a minority of the population. Thus, until more solid tumor-associated antigens or solid tumor-specific MiHA are identified, hematopoietic restricted MiHA co-expressed on solid tumors represent very useful targets for immunotherapy.

Here, two different flow cytometry-based assays were used to study tumor cell recognition. Target cell death was determined with a cytotoxicity assay to study survival kinetics of tumor cells up to 3 days. Cytolytic activation of LRH-1-specific CTL was measured by levels of degranulation. We demonstrated that $LRH\text{-}1^+$ cell lines were susceptible to LRH-1-specific CTL-mediated lysis. Especially, brain tumor cell line DAOY was very efficiently lysed by LRH-1-specific CTL. Lysis of

melanoma cell line BLM and RCC cell line SKRC33 was significantly enhanced by pre-incubation of target cells with inflammatory cytokines IFNγ and TNFα. *P2X5* mRNA expression was not up-regulated in response to inflammatory cytokines, as was previously demonstrated for MiHA BCL2A1 in non-hematopoietic cells.[23] However, cytokine stimulation generally resulted in an increase of HLA-B7 and CD54 expression on tumor cells and higher levels of CD107a expression on LRH-1-specific CTL. Therefore, these results suggest that presence of inflammatory cytokines results in an improved immunological synapse and better T cell activation, which leads to increased target cell lysis. Inflammatory cytokines are known to play a role in anti-tumor immunity.[24] The importance of an inflammatory environment was previously demonstrated in a GVT study in mice, in which secretion of IFNγ was required for eradication of melanomas in vivo by MiHA-specific CTL. Release of IFNγ resulted in inhibition of tumor angiogenesis and up-regulation of MHC class I expression on tumor cells.[25] Importantly, we have demonstrated that *P2X5* is selectively expressed in hematopoietic cells[15] and a subset of solid tumor cells, but not in GVHD target tissues such as skin, liver and gut.[15] In addition, here we show that cytokine treatment has an effect on improved lysis of solid tumor cells, whereas *P2X5* expression remains undetectable in keratinocytes and very low in skin-derived fibroblasts in the presence of inflammatory cytokines.

Despite the lysis stimulating effect of inflammatory cytokines, partial lysis of RCC cell line SKRC18 could not be enhanced by cytokine pre-incubation. SKRC18 cells could only be efficiently lysed by LRH-1-specific CTL after exogenous loading with LRH-1 peptide. This may indicate that the *P2X5* mRNA level, which was just above the threshold of 0.4 (*Table 1*), was not sufficient for complete lysis. In addition, SKRC 18 did not endogenously express HLA-B7 and HLA-B7 expression could not be up-regulated by cytokine pre-incubation. Another explanation may be a defect in intracellular antigen processing. Resistance to lysis due to defects in antigen-processing mechanisms was previously demonstrated for HA-1^{+} solid tumor cell lines.[26] Miyazaki et al describe two HA-1^{H} CTL-resistant cell lines, of which one cell line became susceptible to CTL-mediated lysis after cytokine treatment resulting in restoration of TAP-gene expression. Defects in antigen processing are important mechanisms for metastatic solid tumors to escape from immune-control and may limit the effectiveness of immunotherapy.[27]

In conclusion, our data show that the *P2X5* gene, which encodes MiHA LRH-1, is aberrantly expressed in a subset of solid tumor cell lines and primary solid tumors.

P2X5 expressing tumor types include RCC, melanoma, colorectal carcinoma, brain tumor and breast tumor. Notably, LRH-1$^+$ tumor cells are susceptible to LRH-1 CTL-mediated lysis as shown by flow cytometry-based cytotoxicity assays. This susceptibility may be strongly enhanced by the presence of inflammatory cytokines. Therefore, LRH-1 is an important addition to the panel of hematopoietic-restricted MiHA that are additionally expressed on solid tumor cells and can be exploited for post-transplantation GVT-specific immunotherapy.

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Chapter 7

Minor histocompatibility antigen-specific T cell responses after partial T cell-depleted reduced intensity stem cell transplantation and donor lymphocyte infusion in renal cell carcinoma

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Abstract

Nonmyeloablative allogeneic stem cell transplantation (SCT) can induce remission in patients with renal cell carcinoma (RCC), but this graft-versus-tumor effect is often accompanied by graft-versus-host disease (GVHD). Here, we evaluated feasibility and minor histocompatibility antigen (MiHA)-specific T cell responses in four patients with metastatic RCC who were treated with reduced-intensity conditioning SCT (RIC-SCT) followed by donor lymphocyte infusion (DLI). Two of these patients did not develop GVHD and received DLI to boost graft-versus-tumor immunity. Interestingly, one patient had stable disease and the second patient experienced partial regression of lung metastases. In the patient with stable disease, emergence of SMCY.A2-specific CD8+ T cells was observed after DLI with the potential of targeting SMCY-expressing RCC tumor cells. In the other patient with partial remission, a TCR-Vb4+CD8+ CTL clone emerged following DLI with the capability of targeting RCC cell lines. Functional analysis revealed that this CTL recognizes an HLA-B7-restricted MiHA, which is co-expressed by RCC as well as hematopoietic tumor cells. Furthermore, genotyping analysis for known HLA-B7 presented MiHA showed no mismatches, indicating that TCR-Vb4+CD8+ CTL recognizes a MiHA of yet unknown origin. These findings illustrate that partial T cell-depleted RIC-SCT can induce stable engraftment and MiHA-specific T cell responses in metastatic RCC patients. However, transplantation-related mortality and toxicity due to infections needs to be resolved before MiHA-based T cell immunity can be safely exploited for the benefit of patients with metastatic RCC.

Introduction

Allogeneic stem cell transplantation (SCT) has become the treatment of choice for patients with various hematological malignancies and some studies have also shown that metastatic renal cell carcinoma (RCC) does respond to this therapy.[1] Several studies have explored allogeneic SCT after nonmyeloablative or reduced intensity conditioning (RIC) with or without donor lymphocyte infusions (DLI) as curative treatment for metastatic RCC, and objective responses varied from 0% to 53%.[2-5] However, substantial transplantation-related mortality and toxicity due to graft-versus-host-disease (GVHD) have been observed in these trials. Therefore, further development of allogeneic SCT for solid tumors demands a more specific approach to selectively boost graft-versus-tumor (GVT) reactivity without enhancement of GVHD.

Minor histocompatibility antigens (MiHA) are the target antigens of the GVT response, and expansion of MiHA-specific cytotoxic T lymphocytes (CTL) usually precedes clinical remission of the malignancy in patients treated with DLI.[6,7] However, alloreactive CTL responses induced upon DLI generally lack tumor specificity and are often accompanied by GVHD. Therefore, it would be highly beneficial to direct T cell immunity towards MiHA that are selectively expressed on solid tumor cells. Only a few tissue-restricted MiHA have been described that are aberrantly expressed on solid tumors, including HA-1[8,9], ECGF-1[10], BCL2A1[11], LRH-1 and C19orf48.[12] Of special interest is the HLA-A2-restricted MiHA C19orf48 since it was identified using CTL obtained from RCC patients with tumor regression after SCT.[12] However, further characterization of the target antigens of alloreactive T cells on RCC tumor cells is of great importance for the development of specific post-transplant immunotherapy for metastatic RCC.

Here, we report the clinical responses and MiHA-specific T cell responses in four patients with metastatic RCC treated with partial T cell-depleted RIC-SCT followed by DLI. Presence of MiHA-specific CTL targeting RCC tumor cells *in vitro* could be demonstrated in two patients who experienced tumor regression or stable disease following RIC-SCT and DLI without clear evidence of GVHD. In one patient the T cell response was directed against the HLA-A2-restricted SCMY peptide FIDSYICQV, and in the other patient the response was directed against a yet unidentified HLA-B7-restricted MiHA. These results indicate that MiHA-specific T cell responses may occur after DLI without the induction of GVHD in RCC patients who are treated with partial T cell-depleted RIC-SCT.

Materials and Methods

Patient eligibility

From December 2002 till August 2003, four consecutive patients with metastatic RCC have been included in a phase I feasibility study in which we studied immunologic responses of partial T cell-depleted RIC-SCT followed by DLI. The eligibility criteria for this study included histological proven, clinically progressive metastatic RCC, failure on earlier cytokine based systemic therapy and no other curative treatment available. Further selection criteria were WHO performance status 0-1, availability of an HLA-identical sibling donor willing to serve as stem cell and lymphocyte donor, and normal organ functions. This study was approved by the Local Ethics Committee of Radboud University Nijmegen Medical Centre (RUNMC) and all patients gave written informed consent.

Conditioning regimen, GVHD prophylaxis and DLI

All patients received a partial T cell-depleted RIC-SCT. The conditioning regimen consisted of total lymph node irradiation (TLI) on each of three consecutive days followed by cyclophosphamide 50 mg/kg body weight intravenously on each of four consecutive days (total dose 200 mg/kg bodyweight). TLI consisted of mantle field, inverted Y-fields including the spleen and was given in an accelerated regimen. The inguinal and femoral lymph node regions were also irradiated. A total dose of 12 Gy was delivered in 2 Gy fractions, twice daily, on three consecutive days.

Donors were HLA-identical siblings. Three patients received bone marrow stem cells and one patient peripheral blood stem cells. For GVHD-prophylaxis, bone marrow grafts were depleted from T and B cells using an indirect method of anti-CD2 and anti-CD19 monoclonal antibodies combined with goat-anti-mouse magnetic beads in an Isolex 300i cell selection device (Nexell-Baxter, Irvine, CA, USA). One sibling donor donated peripheral blood stem cells and on this graft CD34 enrichment with immunomagnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany) was performed. Following depletion or enrichment, CD3+ T cells were added back to generate a stem cell graft containing a fixed number of $0.5x10^6$ T cells/kg body weight of recipient. This procedure resulted in grafts containing a median number of $2.7x10^6$ CD34+ cells/kg and a median of $0.5x10^6$ T cells/kg. All patients received cyclosporine A (CsA) 3 mg/kg/day by continuous intravenous infusion from days -1 to +14. Thereafter CsA dose was reduced to 2 mg/kg/day and continued until day 21. Beyond day 21, CsA was administered orally at a dose of 6 mg/kg/day for 8-10 weeks

and tapered off thereafter.

Patients underwent CT-scan of abdomen and chest before, and every 3 months after RIC-SCT to assess disease response. Patients without acute GVHD grade >II, without chronic GVHD and with residual disease received DLI four weeks after discontinuation of immunosuppression. If no GVHD occurred and disease persisted, a second DLI was administered two months later. The first DLI dose was $0.1x10^8$ T cell/kg bodyweight of the recipient, and the second DLI dose was 0.7×10^8 T cells/kg.

Isolation of PBMC subsets and chimerism analysis

Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Hypaque density gradient centrifugation (Pharmacia, Uppsala, Sweden). T cells, NK cells, myeloid blood dendritic cells (mDC), plasmacytoid blood DC (pDC) and monocytes were isolated from the PBMC as described before.[13] Real-time quantitative PCR of single nucleotide polymorphisms (SNP) and/or the SMCY gene was used for the quantification of donor and recipient hematopoietic cells as described previously.[14-16] Briefly; recipient/donor pairs were screened for discriminating SNPs. Quantification is based on real-time PCR with allele-specific primers for DNA-sequences containing the discriminating SNP and target DNA-specific probes. The sensitivity of this method for the demonstration of a minor population is 0.1% to 0.5%, depending on the purity of the sorted population. Analysis of chimerism in T cells was performed in all four patients. Chimerism in other subsets of cells (i.e. NK cells, monocytes, mDC and pDC) was assessed in two RCC patients who were treated with DLI.

Cell isolation and culture

CD8+ CTL lines H and B were isolated from PBMC obtained one and three months after DLI-1, respectively, by weekly stimulation with PBMC obtained before SCT in Iscove's modified Dulbecco's medium (IMDM) (Invitrogen, Carlsbad, California) supplemented with 10% human serum (HS; Sanquin blood bank, Nijmegen, the Netherlands). After initial stimulation, CTL line H, CTL line B and the HLA-B7 alloreactive CTL Kor18 (kindly provided by Prof. dr. E. Goulmy, Department of Immunohematology, Leiden University Medical Center, The Netherlands) ($0.5*10^6$) were cultured in IMDM/10% HS containing irradiated (80 Gy) recipient EBV-LCL ($0.5*10^6$), irradiated (60 Gy) allogeneic PBMC ($0.5*10^6$) from two donors, 100 IU/ml IL-2 (Chiron, Emeryville, CA) and 1 μg/ml PHA-M (Boehringer, Alkmaar, the Netherlands). CTL lines were used in cytotoxicity and stimulation assays either 7

days after stimulation or 1 day after thawing a frozen aliquot. All cell lines and primary cells were cultured in IMDM/10% FCS. EBV-LCL from the CEPH families were provided by Dr. P. Martin (Department of Immunology, Fred Hutchinson Cancer Research Center, Seattle, Washington, USA) or purchased from the Coriell Institute for Medical Research (Camden, NJ). SKRC cell lines were kindly provided by Dr. E. Oosterwijk (Department of Urology, RUNMC, The Netherlands).

IFNγ secretion assay

IFNγ producing CTLs were detected and isolated using the IFNγ secretion assay (Miltenyi Biotec). Briefly, $1x10^6$ CTLs were incubated in a 24-well plate with $1x10^6$ irradiated (30 Gy) recipient EBV-LCL in a total volume of 2 mL IMDM/10% HS. After 16 hours of incubation at 37°C, cells were harvested, washed with PBS plus 0.5% FCS and 5mM EDTA, and labeled at a concentration of 10^8 cells/ml with 50 µg/ml Ab-Ab conjugates directed against CD45 and IFNγ for 10 minutes on ice. Subsequently, cells were diluted with IMDM/10% FCS at $1x10^5$ cells/ml and allowed to secrete IFNγ for 45 minutes at 37°C. After the cytokine-capturing period, cells were collected, resuspended at a concentration of 10^8 cells/ml in PBS/0.5% FCS/5 mM EDTA, and stained with 5 µg/ml PE–conjugated anti-IFNγ mAb and FITC-conjugated CD8 mAb for 20 minutes at 4°C. Finally, cells were analyzed and isolated by cellsorting using an Epics Elite flow cytometer (Beckman Coulter, Fullerton, California).

Flow cytometry

CTL were phenotyped using a panel of TCR-Vb-specific, CD8 (LT8) (Proimmune, Oxford, UK) and CD3 (UCHT1) monoclonal antibodies (Beckman Coulter). After washing, cells were resuspended in PBS/0.5%BSA and analyzed using the Coulter FC500 flow cytometer (Beckman Coulter). PE- and APC-labeled SMCY.A2 tetramers containing HLA-A2-restricted peptide FIDSYICQV were produced as described previously.[17]. PE-labeled tetramers for the HLA-A2-restricted EBV peptide GLCTLVAML were purchased from Beckman Coulter. PBMC or CTL lines were incubated with 20 µg/ml tetramer for 15 min at room temperature. After washing with PBS/0.5% BSA, cells were labeled with AlexaFluor700-conjugated CD8 (Invitrogen) in combination with FITC-conjugated CD4, CD14, CD16 and CD19 (Beckman Coulter) for 30 min at 4°C. Finally, cells were washed and resuspended in PBS/0.5% BSA containing 0.2 µM Sytox Blue marking dead cells and analyzed using the Cyan flow cytometer (Beckman Coulter). Tetramer staining using both APC and PE showed double

positive events, allowing to discriminate optimal between background staining and positive cells. All FITC-positive cells were gated into a dump channel and were excluded from further analysis.

Retroviral transduction of HLA-B*0702 in cell lines and CTL stimulation assay

HLA-B*0702 cDNA (provided by Prof. dr. E. Goulmy) was subcloned in the LZRS-IRES-EGFP vector. The resulting LZRS-HLA-B*0702-IRES-EGFP vector was used to generate a stable producer cell line. Retroviral transduction was performed using non-tissue culture-treated 35-mm^2 dishes (Becton Dickinson) coated with 10 μg/ml retronectin (Takara Biomedicals). In brief, 10^6 target cells were resuspended in 2 ml virus supernatant and transferred to retronectin-coated dishes. After 24 h of incubation, cells were collected and incubated with fresh virus supernatant. Finally, transduced cells were cultured for 5 additional days before use in CTL stimulation assays as described previously.[18] Release of IFNγ was determined by ELISA (Pierce Endogen, Rockford, IL).

Table 1: Patient characteristics

Patient	Age/sex	Histology	Metastases	Previous therapy
UPN 651	57/M	Clear cell	Lung and mediastinum	IFN-α
UPN 677	53/M	Papillary	Soft tissue	IL-2, G250 mAb and RT (2 cycles)
UPN 705	60/M	Clear cell	Lung, lymph node, thyroid, soft tissue	IFN-α with 6 Retinoic acid and resection of metastases
UPN 686	49/M	Clear cell	Lung	IL-2, IFN-α, 5FU (2 cycles) and dendritic cell vaccination

IFN-α indicates interferon-α; IL-2 indicates interleukin-2, G250 mAb indicates monoclonal antibody against G250; RT indicates radiotherapy; 5FU indicates 5-fluorouracil.

Flow cytometry-based cytotoxicity studies

Flow cytometry-based cytotoxicity assays were performed as previously described[19] with minor adaptations. HLA-B7$^+$ cell lines were labeled with 2.5 μM carboxyflu-

orescein diacetate succimidyl ester (CFSE; Molecular Probes Europe, Leiden, The Netherlands). Alternatively, HLA-B7– cell lines were retrovirally transduced with LZRS-HLA-B*0702-IRES-EGFP as previously described and used as targets without CFSE-labeling. Target cells (1×10^4) were co-cultured with unlabelled effector cells (3×10^4) at an E:T ratio of 3:1 in a total volume of 200 µl IMDM/10% FCS containing 25 U/ml IL-2 in 96-wells flat-bottom plates. After 1-3 days of co-culture, cells were harvested and 7-amino-actinomycin D (7AAD; Sigma-Aldrich, St Louis, MO) was added. Numbers of viable target cells were quantified by flow cytometry.

Results

Clinical description of transplanted RCC patients

Four patients with progressive metastatic RCC after treatment with at least one line of systemic therapy were treated with RIC-SCT (*Table 1*). Three patients had a clear cell and one patient had a papillary cell pathological subtype. The age of these four patients ranged from 49 to 60 years. All patients had undergone a tumor nefrectomy at a median of 4.9 years (range 2.8-11.1 years) before transplantation. No autologous RCC cell lines were available from the four included patients.

All four patients had donor-engraftment after RIC-SCT. The median time to reach leukocytes >1.0 x 10^9/L was 13 days (range 9-14 days), and the median time to reach a number of platelets > 20 x 10^9/L was 11 days (range 9-17 days), although one patient did not recover without transfusions. Only one patient (UPN 705) developed acute GVHD grade II after early discontinuation of CsA because of multi-organ failure including renal failure (*Table 2*). Furthermore, another patient (UPN 651) developed limited chronic GVHD. Two patients (UPN 677 and UPN 686) did not develop acute or chronic GVHD, and received DLI after discontinuation of immunosuppression with CsA. Patient UPN 677 was treated with two DLIs of 0.1 and 0.7x10^8 T cells/kg, respectively, and UPN 686 received one DLI of 0.1 x10^8 T cells/kg.

The survival after RIC-SCT of these four patients was 1.3, 5.0, 8.5 and 8.8 months, respectively (*Table 2*). Our first patient (UPN 651) presented with cerebral metastases shortly after RIC-SCT, and was treated with dexamethasone and radiotherapy. This patient died five months after RIC-SCT from progressive meningo-encephalitis during treatment with corticosteroids because of chronic GVHD and cerebral metastases. Patient UPN 705 developed multi-organ failure shortly after allografting and died from this cause one month after RIC-SCT, before the first evaluation of disease

response. The two patients who received DLI achieved a stable disease and a partial remission of pulmonary metastases, respectively.

Table 2: T cell Chimerism, GVHD, DLI and outcome

Patient	T cell chimerism (% recipient cells)					GVHD acute	GVHD chronic	DLI($x10^8$ T cells/kg)	Outcome
	d+30	d+60	d+90	Before DLI	After DLI				
UPN 651	3%	1%	2%	na	na	no	limited	No	PD, died from meningo-encephalitis during dexamethasone (5 months)
UPN 677	6%	1%	1%	0%	0%	no	no	0.1 0.7	SD, died from CMV-pneumonitis (8.5 months)
UPN 705	0%	na	na	na	na	gr II	na	No	NE, died from multi-organ failure (1.3 months)
UPN 686	7%	47%	43%	ne	0%	no	no	0.1	PR, died from invasive fungal infection (8.8 months)

Abbreviations: GVHD, graft-versus-host-disease; DLI, donor lymphocyte infusion; PD, progressive disease, SD stable disease; NA, not applicable; NE, not evaluated; PR partial remission.

The first evaluation in patient UPN 677 at three months after RIC-SCT showed stable disease, which was paralleled by expansion of $CD8^+$ T cells during tapering off CsA (*figure 1a*). However, one month later this patient developed encephalopathy, which was attributed to EBV-reactivation. PCR for EBV was positive in peripheral blood (200 and 400 copies/ml) and liquor (2,000 copies/ml). CT-scans of chest, abdomen and cerebrum showed no lymphadenopathy. DLI (0.1×10^8 T cells/kg) was already scheduled and administered to reconstitute anti-viral T cell immunity at five months after RIC-SCT. Following this first DLI, circulating EBV-specific $CD8^+$ T cells could

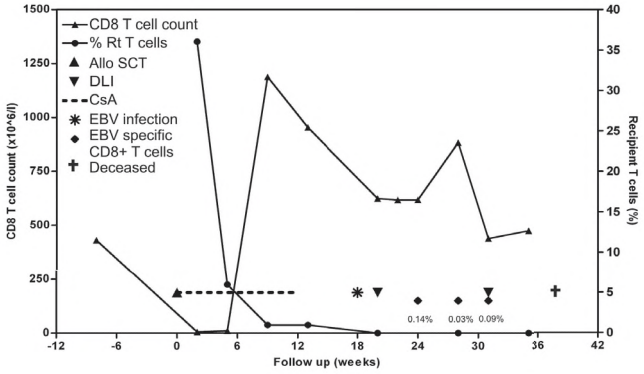
be detected up to 11 weeks using tetramers against the GLCTLVAML epitope (*Figure 1*a). The patient recovered from the encephalopathy and EBV-PCR became negative. At seven months after RIC-SCT a second DLI ($0.7x10^8$ T cells/kg) was administered and the tumor remained stable without occurrence of GVHD. However, at 8.5 months after RIC-SCT, patient UPN 677 developed progressive dyspnoe and died from CMV-pneumonitis. Post-mortal examination showed CMV-pneumonitis, but no signs of GVHD.

Patient UPN 686 developed Candida blood stream infection 2 weeks after RIC-SCT, which recovered upon treatment with Fluconazole. Interestingly, evaluation of disease response 3 months after RIC-SCT showed regression of the three pulmonary metastases. This patient received DLI ($0.1x10^8$ T cells/kg) at 3 months after RIC-SCT (*Figure 1b*). Three months later (i.e. 6 months after RIC-SCT) CT-scan evaluation showed stable disease. Unfortunately, this patient died from invasive fungal infection 9 months after RIC-SCT despite treatment with Fluconazole and caspofungin. Post-mortal examination showed multiple histological confirmed fungal lesions in lungs, liver, spleen and kidney. Furthermore, two lesions, each of 1 cm with vital tumor cells, were found in the lungs but no signs of GVHD.

Clinical evidence for alloreactivity towards host cells

All patients eventually achieved complete donor chimerism in the T cell fraction (*Table 2*). UPN 705 was complete donor chimera in T cells at 30 days after RIC-SCT, whereas UPN 651, 677 and 686 were mixed chimeric with only 3%, 6% and 7% recipient T cells, respectively. In patient UPN 677 the fraction recipient T cells further declined and converted to complete donor T cell chimerism before DLI, which was given on day 141 (*Figure 1a*). In contrast, myeloid DC and monocytes remained mixed chimeric with 25-75% recipient cells until conversion to complete donor at day 141 before DLI (*Table 3*). A different chimerism pattern was observed in patient UPN 686 (*Table 2* and *3*). This patient was mixed chimeric with around 45% recipient T cells at 60 and 90 days after RIC-SCT, while percentages of recipient myeloid DC and monocytes were only 8% and 6%, respectively. Interestingly, both T cells and APCs had converted to complete donor chimerism 3 months after DLI. Furthermore, the conversion to complete donor chimerism occurred in both patients without signs of GVHD.

a



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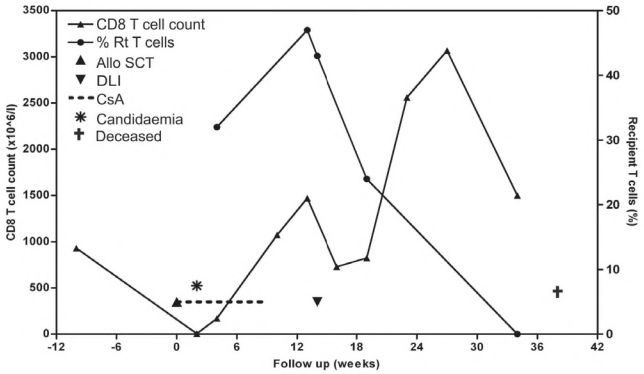

Figure 1: Longitudinal follow-up of CD8+ T cells in peripheral blood from metastatic RCC patients UPN 677 and 686 in relation to clinical outcome. (a) Patient UPN 677: the percentages of recipient T cells (right y axis) are compared with the CD8+ T cell count $\times 10^6$ per liter peripheral blood (left y axis). Administration of SCT and DLI 1-2 are indicated by ▲ and ▼, respectively. Treatment interval with CsA is shown by the dotted line. Time points of confirmed EBV infection, occurrence of EBV-specific T cells (depicted as % from total CD8+ T cell population) and death are indicated. (b) Patient UPN 686: the percentages of recipient T cells (right y axis) are compared with the CD8+ T cell count $\times 10^6$ per liter peripheral blood (left y axis). Administration of SCT and DLI are indicated by ▲ and ▼, respectively. Treatment interval with CsA is shown by the dotted line. Time points of confirmed candidemia and death are indicated.

In parallel, reconstitution of lymphocyte subsets was studied in both RCC patients treated with DLI (i.e. UPN 677 and UPN 686). Total CD3+ T cells recovered to normal values at two months. However, the CD3+ T cell fraction consisted predominantly

of CD8+ T cells, and CD4+ T cell counts did not recover to pre-transplantation values during follow-up (data not shown). Patient UPN 686 showed two expansions of CD8+ T cells after RIC-SCT (*Figure 1b*). The first expansion occurred during tapering of CsA and before the first DLI. This expansion correlated in time with regression of pulmonary metastases. The second CD8+ T cell expansion began 23 weeks after RIC-SCT and nine weeks after DLI, respectively. Patient UPN 677 also showed two expansions of CD8+ T cells after RIC-SCT (*Figure 1a*). The first occurred 9 weeks after RIC-SCT also during tapering of CsA. A second expansion of $CD8^+$ T cells was observed 28 weeks after RIC-SCT and eight weeks after DLI. Collectively, the chimerism kinetics and expansions of CD8+ T cells were indicative for T cell alloreactivity towards hematopoietic cells, but episodes with viral infections may interfere.

Table 3: Chimerism in myeloid dendritic cells and monocytes

Patient	Myeloid dendritic cell chimerism (% recipient cells)					Monocyte chimerism (% recipient cells)				
	d+30	d+60	d+90	Before DLI	After DLI	d+30	d+60	d+90	Before DLI	After DLI
UPN 677	37%	31%	75%	1%	0%	25%	36%	40%	0%	0%
UPN 686	2%	8%	nd	nd	0%	2%	6%	7%	nd	3%

Assessing for alloreactive MiHA-specific CD8+ CTL after transplantation

Because objective signs of T cell alloreactivity were present in RCC patients UPN 677 and UPN 686, we investigated whether MiHA-specific CD8+ T cells targeting RCC tumor cells could be isolated from these patients. Therefore, we stimulated CD8+ T cells obtained 4 weeks and 12 weeks, respectively, after the first DLI with irradiated recipient PBMC obtained pre-SCT. After two restimulations in the presence of IL-2, the specificity of the obtained T cell lines was tested in an IFNγ secretion assay. We detected approximately 2% IFNγ-secreting CD8+ T cells in both T cell cultures (*Figure 2a*). These IFNγ+ CD8+ T cells were sorted by flow cytometry and weekly restimulated with irradiated recipient EBV-LCL. The resulting CD8+ T cell lines, termed CTL line H (from UPN 677) and CTL line B (from UPN 686), displayed significant IFNγ production against recipient EBV-LCL, but not towards donor EBV-

LCL, indicating the recognition of disparate MiHA (*Figure 2b*). These data indicate that MiHA-specific CD8+ T cells were present *in vivo* in RCC patients UPN 677 and UPN 686 following allogeneic RIC-SCT and DLI.

Emergence of SMCY-specific CTLs in a RCC patient after DLI

Next, we determined whether CTL line H contained dominant T cell populations using TCR-Vb PCR and flow cytometry. This analysis revealed TCR-Vb1+CD8+ cells to be the dominant T cell population in CTL line H (data not shown). In parallel, we performed functional assays to determine HLA-restriction and phenotype frequency of the recognized MiHA. Specific IFNγ production by CTL line H was substantially inhibited by anti-HLA class I and anti-HLA-A2 antibodies, but not by antibodies against anti-HLA-B/C and anti-HLA class II (*Figure 3a*). Furthermore, testing EBV-LCL from unrelated HLA-A2+ individuals revealed that 3 out of 9 individuals were recognized, which were all of male origin (*Figure 3b*). These observations suggest that the dominant TCR-Vb1+CD8+ T cells in CTL line H recognize an HLA-A2-restricted HY antigen.

So far, only one HLA-A2-restricted Y chromosome-encoded MiHA has been identified, which is the SMCY.A2 epitope FIDSYICQV. Therefore, we stained CTL line H with PE- and APC-conjugated SMCY.A2 tetramers and found around 12.5% tetramer+ CD8+ T cells (*Figure 3c*). These SMCY.A2 tetramer+ T cells confirmed to be TCR-Vb1+ by flow cytometry (data not shown). A greater than 95% pure population of SMCY.A2 tetramer+ CTLs was isolated by flow cytometry allowing further characterization of its cytotoxic potential (*Figure 3c*). Flow cytometry-based cytotoxicity assays revealed that the TCR-Vb1+ SMCY.A2 CTL induced high levels of cytotoxicity against HLA-A2+ peptide-loaded HY- donor EBV-LCL as well as HY+ recipient EBV-LCL (*Figure 3d*).

To evaluate for SMCY.A2-reactive CD8+ T cells *in vivo*, PBMC of patient UPN 677 collected post DLI-1 were stained with SMCY.A2 tetramers. This analysis showed that SMCY.A2-specific CD8+ T cells became detectable in the post-DLI setting (*Figure 3e*), constituting 3.6%, 2.1% and 2.4% of the CD3+CD8+ T cell population collected at week +24, +28 and +31, respectively. Collectively, these data show that SMCY.A2 CD8+ T cells expanded in RCC patient UPN 677 after DLI with the potential of targeting SMCY-expressing RCC tumor cells in the absence of GVHD.

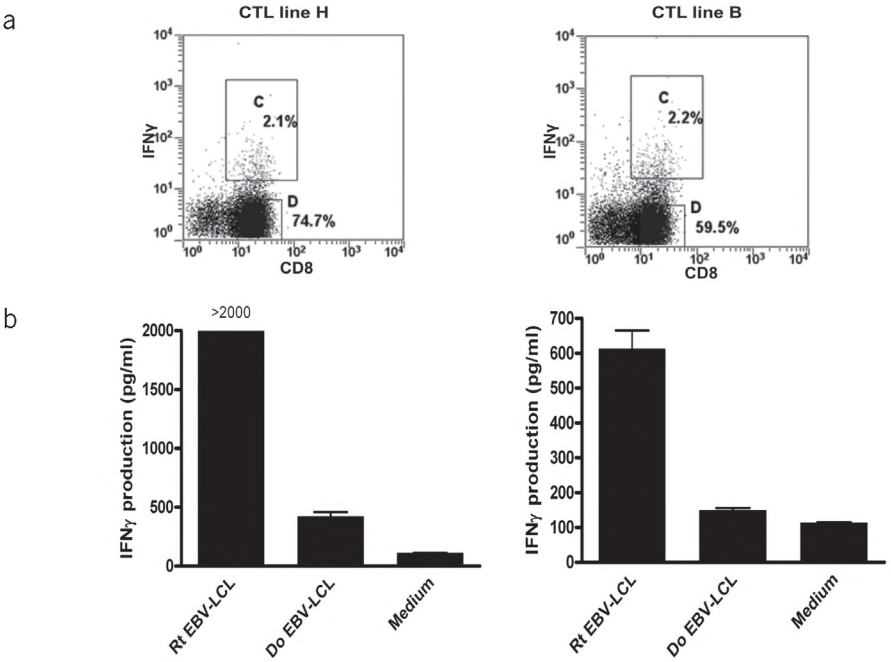

Figure 2: Generation of MiHA-reactive CD8+ CTL lines from patients with RCC after allogeneic RIC-SCT. (a) Detection of IFNγ-secreting CD8+ T cells in T cell lines generated from patient UPN 677 and UPN 686 after co-culture with EBV-LCL of the recipient. Stimulated T cells were stained with PE-conjugated IFNγ detection reagent and FITC-conjugated anti-CD8 mAb, and analyzed by flow cytometry. (b) Production of IFNγ by CTL lines H and B upon stimulation with recipient (Rt) EBV-LCL, donor (Do) EBV-LCL or medium. Data are displayed as mean IFNγ release ± SD of triplicate wells.

Expansion of TCR-Vb4+CD8+ cells targeting an HLA-B7-restricted MiHA after DLI

Similarly, we determined the dominant T cell populations in CTL line B using TCR receptor analysis. PCR and flow cytometry showed a predominant TCR-Vb4+CD8+ cells in CTL line B (data not shown). Subsequently, TCR-Vb4+CD8+ cells were sorted and cultured resulting in pure population of more than 95% (*Figure 4a*). This TCR-Vb4+CD8+ CTL, designated CTL B1, mediated specific IFNγ production against recipient EBV-LCL, but not towards donor EBV-LCL (*Figure 4b*). Release of IFNγ could be completely inhibited by anti-HLA class I and anti-HLA-B/C antibodies, but not by antibodies against anti-HLA-A2 and anti-HLA class II (*Figure 4b*). Testing of EBV-LCL from unrelated individuals sharing expression of HLA-B7 with the recipient, and EBV-LCL from an HLA class I-mismatched individual that were transduced

with HLA-B*0702, revealed that CTL B1 recognizes an HLA-B7-restricted MiHA (*Figure 4c*). Genotyping analysis of recipient UPN 686 and his transplant donor for known MiHA presented by HLA-B7 (i.e. LRH-1, ECGF, and UGT2B17) showed no mismatches, indicating that TCR-Vb4+CD8+ CTL B1 recognizes a novel HLA-B7-restricted MiHA.

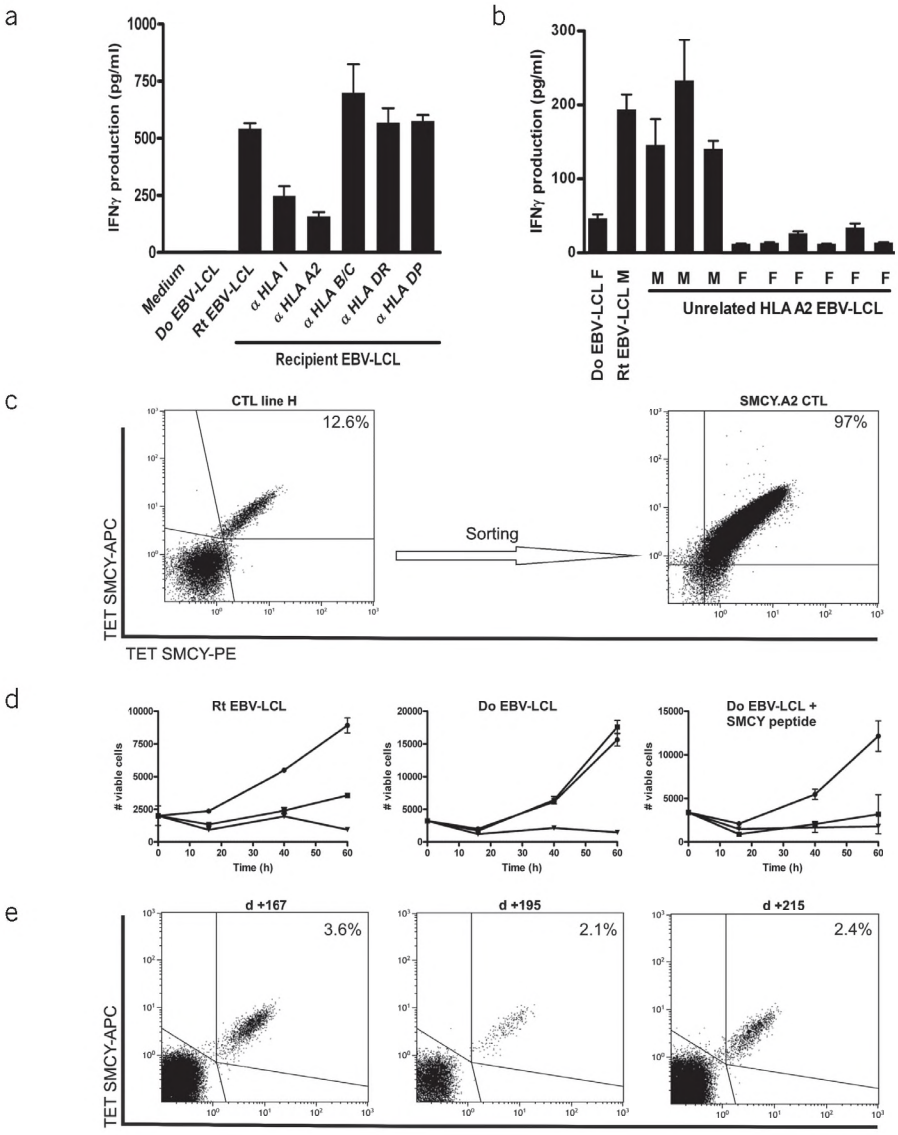

Figure 3: CD8+ T cells reactive with the SMCY.A2 peptide developed after DLI in RCC patient UPN 677. (a) HLA-restriction was determined by the production of IFNγ released by CTL line H upon stimulation with recipient EBV-LCL in the presence of HLA blocking antibodies. Data are displayed as mean IFNγ release ± SD of triplicate wells. (b) Production of IFNγ by CTL line H stimulated with EBV-LCL of 9 HLA-A2+ unrelated individuals, showing recognition of 3 out of 9 EBV-LCL of only male origin. Data are displayed as mean IFNγ release ± SD of triplicate wells. (c) Flow cytometry analysis of CD8+ CTL line H simultaneously stained PE- and APC-conjugated SMCY.A2 tetramer, CD8 AlexaFluor 700, CD4, CD14, CD16 and CD19 FITC and Sytox Blue. CD8+ T cells were gated on FITC- and Sytox Blue- cells. The percentage of SMCY.A2 tetramer-binding cells among viable CD8+ T cells was 12.76%. These SMCY.A2 tetramer+ CD8+ T cells were sorted and expanded resulting in a >95% pure population. (d) Specific reactivity of SMCY.A2-specific CTL from patient UPN 677 against recipient (Rt) and donor (Do) EBV-LCL, and donor EBV-LCL pulsed with peptide FIDSYICQV. (e) Detection of SMCY.A2-specific CD8+ T cells in peripheral blood of RCC patient UPN 677. PBMC collected 167, 195 and 215 days post DLI-1 were simultaneously stained with PE- and APC-conjugated SMCY.A2 tetramer, CD8 AlexaFluor 700, CD4, CD14, CD16 and CD19 FITC and Sytox Blue. Subsequently, cell populations were analyzed by flow cytometry. Cells were gated on CD8+FITC-Sytox Blue- lymphocytes, and the percentage of tetramer-binding cells among CD8+ T cells is depicted in the dot plots.

To investigate whether the MiHA targeted by CTL B1 is expressed by RCC tumor cells, we performed flow cytometry-based cytotoxicity assays, which facilitates determination of target cell proliferation and death by both rapid and more slowly T cell effector mechanisms.[19] Using this *in vitro* assay, we observed that CTL B1 efficiently lysed recipient EBV-LCL, whereas no cytotoxicity was observed against donor EBV-LCL (*Figure 5a*). Interestingly, regarding the RCC tumor cell lines, we observed that CTL B1 significantly targets the HLA-B7+ RCC cell lines SKRC24 and SKRC33 (*Figure 5b).* In addition to these RCC cell lines, we also analyzed several cell lines from other tumor types. This analysis revealed that CTL B1 also recognizes and kills HLA-B7+ brain cancer cells (DAOY cell line), multiple myeloma cells (UM1 cell line) and AML cells (THP-1 cell line) (*Figure 5c*). Allo-HLA-B7-specific CTL lysed efficiently all solid and hematological tumor cell lines tested (*Figure 5b and c*). Finally, to evaluate for TCR-Vb4+CD8+ CTL B1 in vivo, PBMC of patient UPN 686 collected up to 20 weeks post-DLI were co-stained with anti-CD8 and anti-Vb4 antibodies. Flow cytometric analysis showed that TCR-Vb4+ CD8+ T cells could be detected at 2, 4, 9, 13 and 20 weeks post-DLI (*Figure 6*).

These data show that TCR-Vb4+CD8+ CTL B1 emerged in RCC patient UPN 686 following DLI with the capability of targeting RCC metastases in the absence of GVHD.

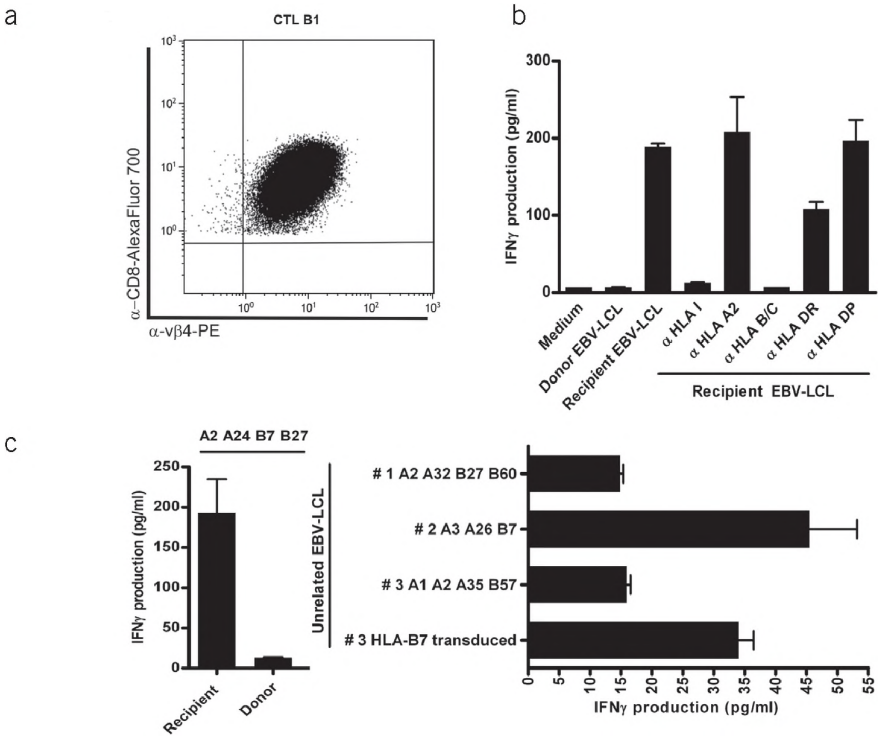

Figure 4: Detection of RCC-reactive CD8+ T cells in peripheral blood of RCC patient 686 after DLI. (a) Flow cytometry analysis of CTL B1 showing a >95% pure population of CD8+TCR-Vb4+ T cells. (b) HLA-restriction was determined by the production of IFNγ released by CTL B1 upon stimulation with recipient EBV-LCL in the presence of HLA blocking antibodies. Data are displayed as mean IFNγ release ± SD of triplicate wells. (c) Production of IFNγ by CTL B1 stimulated with recipient EBV-LCL, EBV-LCL of an unrelated individual (#2) sharing HLA-B7 with the recipient, and an EBV-LCL of an HLA class I-mismatched individual (#3) that was transduced with HLA-B*0702. Data are displayed as mean IFNγ release ± SD of triplicate wells.

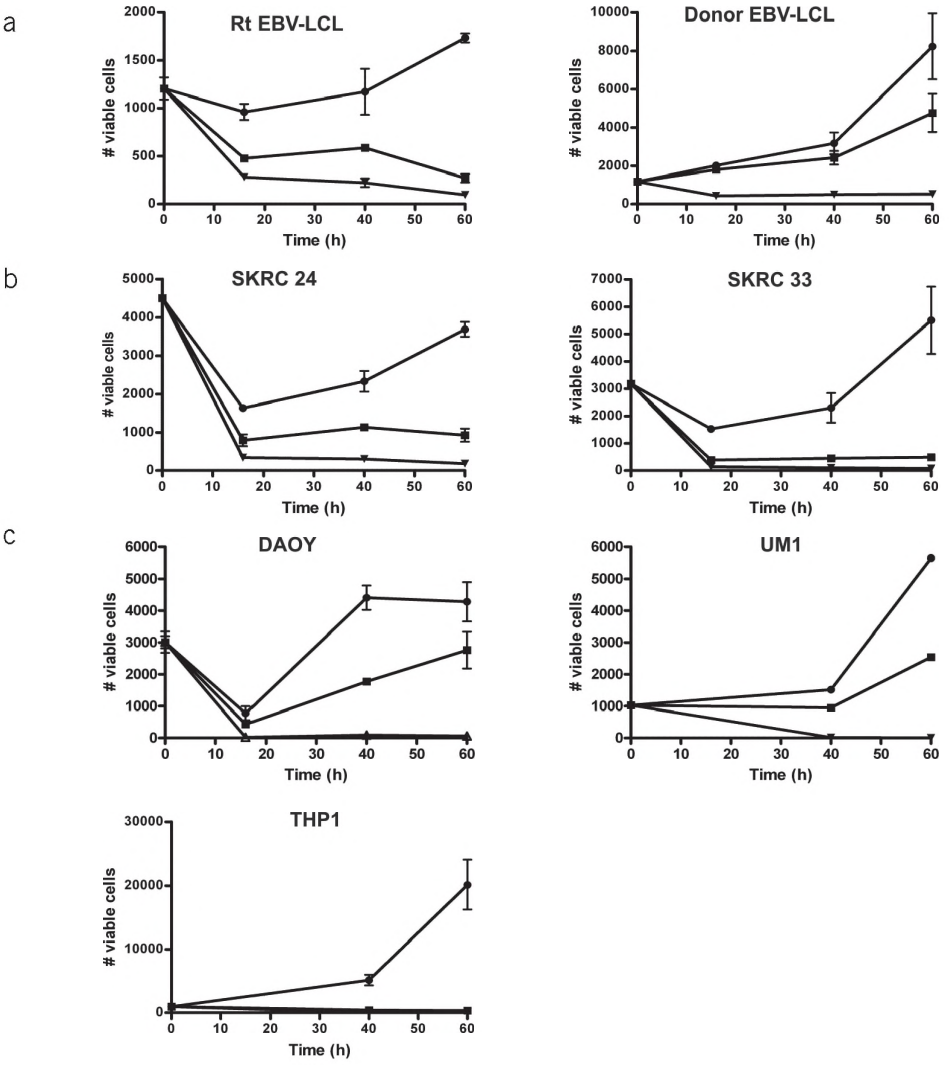

Figure 5: Cytotoxicity of CD8+TCR-Vb4+ CTL B1 against RCC and hematological tumor cell lines. Survival of target cell lines in flow cytometry-based cytotoxicity assays was determined after incubation with CTL B1 (■), allo-HLA-B7 CTL (▼; positive control) or medium only (●) in the presence of 25 U/ml IL-2. Survival of viable CFSE-labeled target cells is shown from (a) donor and recipient EBV-LCL, (b) the HLA-B7+ RCC cell lines SKRC24 and SKRC33, and (c) the HLA-B7+ brain tumor cell line DAOY, AML cell line THP-1and multiple myeloma cell line UM1 in the absence or presence of CTLs at an E:T ratio of 3:1. Data are depicted as mean ± SD of triplicate wells.

Discussion

This feasibility study evaluated the clinical and immunologic responses of patients with metastatic RCC treated with partial T cell-depleted RIC-SCT followed by DLI. All four patients achieved complete donor engraftment that was preceded by mixed chimerism in two patients. One patient converted to complete donor chimerism after discontinuation of immunosuppression and one patient became complete donor chimeric after DLI. Patterns of donor engraftment differed between these two patients, one patient showed first T cell-engraftment and the other patient rapid myeloid engraftment. These differences may be related to the baseline level of immunosuppression. Chimerism analysis in patients treated with fludarabine-cyclophosphamide as conditioning followed by non T cell-depleted grafts has demonstrated rapid T cell-engraftment followed by more gradual myeloid engraftment.[20]

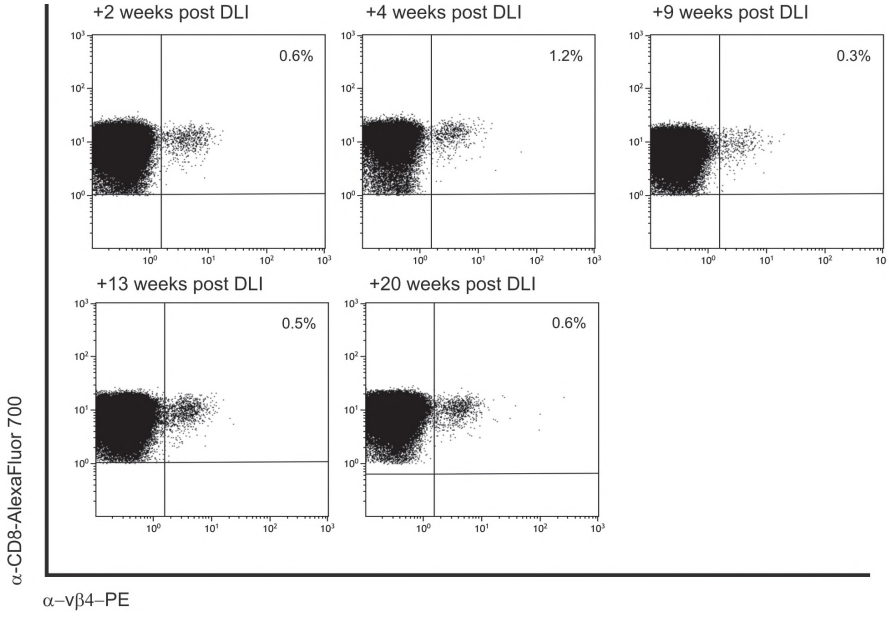

Figure 6: Detection of CD8+TCR-Vb4+ T cells in peripheral blood of RCC patient UPN 686. PBMC collected 2, 4, 9, 13 and 20 weeks post DLI were stained with anti-TCR-Vb4 PE, CD8 AlexaFluor 700, CD4, CD14, CD16 and CD19 FITC and Sytox Blue. Subsequently, cell populations were analyzed by flow cytometry. Cells were gated on CD8+FITC-Sytox Blue- lymphocytes, and the percentage of TCR-Vb4+ cells among CD8+ T cells is depicted in the dot plots.

The occurrence of GVHD was limited in this pilot study. None of the patients developed aGVHD >grade II or extensive chronic GVHD. Earlier studies with T cell replete RIC-SCT have shown that approximately half of the patients develop grade II-IV aGVHD.[2;4;5;21-25] Two patients without GVHD were treated with DLI without development of GVHD. One of these patients had stable disease and the second patient showed regression of pulmonary metastases. Response rates in earlier studies ranged from 0-53%.[2;4;5;25] Although previous studies have shown promising results with complete responders, no objective responses were observed in a subsequent multicentre study.[26] In our study, transplantation-related mortality was high despite the low incidence of GVHD. Two patients died from infectious complications and one patient died from multi-organ failure. A causative role in this unfavorable outcome for the treatment with immune-modulating agents before and the tumor burden at the time of RIC-SCT cannot be excluded.

It has been recognized that alloreactive T cell responses are essential in the GVL effect in hematological malignancies. Also for solid tumors, donor T cells are thought to play a crucial role in GVT immunity. In this study, we demonstrate emergence of SMCY.A2-specific CTL in RCC patient UPN 677 after DLI without the clinical manifestations of GVHD. Furthermore, flow cytometry-based cytotoxicity assays revealed that the SMCY.A2 CTL induced high levels of cytotoxicity against HLA-A2+ HY+ target cells. Interestingly, Hambach et al. showed that SMCY.A2-specific CTL are capable to efficiently target solid tumor cells in a three-dimensional micro tumor model.[27] Therefore, our clinical findings suggest that emergence of SMCY.A2 CTL may have played a role in the stabilization of tumor growth after allogeneic SCT and DLI in patient UPN 677. However, we did not study the quantitative contribution of this SMCY.A2 CTL in comparison to other MiHA-specific CTL in the conversion of chimerism and potential tumor stabilization. Several reports have shown that patients responding after allogeneic SCT contain tumor-reactive T cell clones directed against multiple MiHA.[28;29] Interestingly, high numbers of SMCY.A2 CD8+ T cells up to 3.6% of the total CD8+ T cell population in patient UPH 677 did not induce clinical signs of GVHD to either skin, liver or gut. Earlier studies have shown that SMCY is ubiquitously expressed, and SMCY-specific CTL responses have been associated with GVHD.[30;31] However, GVHD is a multifactorial disease process to which many factors contribute, and we speculate that lack of other inflammatory triggers prevented the development of acute GVHD.

In addition, we isolated a TCR-Vb4+CD8+ CTL, designated CTL B1, targeting a

novel HLA-B7-restricted MiHA from RCC patient UPN 686. This CTL B1 emerged after DLI at the time of conversion of chimerism. Flow cytometry-based cytotoxicity assays revealed that TCR-Vb4+CD8+ CTL B1 mediated efficient lysis of HLA-B7+ RCC cell lines. Unfortunately, we could not test cytotoxicity against autologous RCC-tumor cells, since autologous tumor material was not available. In addition to recognition of allogeneic RCC cell lines, CTL B1 also mediated cytotoxicity against HLA-B7+ brain tumor, multiple myeloma and AML cell lines. These data indicate that the HLA-B7-restricted MiHA recognized by TCR-Vb4+CD8+ CTL B1 is co-expressed by solid tumor and hematopoietic cell types. More detailed knowledge about the tissue distribution and specificity of this novel HLA-B7 presented MiHA awaits molecular identification of the encoding gene.

Furthermore, the discovery of antigenic targets of alloreactive T cells in transplanted RCC patients may allow the development of tumor-specific posttransplantation strategies such as vaccination or adoptive T cell transfer. Two earlier studies have identified target antigens at the molecular level using CD8+ CTL isolated from patients with metastatic RCC treated with allogeneic SCT. Tykodi et al. identified an HLA-A*0201-restricted MiHA, which is encoded by the C19orf48 gene located on chromosome 19q13.[12] In addition, recognition of the HERV-E antigen by alloreactive T cells was described by Takahashi et al. [32] Not only tumor-specific MiHA are interesting for these purposes, but also hematopoietic-restricted MiHA with aberrant expression in tumor cells may be useful for this strategy. Aberrant expression of hematopoietic-restricted MiHA by solid tumor cells has been observed for HA-1, ECGF-1, BCL2A1 and LRH-1.[8-11] Interestingly, adoptive transfer of a single dose of HA-1 CTLs was effective in eradicating disseminated tumors in a mouse model.[27] The feasibility of adoptive immunotherapy with ex vivo-generated HA-1-specific CTLs has also been studied in patients with leukemia.[33] Alternatively to adoptive transfer of MiHA-specific CTLs, patients could be vaccinated with peptides or DCs loaded with peptides or electroporated with MiHA-encoding mRNA. The use of mRNA-electroporated DC to stimulate MiHA-specific T cell responses ex vivo has been recently explored by us with LRH-1 encoding P2X5 mRNA.[34] This study indicated that in LRH-1+ patients transplanted with LRH-1- donors the GVT response can be enhanced with DC electroporated with LRH-1 encoding mRNA. However, the potency of MiHA-based immunotherapy has to be further explored in clinical trials. Currently, the role of allogeneic RIC-SCT for the treatment of metastatic RCC patients is still unclear. New developments in the treatment of metastatic RCC have

significantly changed the treatment strategies for these patients.[35-38] However, these novel drugs do not cure patients and the incidence of complete remissions is low. Therefore new strategies should continue to be explored, and adoptive cellular therapy remains a promising option.

In conclusion, we describe the engraftment, clinical responses and T cell responses after partial T cell-depleted RIC-SCT followed by DLI in patients with metastatic RCC. Our transplant procedure resulted in stable engraftment, manageable GVHD and objective clinical responses in two patients. SMCY-specific CD8+ T cells were identified in a patient with stable disease, and CD8+ T cells targeting a novel HLA-B7-restricted MiHA in the second patient with partial regression. Although the role of allogeneic RIC-SCT in RCC may be limited in the near future, this study shows that the approach of partial T cell-depleted SCT followed by DLI induces MiHA-specific T cell responses potentially targeting RCC tumor cells. Furthermore, the identification of antigenic targets of alloreactive T cells remains most important for a further understanding of the GVT response and for development of strategies to target tumor cells selectively after allogeneic SCT.

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Chapter 8

Summary and general discussion

Summary and general discussion

Allogeneic stem cell transplantation (SCT) is an established treatment modality for several hematologic malignancies. The curative effect of allogeneic SCT is mediated by the graft-versus-tumor (GVT) response, an immune reaction of donor-derived T cells recognizing minor histocompatibility antigens (MiHA) on malignant cells of the recipient. Unfortunately, this beneficial GVT response is often accompanied by graft-versus-host-disease (GVHD), which is an allo-immune reaction against skin, mucosa (gastro-intestinal tract, eyes and lung) and liver. Complete or partial depletion of T cells from the stem cell graft is an effective method to reduce GVHD in the setting of myeloablative conditioning. Earlier studies have shown that partial T cell depletion followed by pre-emptive post-transplantation immunotherapy with donor lymphocyte infusion (DLI) is effective to prevent relapses after allogeneic myeloablative SCT in high risk patients.[1] However, the efficacy of DLI varies among different hematological malignancies. DLI results in complete molecular remission in 70-80% of patients with a relapse of CML in chronic phase, but patients with relapsed leukemia or MM respond in only 10-40% of cases. The mechanisms responsible for the different DLI-mediated anti-tumor responses between various disease types are still not completely understood and further identification of these mechanisms may help to develop new strategies of post-transplantation immunotherapy. Antigen presenting cells (APC) play an important role in the initiation of alloreactive CD8+ T cell responses following allogeneic SCT. Mouse models have shown that early after SCT, when there are residual recipient APC, direct presentation of antigens derived from genes co-expressed by recipient APC and malignant cells is sufficient to induce effective GVT reactivity.[2;3] In a MHC-mismatched mouse-model, DLI administration to mixed chimeras produced an improved leukemia-free survival compared to DLI in complete donor chimeras.[4] Strategies aiming at the APC component of the GVL response are for example vaccination with professional APC: dendritic cells. In this

thesis, we studied the role of recipient-derived dendritic cells (DC) in GVT reactivity in CML patients, and started a feasibility study of recipient-derived DC-vaccination in MM patients with residual disease after reduced intensity conditioning (RIC)-SCT and DLI.

The insight in the importance of the GVT response in the curative potential of allogeneic SCT has led to the development of conditioning strategies with reduced intensity to reduce the toxicity of the chemotherapy and radiotherapy. These conditioning regimens are more immunosuppressive than myeloablative and allow engraftment of the donor immune system. Although these strategies have reduced transplantation related mortality (TRM), GVHD remains a key issue affecting quality of life in the non T cell-depleted setting. Therefore, we studied a new approach of partial T cell-depleted transplantation followed by DLI in a RIC-setting in patients with malignant lymphoma, multiple myeloma (MM) and metastatic renal cell carcinoma (RCC). An additional step to improve immunotherapy after SCT is to separate GVT from GVHD with adoptive immunotherapy. Adoptive immunotherapy aims at targeting MiHA with expression limited to malignant cells. In case of hematological malignancies, MiHA with expression on normal and malignant hematopoietic cells can be used, since after allogeneic SCT hematopoiesis will be of donor origin. To study the feasibility of this approach for metastatic solid tumors, we analyzed the aberrant expression of the hematopoietic-restricted MiHA LRH-1 in solid tumor cells and studied MiHA-specific CTL responses in two patients treated with RIC-SCT for RCC.

In *chapter 2* we describe the study that was performed to elucidate the mechanisms that determine the effectiveness of DLI in patients with relapsed CML in relation to DC chimerism. We hypothesized that the presence of recipient APC may be an important factor for the induction of DLI-induced GVL reactivity. Absolute numbers of DC-subsets and chimerism in T cells and two subsets of DC (i.e. myeloid DC and plasmacytoid DC) were analyzed. Based on T cell and DC chimerism we identified three groups. Patients with a molecular relapse (n=4) who showed complete donor chimerism in T cells and DC subsets attained complete molecular remission without significant GVHD. The second group consisted of six patients with complete donor chimerism in T cells but mixed chimeric in DC subsets. Also these patients, a complete molecular remission was achieved, but in this case associated with GVHD in 4 out of 6 and cytopenias in 3 out of 6 patients. The third group (n=5) showed mixed chimerism in T cells and complete recipient chimerism in MDC, of whom only 2 out

of 5 patients entered complete molecular remission. Absolute numbers of MDC and PDC did not correlate with induction of alloreactivity. Interestingly, the combination of donor chimerism in T cells and the presence of recipient DC subsets induces efficient GVL reactivity in patients with a cytogenetic or hematological relapse, in association with GVHD in 4/6 (66%) of the patients. In contrast, in patients with a molecular relapse low-dose DLI can induce mild alloreactivity, which is sufficient to induce complete molecular remission without significant GVHD. These data partly support our hypothesis on the important role of recipient-derived DC and suggests that the balance between T cell and DC chimerism may be important in the initiation of an allo-immune response after DLI.

In *Chapter 3*, we describe a retrospective study on partial T cell-depleted myeloablative SCT in MM followed by pre-emptive DLI to boost graft-versus-myeloma (GVM) reactivity. From 1997 to 2001 relatively young MM patients (< 55 years of age) were offered an allogeneic SCT, in the case an HLA-identical sibling donor was available, in first line of treatment according to the HOVON-24 protocol. This study was performed to analyze the role of pre-emptive DLI as post-transplantation immunotherapy. Thirteen of the 24 patients actually received pDLI according to the protocol. With a median follow-up of 67 months, 7 of the 24 patients (29%) were in continuous complete remission (CCR) and all seven patients had received pDLI. Despite the favorable outcome in patients receiving pDLI, transplantation related mortality (TRM) of the myeloablative SCT was high (29%) as observed in all myeloablative studies in MM. The high TRM was a reason that myeloablative transplants are currently no longer performed. Nevertheless, this retrospective analysis was the basis of the development of a new approach of partial T cell-depleted allogeneic SCT in the RIC-setting, combined with pre-emptive immunotherapy, which is described in *chapter 4*.

In the studies described in *chapter 4*, the applicability of partial T cell-depleted RIC-SCT in MM followed by pDLI as post-transplantation immunotherapy was further explored. As a novel approach we incorporated recipient-derived DC vaccination in the post-transplantation strategy for patients with residual disease after two DLIs. Studies in mouse models demonstrated that recipient DC play a pivotal role in the initiation of alloreactive CD8+ T cell-mediated immunity against leukemia.[3;5] Moreover, the presence of recipient DC in the setting of mixed chimerism has a positive

impact on the effectiveness of DLI.[4] Since recipient DC and myeloma tumor cells are both derived from the patient hematopoietic system, immune responses induced by recipient-derived DC may enhance GVM. In this study, twenty MM patients received partial T cell-depleted RIC-SCT after autologous SCT after which they all engrafted successfully. Fourteen patients received post-transplantation immunotherapy, of whom 8 patients received only pDLI, 5 patients both pDLI and DC-vaccination, and 1 patient only DC-vaccination because the donor was no longer available. DC-vaccination was associated with limited toxicity and none of these patients developed GVHD. TRM at one year was low (10%) and the overall survival was 84% with median follow-up of 27 months. Genotyping for known MiHA in the vaccinated recipients and their donors did identify MiHA-mismatches that could be involved in GVM and GVHD, but specific T cells against these known MiHA using tetramer staining could not be detected. Importantly, this study demonstrated that partial T cell-depleted RIC-SCT is feasible with much lower TRM than myeloablative SCT. Furthermore, vaccination with recipient-derived DC was safe, immunogenic and did not induce GVHD. Early disease-free survival was promising but it is yet unclear whether DC vaccination played an essential role in the clinical outcome.

In *Chapter 5*, we present studies on the feasibility of partial T cell-depleted RIC-SCT combined with DLI for relapsed transformed non-Hodgkin's lymphoma, follicular lymphoma, mantle cell lymphoma, and chronic lymphocytic leukemia. In contrast to the MM study, with preceding autologous transplant that induces immunosuppression, in these patients we applied a two-step approach with pretransplant host T cell-depletion with cyclophosphamide and fludarabine followed by the transplant conditioning within three weeks. This study demonstrated that partial T cell-depleted RIC-SCT with pre-transplant host T cell depletion results in fast and stable engraftment. All patients transplanted with stem cells from an HLA-identical sibling donor developed complete donor chimerism during follow-up. The induction course to deplete host T cells seems essential, because omitting this induction course resulted in a primary take failure in two patients. Both patients received a transplant from an unrelated donor after conditioning with cyclophosphamide, fludarabine and antithymocyte globulin (ATG) without host T cell depletion before conditioning. Partial T cell-depletion in vitro in combination with a relatively short course of cyclosporine A (CsA) resulted in aGVHD grade ≥II in only 19% of the patients, which was manageable with corticosteroids, CsA and ultraviolet B therapy for GVHD limited to

the skin. Main complications were related to viral reactivations including varicella zoster infections and EBV-associated lymphoproliferative disease. The relapse rate was low, even in patients with transformed NHL and despite the fact that only 19% of the patients received pDLI. Overall survival and lymphoma-free survival were 83% and 74%, respectively. These findings indicate that partial T cell depleted RIC-SCT is feasible if host T cell depletion is applied before the conditioning. Because the main complications were viral-related and the relapse rate was low, additional improvement may be achieved by adding strategies aiming at immune reconstitution such as transfer of virus-specific lymphocytes.

The development of RIC regimens has made it possible to explore the GVT effect in patients with metastatic solid tumors. Several reports have described partial or complete remissions; however the ultimate success rate (complete remissions) is limited. Furthermore, in most patients responses are accompanied by severe GVHD, which impairs the quality of life. Also in solid tumors, the GVT effect is most probably mediated by donor T cells recognizing MiHA on the malignant cells of the recipient. Therefore, directing donor T cell immunity to MiHA that are selectively expressed on solid tumor cells or are expressed on hematopoietic cells with aberrant expression on solid tumor cells may be a good strategy to selectively boost GVT in the absence of GVHD. In 2005, our group identified a hematopoietic-restricted MiHA, designated LRH-1, which elicits an HLA-B7-restricted CTL-response in leukemia patients treated with DLI.[6] In *chapter 6* we investigated whether the *P2X5*-encoded LRH-1 epitope is expressed on solid tumor cell lines and primary solid tumor cells. Aberrant expression of *P2X5* was observed in a significant portion of solid tumor cell lines and primary tumor cells, including RCC, melanoma, colorectal carcinoma, brain cancer and breast cancer. Furthermore, flow cytometry-based cytotoxicity assays showed that *P2X5*-expressing solid tumor cells are susceptible to LRH-1 CTL-mediated lysis. These data indicate that LRH-1 is an interesting hematopoietic-restricted MiHA that could be used in the development of MiHA-specific adoptive immunotherapy for solid tumors.

The strategy of partial T cell-depleted RIC-SCT followed by DLI was also studied by us in patients with metastatic RCC. In *Chapter 7*, we report the clinical and immunological responses of this approach in four patients with metastatic RCC. All four patients achieved complete donor engraftment that was preceded by mixed chimer-

ism in two patients. None of the patients developed aGVHD >grade II or extensive chronic GVHD. Two patients without GVHD were treated with DLI, which did not result in GVHD. Both patients responded, one patient had stable disease and one patient showed regression of pulmonary metastases. Interestingly, MiHA-specific CTL targeting RCC tumor cells could be isolated from these two patients. In one patient with stable disease, the CTL response was directed against the HLA-A2-restricted SCMY peptide, which is a Y-chromosome encoded antigen. In the other patient with partial remission, the CTL response was directed against an HLA-B7-restricted MiHA of unknown origin. These results indicate that MiHA-specific T cell responses targeting RCC cells can be mounted by DLI without the induction of GVHD in RCC patients who are treated with partial T cell-depleted RIC-SCT followed by DLI.

In conclusion, these studies describe strategies to enhance the beneficial GVT effect of allogeneic SCT while reducing GVHD, morbidity and mortality of the SCT. The combination of partial T cell-depleted RIC-SCT followed by DLI was most effective in patients with lymphoma and CLL. In our study, we included patients with chemotherapy-sensitive disease since all had reached at least PR before RIC-SCT. It is known from the literature that allogeneic SCT is much less effective in chemotherapy refractory disease. Although partial T cell-depleted RIC-SCT after host T cell-depletion is a highly immunosuppressive approach, infectious complications in our transplanted cohort were comparable with other studies in the RIC-setting. Further improvement may be achieved by longer prophylaxis with valaciclovir to prevent varicella zoster reactivation and the transfer of virus-specific T cells for viral reconstitution. In MM, TRM decreased significantly after partial T cell-depleted RIC-SCT. However, relapse of MM still occurred in a substantial number of patients despite pDLI and recipient-derived DC-vaccination after two DLIs. We showed that recipient DC vaccination after RIC-SCT is feasible and safe; however MiHA-specific T cell responses have not yet been identified in DLI-responding patients. Although the use of recipient-derived DC is not completely investigated yet, a next step to enhance the antitumor effect is the vaccination with mature donor-derived DC electroporated with MiHA-encoding mRNA.
More challenging is the role of RIC-SCT in metastatic RCC. Four patients were treated with partial T cell-depleted RIC-SCT followed by DLI and these patients had progressive and non-chemotherapy responsive disease. In two patients, we showed MiHA-specific T cell-responses after DLI. Also for this patient-group adoptive im-

munotherapy targeting tumor-specific MiHA would be an interesting approach. In our pre-clinical study, aberrant *P2X5*-expression was observed in several solid tumor cell lines including RCC. However, new developments in treatment of RCC with tyrosine-kinase inhibitors have significantly changed the treatment strategies for these patients. Currently the role of allogeneic SCT in this disease is unclear, but it still has the potential to contribute to the dismal prognosis that these patients continue to face.

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Chapter 9

Nederlandse samenvatting
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Nederlandse samenvatting

Allogene stamceltransplantaties (SCT) vormen een belangrijke behandelingsoptie voor verschillende hematologische maligniteiten. De mogelijkheid tot genezing door allogene SCT wordt toegeschreven aan het graft-versus-tumor (GVT) effect, een immunologische reactie van T cellen van de donor die middels minor histocompatibiliteits antigenen (MiHA) de tumorcellen van de patiënt herkennen. Helaas gaat deze GVT reactie vaak ook gepaard met graft-versus-host-disease (GVHD), ofwel omgekeerde afstotingsziekte. Hierbij richt de immunologische reactie van het donor immuunsysteem zich tegen gezonde weefsels, met name de huid, slijmvliezen (tractus digestivus, ogen en longen) en de lever. Complete of gedeeltelijke verwijdering van T cellen (partiële T cel-depletie) uit het transplantaat is een effectieve methode om GVHD te verminderen, maar verhoogd tegelijkertijd de recidief kans. Eerdere studies tonen aan dat partieel T cel-gedepleteerde allogene SCT, gevolgd door preemptieve immuuntherapie met donorlymfocyten infusie (DLI), bij het van tevoren ontbreken van significante GVHD, een effectieve aanpak is in hoogrisico patiënten.[1] Het effect van DLI ter bestrijding van het terugkomen van de ziekte varieert voor de verschillende hematologische maligniteiten. In Chronische Myeloide Leukemie (CML) leidt DLI in 70-80% van de patiënten met recidief CML in chronische fase tot een complete moleculaire remissie, maar bij patiënten met recidief Acute Leukemie of Multipel Myeloom (MM) is dit percentage slechts 10-40%. De mechanismen die verantwoordelijk zijn voor de DLI-gemedieerde anti-tumor activiteit tussen de verschillende maligniteiten zijn nog niet opgehelderd. Inzicht in deze mechanismen zijn belangrijk voor de ontwikkeling van betere posttransplantatie immuuntherapie. Antigeen presenterende cellen (APC) spelen een belangrijke rol bij het op gang brengen van alloreactieve CD8+ T cel responsen na allogene SCT. In muizenmodellen is aangetoond dat vroeg na de SCT, wanneer er ook nog ontvanger APC aanwezig zijn, directe presentatie van hematopoietisch-specifieke MiHA op de APC aan donor

T cellen aanwezig is, hetgeen voldoende is om een effectieve GVT reactiviteit ten aanzien van normale en maligne hematopoietische cellen te induceren.[2;3] Dit wordt ondersteund door onderzoek in muismodellen waarbij DLI in muizen met gemengd chimerisme resulteert in een betere leukemievrije overleving vergeleken met DLI in complete donor chimeren.[4] Vaccinaties met professionele APC, de zogenaamde dendritische cellen (DCs), vormen een strategie gericht op de antigeenpresenterende component van de GVT respons.

In dit proefschrift hebben we de rol bestudeerd van DCs, afkomstig van de ontvanger, in de GVT reactiviteit in CML patiënten en zijn we een haalbaarheidsstudie gestart naar ontvanger DC-vaccinatie in MM patiënten met residuale ziekte na "reduced intensity conditioning" (RIC)-SCT en DLI.

Het inzicht dat vooral de GVT respons verantwoordelijk is voor het curatieve vermogen van allogene SCT, en niet de chemotherapie in de conditionering, heeft geleid tot de ontwikkeling van minder intensieve conditioneringstrategieën. Deze conditioneringschema's zijn vooral immuunsuppressief van aard met als belangrijkste doel dat de donorcellen worden geaccepteerd door de ontvanger. Door de vermindering in toxiciteit van de conditionering is de transplantatie gerelateerde mortaliteit (TRM) afgenomen, maar de mate en ernst van GVHD wordt hiermee niet beïnvloed en juist deze GVHD speelt een belangrijke rol in de kwaliteit van leven na transplantatie. Om deze reden hebben we bij het onderzoek beschreven in dit proefschrift gekozen voor een nieuwe benadering bestaande uit RIC allogene SCT gecombineerd met partiële T cel depletie, gevolgd door DLI in patiënten met maligne lymfoom, MM en gemetastaseerd niercelcarcinoom.

Een volgende stap is de posttransplantatie immuuntherapie meer specifiek te richten op tumorcellen (GVT) en hierbij de gezonde weefsels (GVHD) te sparen. Dit wordt getracht door immuuntherapie toe te passen die zich specifiek richt zich op MiHA die alleen maar op de tumorcellen voorkomen. In het geval van hematologische maligniteiten maakt het niet uit of deze MiHA ook op normale hematopoietische cellen voorkomen, immers de hematopoiese wordt vervangen door donor cellen. Om de mogelijkheden van deze benadering te bestuderen bij gemetastaseerde solide tumoren hebben we expressie van het MiHA LRH-1, een antigeen dat op normale hematopoietische cellen voorkomt, bestudeerd op solide tumorcellen. Tevens werden MiHA-specifieke cytotoxische T cel reacties bestudeerd in twee patiënten met gemetastaseerd niercelcarcinoom behandeld met RIC-SCT gevolgd door DLI, met als doel de geïsoleerde T cellen te gebruiken om de herkende MiHA te identificeren.

In *Hoofdstuk 2* beschrijven wij een studie naar de mechanismen van DLI in patiënten met een recidief CML door bepaling van het chimerisme in de dendritische cellen. Onze hypothese was dat aanwezigheid van DCs van de patiënt een belangrijke factor zou vormen voor de inductie van DLI geïnduceerde GVT reactiviteit. De absolute aantallen myeloide en plasmacytoide DC en hun chimerisme evenals het T cel chimerisme werden geanalyseerd. Op basis van het T cel chimerisme en DC chimerism identificeerden wij drie groepen. Patiënten met een moleculair recidief (n=4) hadden compleet donor chimerisme in de T cellen en DC-subpopulaties en bereikten na DLI allen een complete moleculaire remissie zonder significante GVHD. De tweede groep bestond uit zes patiënten met compleet donor chimerisme in T cellen maar gemengd chimerisme in de DC-subpopulaties. Ook in deze 6 patiënten werden complete moleculaire remissies bereikt, maar dit ging gepaard met duidelijke GVHD in 4 van de 6 patiënten en cytopenieën in 3 van de 6. De derde groep (n=5) had een gemengd chimerisme in de T cellen en complete ontvanger (recipiënt) chimerisme in myeloide DC. In deze groep bereikten slechts 2 van de 5 patiënten een complete moleculaire remissie. Deze resultaten toonden aan dat de combinatie van donor chimerisme in de T cellen en de aanwezigheid van recipiënt DC-subpopulaties kan leiden tot een efficiënte GVL reactiviteit, echter met GVHD in 4/6 (66%) van de patiënten. Terwijl lage dosis DLI in CML patiënten met een moleculair recidief kan leiden tot milde alloreactiviteit, welke echter voldoende is om een complete moleculaire remissie te bereiken en zonder GVHD. Deze data ondersteunen deels onze hypothese betreffende de belangrijke rol van ontvanger DCs en suggereren dat de balans tussen T cel- en DC chimerisme mogelijk van belang is in de initiatie van een allo-immuun response na DLI.

Hoofdstuk 3 betreft een retrospectieve studie naar partieel T cel-gedepleteerde myeloablatieve SCT in MM gevolgd door pre-emptieve DLI met als doel de graft-versus-myeloma (GVM) reactiviteit te versterken. Jonge MM patiënten (< 55 jaar) met een HLA-identieke familie donor, kwamen in de periode van 1997 tot 2001 in aanmerking voor een allogene SCT als eerstelijns behandeling in het kader van de HOVON-24 studie. Doel van onze studie was de bijdrage van pre-emptieve DLI te evalueren. Dertien van de 24 patiënten ontvingen daadwerkelijk pDLI als gepland in het protocol. Met een mediane follow-up van 67 maanden waren 7 van de 24 patiënten (29%) in een continue complete remissie (CCR) en alle zeven behoorden tot de groep van 13 patiënten die pDLI ontvingen. Ondanks de gunstige uitkomsten voor

de patiënten die behandeld werden met pDLI, was de transplantatie gerelateerde mortaliteit (TRM) van de myeloablatieve SCT hoog (29%) zoals ook beschreven in andere myeloablatieve studies in MM. Om die reden zijn wij in 2001 gestopt met myeloablatieve transplantatie in MM. Wel vormde deze retrospectieve analyse de basis voor de ontwikkeling van een nieuwe benadering, partieel T cel-gedepleteerd allogene SCT in de RIC-setting gevolgd door pre-emptieve DLI en nieuwere vormen van immuuntherapie.

In *hoofdstuk 4* beschrijven we de resultaten van bovengenoemde benadering, partieel T cel-gedepleteerd RIC-SCT in MM gevolgd door pre-emptieve DLI. Als nieuwe post-transplantatie immuuntherapie hebben we ontvanger DC vaccinatie toegevoegd voor patiënten met nog aantoonbare ziekte na de stamceltransplantatie en twee maal DLI. Zoals beschreven laten muizenmodellen zien dat ontvanger APC's een sleutelrol spelen in de initiatie van alloreactieve CD8+ T cel-gemedieerde immuunresponsen tegen leukemie.[3;5] Bovendien heeft de aanwezigheid van ontvanger DCs mogelijk een positieve bijdrage aan de effectiviteit van de DLI.[4] Omdat ontvanger DCs en multiple myeloom plasmacellen beiden afkomstig zijn van het hematopoietische systeem van de patiënt, zouden tumor responsen geïnduceerd door ontvanger DCs tevens het GVM effect kunnen versterken. Twintig MM patiënten werden behandeld met een partieel T cel-gedepleteerde RIC-SCT na een voorafgaande inductie behandeling inclusief hoge dosis melfalan en een autologe SCT. In geen enkele patiënt trad rejectie van het transplantaat op. Veertien patiënten ontvingen posttransplantatie immuuntherapie, 8 alleen pDLI, 5 patiënten zowel pDLI als een DC-vaccinatie, en 1 patiënt kreeg alleen een DC-vaccinatie, dit omdat de stamceldonor niet meer beschikbaar was. De toxiciteit van DC-vaccinatie was beperkt en er werd bij geen van de patiënten GVHD gezien. Tevens bleek DC-vaccinatie een T cel response te induceren tegen het controle eiwit KLH in de gevaccineerde patiënten. De transplantatie gerelateerde mortaliteit was laag (10%) vergeleken met de myelo-ablatieve benadering en de overleving na een mediane follow-up van 27 maanden was 84%. Of de posttransplantatie immuuntherapie met DC bijdraagt in de ziektevrije overleving is uit deze gegevens niet vast te stellen. Bij genotypering voor bekende MiHA in de gevaccineerde patiënten en hun donoren werden mismatches gevonden die betrokken zouden kunnen zijn bij het GVM effect en GVHD, maar specifieke T cellen tegen deze bekende MiHA-mismatches konden met het gebruik van tetrameren in het bloed niet worden aangetoond. Deze studie laat zien dat partieel T cel-gedepleteerd RIC-SCT in de praktijk

toepasbaar is met een veel lagere TRM dan myeloablatieve SCT. Tevens bleek dat vaccinatie met ontvanger DCs veilig en immunogeen is maar niet leidde tot GVHD. De vroege ziektevrije overleving lijkt goed maar het is onduidelijk in hoeverre DC vaccinatie daadwerkelijk een rol heeft gespeeld in deze klinische resultaten.

In *hoofdstuk 5* is een vergelijkbare benadering van partieel T cel-gedepleteerd RIC-SCT gecombineerd met DLI toegepast bij patiënten met een recidief getransformeerd non-Hodgkin's lymfoom, folliculair lymfoom, mantelcel lymfoom, en chronische lymfatische leukemie. In tegenstelling tot de studie in MM, waarbij voorafgaand een autologe transplantatie wordt uitgevoerd welke naast reductie van de ziekteactiviteit ook leidt tot immuunsuppressie, hebben we in deze patiënten een tweestaps benadering gekozen om rejectie van het transplantaat tegen te gaan. Voorafgaand aan de transplantatie werd het aantal T cellen in de patiënt gereduceerd door een kuur met cyclofosfamide en fludarabine. Indien er een reductie was in het aantal CD4 cellen tot minder dan $0{,}05x10^9$/L, dan werd de behandeling binnen drie weken voortgezet met een hogere dosis fludarabine en cyclofosfamide als conditionering. Gebruikmakend van deze strategie zagen we een snelle en stabiele acceptatie van het transplantaat waarbij alle patiënten met een HLA-identieke sibling donor een compleet donor chimerisme ontwikkelden gedurende follow-up. De inductiekuur, met als doel de T cellen in de ontvanger te verminderen lijkt essentieel. Het weglaten van deze inductiekuur heeft in 2 patiënten met een onverwante donor geleid tot primaire rejectie, ondanks dat deze patiënten in vivo nog eens een extra T cel depletie ondergingen met anti-thymocyten globuline (ATG). Partiële T cel-depletie in vitro gevolgd door een relatief korte behandeling met cyclosporine A (CsA) leidde tot acute GVHD>graad 2 in slechts 19% van de patiënten. Deze acute GVHD kon in alle gevallen adequaat behandeld worden met corticosteroïden, CsA en ultraviolet B therapie bij GVHD beperkt tot de huid. De belangrijkste complicaties werden veroorzaakt door virus reactivaties, met name varicella zoster infecties en EBV-geassocieerde lymfoproliferatieve aandoeningen. Het aantal recidieven was laag, zelfs in de patiënten met een getransformeerd NHL en ondanks het feit dat slechts 19% van de patiënten uiteindelijk DLI hebben ontvangen. De totale overleving en lymfoomvrije overleving waren 83% and 74%, respectievelijk bij een mediane follow-up van 19 maanden. Deze bevindingen laten zien dat partieel T cel-gedepleteerd RIC-SCT toepasbaar is op voorwaarde dat voldoende host T cel-depletie wordt toegepast voor start van de uiteindelijke conditionering. Verbetering van de immuunreconstitutie

na de RIC-SCT zoals transfer van virusspecifieke lymfocyten lijkt noodzakelijk, omdat de belangrijkste complicaties gerelateerd waren aan virale problematiek na de transplantatie.

De ontwikkeling van RIC strategieën heeft het mogelijk gemaakt om het GVT effect ook te onderzoeken bij patiënten met gemetastaseerde solide tumoren. Diverse onderzoeken laten partiële of complete remissies zien alhoewel het uiteindelijke genezingspercentage toch nog beperkt is. Daarnaast gaat een eventuele respons tegen de ziekte in de meeste patiënten gepaard met ernstige GVHD, die vooral de kwaliteit van leven sterk negatief beïnvloedt. Ook bij solide tumoren wordt het GVT effect zeer waarschijnlijk gemedieerd door donor T cellen die MiHA herkennen op de tumorcellen van de recipiënt. Daarom zou het ook voor solide tumoren een interessante strategie zijn om ter vermindering van de GVHD en verbetering van het GVT effect de donor T cel-reactiviteit te richten op MiHA die uitsluitend tot expressie komen op de tumorcellen of eventueel tegelijkertijd op de tumorcellen en de hematopoietische cellen. In 2005 werd binnen ons laboratorium een hematopoietisch-gerestricteerde MiHA geïdentificeerd, LRH-1 genaamd. Deze MiHA leidt tot een HLA-B7-gerestricteerde CTL respons in leukemie patiënten behandeld met DLI.[6] In *hoofdstuk 6* hebben we onderzocht of het LRH-1 epitope, gecodeerd door het *P2X5* gen, tot expressie komt op solide tumor cellijnen en primaire tumorcellen. We vonden afwijkende expressie van *P2X5* in een significant deel van de tumor cellijnen en primaire tumorcellen, inclusief niercelcarcinoom, melanoom, colorectaal carcinoom, hersentumoren en mammacarcinoom. Bovendien lieten flow cytometrische cytotoxiciteits assays zien dat cellen van solide tumoren die *P2X5* tot expressie brengen gelyseerd worden door de LRH-1 CTL. Deze data wijzen erop dat LRH-1 een interessante hematopoietisch-gerestricteerde MiHA is die ook gebruikt zou kunnen worden in de ontwikkeling van MiHA-specifieke adoptieve immuuntherapie gericht tegen solide tumoren.
De strategie van partieel T cel-gedepleteerd RIC-SCT gevolgd door DLI hebben we ook toegepast bij patiënten met gemetastaseerd niercelcarcinoom. In *Hoofdstuk 7* beschrijven we de klinische en immunologische responsen in vier patiënten. Alle vier werden compleet donor chimeer voorafgegaan door gemengd chimerisme in twee patiënten. Geen van de patiënten ontwikkelde acute GVHD >graad II of uitgebreide chronische GVHD. Twee patiënten zonder GVHD werden vervolgens behandeld met DLI en ook dit leidde niet tot GVHD. Beide patiënten lieten een geringe anti-

tumor respons zien; een patiënt had stabiele ziekte en een patiënt had regressie van longmetastasen. Van deze twee patiënten konden we MiHA-specifieke CTL isoleren die gericht waren tegen RCC tumorcellen. In de patiënt met stabiele ziekte was de CTL respons gericht tegen het HLA-A2-gerestricteerde SCMY peptide, wat een Y-chromosoom gecodeerd MiHA is. In de patiënt met een partiële remissie was de CTL response gericht tegen een nog onbekend HLA-B7-gerestricteerd MiHA, waarvan we de identiteit proberen te achterhalen. Deze resultaten wijzen erop dat in patiënten met een gemetastaseerd niercelcarcinoom die behandeld zijn met een partieel T cel-gedepleteerd RIC-SCT gevolgd door DLI, MiHA-specifieke T cel-responsen worden opgewekt waarbij RCC cellen herkend worden na DLI zonder dat er hierbij GVHD is opgetreden.

De studies in dit proefschrift beschrijven een aanpak om enerzijds het gewenste GVT effect van allogene SCT te versterken en anderzijds de GVHD, morbiditeit en mortaliteit van de SCT te reduceren. De combinatie van een partieel T cel-gedepleteerd RIC-SCT gevolgd door DLI was het meest effectief in patiënten met lymfoom en CLL. In deze studie werden alleen patiënten met chemotherapiegevoelige ziekte geincludeerd waarbij alle patiënten tenminste een partiële remissie hadden bereikt voorafgaand aan de RIC-SCT. Het is bekend uit de literatuur dat allogene SCT veel minder effectief is in chemotherapierefractaire ziekte. In de studie met MM patiënten was de TRM duidelijk minder na een partieel T cel-gedepleteerde RIC-SCT. Echter een substantieel aantal patiënten ontwikkelde een recidief MM ondanks pre-emptieve DLI en recipiënt DC-vaccinatie in combinatie met DLI. We hebben laten zien dat ontvanger DC vaccinatie na RIC-SCT veilig is en haalbaar. Specifieke MiHA-specifieke T cel responsen hebben we tot nu toe nog niet kunnen aantonen, ook niet in patiënten die na DLI een klinische respons vertoonden. Hoewel het gebruik van ontvanger afkomstige DC nog niet volledig is onderzocht, zou een volgende stap kunnen zijn om het antitumor effect te versterken door vaccinatie met donor DC beladen met MiHA peptiden of geëlectroporeerd met MiHA-coderend mRNA. Dergelijke vaccins kunnen ook ingezet worden om het GVT effect te versterken in patiënten met acute leukemie, lymfoom en CLL.
Nog meer uitdagend is het verbeteren van de immuuntherapie na RIC-SCT in gemetastaseerd RCC. Vier patiënten met progressieve en niet-chemotherapie gevoelige ziekte werden behandeld met een partieel T cel-gedepleteerd RIC-SCT gevolgd door DLI. In twee patiënten hebben we MiHA-specifieke T cel-responsen aangetoond na

DLI. Ook voor deze patiëntengroep zou posttransplantatie immuuntherapie gericht op tumorspecifieke MiHA een interessante benadering kunnen zijn. In onze preklinische studie hebben we afwijkende *P2X5*-expressie gevonden in verschillende typen solide tumoren inclusief niercelcarcinoom. Nieuwe ontwikkelingen in de behandeling van het niercelcarcinoom met tyrosine-kinase remmers hebben de behandelstrategieën voor deze patiënten significant veranderd, maar nog niet tot curatie geleid. Thans is de rol van allogene SCT in deze ziekte nog onduidelijk, maar heeft nog altijd de potentie om bij te dragen aan een verbetering van de sombere prognose waar deze patiënten uiteindelijk mee geconfronteerd worden.

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Curriculum Vitae

Tjakiena Henriëtte Levenga werd geboren op 23 maart 1970 te Veendam. Zij behaalde het VWO diploma in 1988 aan het Ubbo Emmius Lyceum te Stadskanaal. Vanaf 1988 studeerde zij geneeskunde aan de Rijks Universiteit Groningen. In 1996 liep zij haar oudste coschap in het Slotervaart ziekenhuis te Amsterdam en in datzelfde jaar behaalde zij het artsexamen. Daarna was zij werkzaam als arts-assistent Interne Geneeskunde (AGNIO) in het Slotervaart Ziekenhuis en later in het Medisch Spectrum Twente te Enschede. In januari 1998 begon zij met haar opleiding tot internist in het Slingeland Ziekenhuis te Doetinchem (opleider dr. F. de Vries). In 2000 werd de opleiding voortgezet in het Universitair Medisch Centrum (UMC) St Radboud te Nijmegen (opleiders: prof. dr. J.W.M. van der Meer, prof. dr. P.M.J. Stuyt en dr. J. de Graaf). In november 2002 is zij gestart met het aandachtsgebied Medische Oncologie in deeltijd (opleider prof. dr. P.H.M. De Mulder†) in combinatie met het onderzoek beschreven in dit proefschrift. In maart 2003 vond de registratie als internist plaats, gevolgd door registratie als medisch oncoloog in maart 2005. In november 2004 werd gestart met het aandachtgebied hematologie (opleider prof. dr. T.J.M. de Witte), gevolgd door registratie als Hematoloog in 2008. Vanaf november 2009 is zij werkzaam als internist hemato-oncoloog in het Groene Hart Ziekenhuis te Gouda. Zij woont samen met Arie van den Berg. Samen hebben zij drie kinderen, Loes (2006), Lars (2007) en Nanno (2007).

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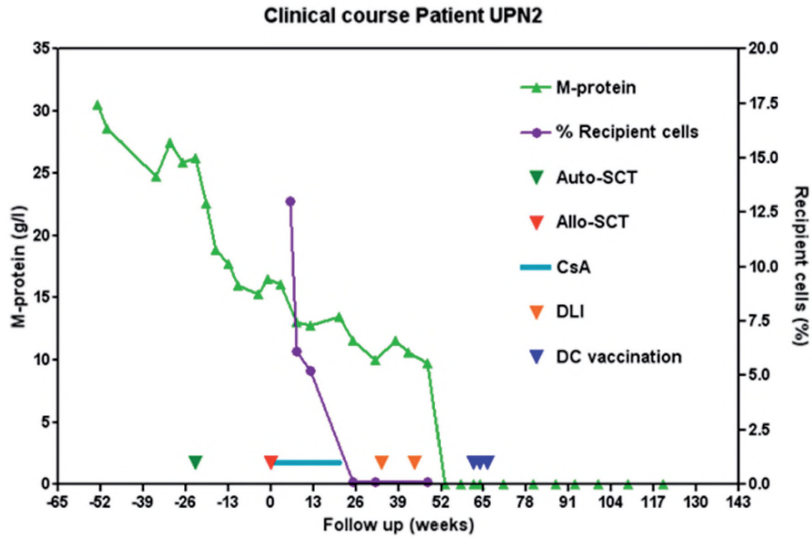
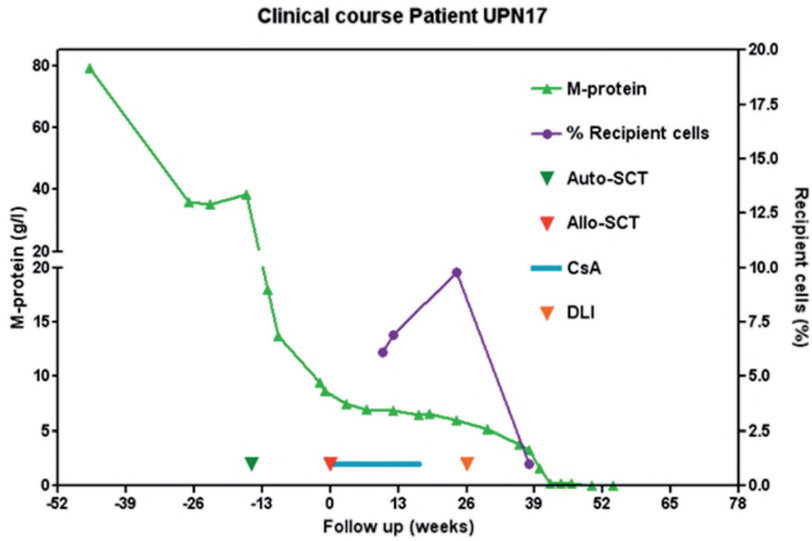
Colour figures

a



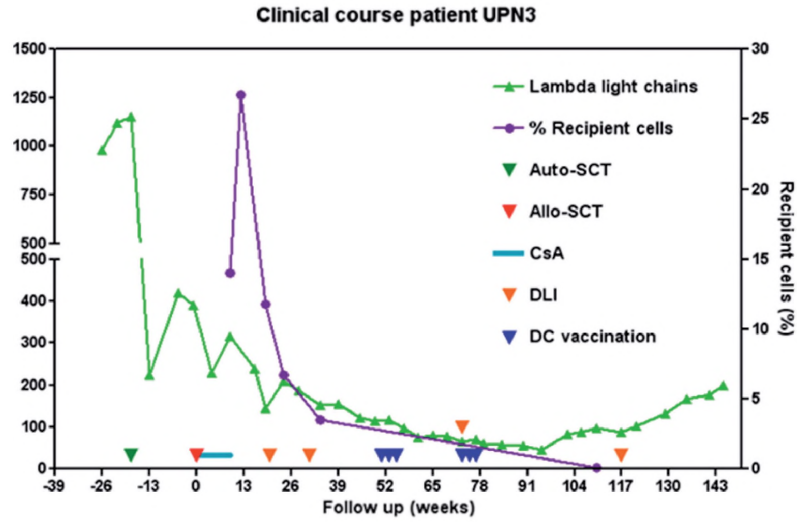
b



Chapter 4

Figure 2: Clinical course of patient UPN2 (a) and patient UPN17 (b). The Y-axis on the left shows disease load as measured by serum free light chains or M-protein, and is shown with the green line. The Y-axis on the right shows the percentage recipient cells in peripheral blood and is shown with a purple line. Triangle in blue indicates autologous SCT, triangle in red indicates RIC-SCT, triangle in orange indicate DLI and triple-triangle in blue indicate 1 cycle of DC vaccinations.

a



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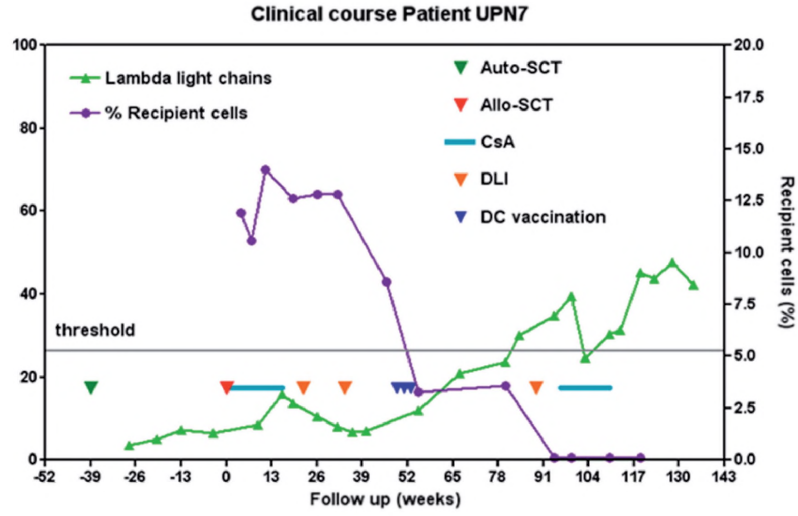
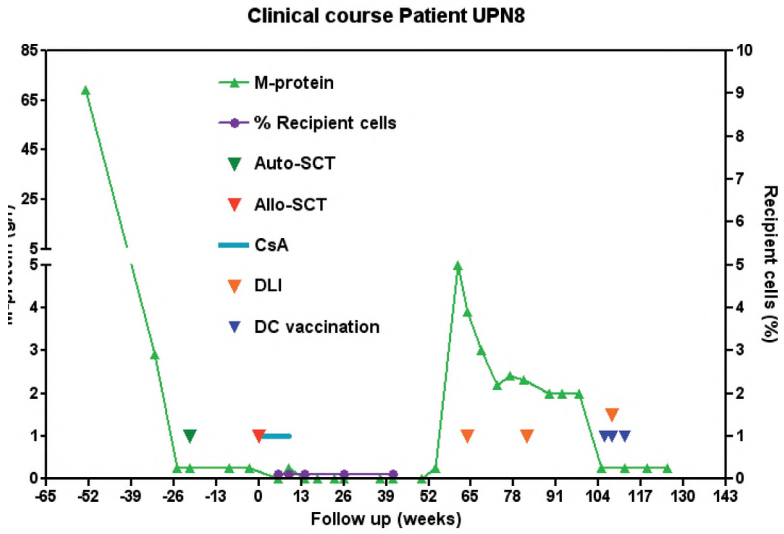


Chapter 4

Figure 6: Clinical course of patient UPN3 (a) and patient UPN7 (b). The Y-axis on the left shows disease load as measured by serum free light chains or M-protein, and is shown with the green line. The Y-axis on the right shows the percentage recipient cells in peripheral blood and is shown with a purple line. Triangle in blue indicates autologous SCT, triangle in red indicates RIC-SCT, triangle in orange indicate DLI and triple-triangle in blue indicate 1 cycle of DC vaccinations.



Chapter 4

Figure 7: Clinical course of patient UPN8. The Y-axis on the left shows disease load as measured by serum free light chains or M-protein, and is shown with the green line. The Y-axis on the right shows the percentage recipient cells in peripheral blood and is shown with a purple line. Triangle in blue indicates autologous SCT, triangle in red indicates RIC-SCT, triangle in orange indicate DLI and triple-triangle in blue indicate 1 cycle of DC vaccinations.