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Evaluation of donor lymphocyte infusions after allogeneic stem cell transplantation

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Citation
Eefting, M. (2018, December 13). *Evaluation of donor lymphocyte infusions after allogeneic stem cell transplantation*. Retrieved from https://hdl.handle.net/1887/67416

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Author: Eefting, M.
Title: Evaluation of donor lymphocyte infusions after allogeneic stem cell transplantation
Issue Date: 2018-12-13

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Matthias Eefting

EVALUATION OF DONOR LYMPHOCYTE INFUSIONS AFTER ALLOGENEIC STEM CELL TRANSPLANTATION

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Cover image: First steps, after Millet, by Vincent van Gogh.

Layout and printed: Optima Grafische Communicatie, Rotterdam, the Netherlands
(www.ogc.nl)
ISBN: 978-94-6361-166-4

EVALUATION OF DONOR LYMPHOCYTE INFUSIONS AFTER ALLOGENEIC STEM CELL TRANSPLANTATION

Proefschrift

ter verkrijging van
de graad van doctor aan de Universiteit Leiden,
op het gezag van de Rector Magnificus prof. Mr. C.J.J.M. Stolker,
volgens besluit van het College voor Promoties
te verdedigen op
donderdag 13 december 2018
klokke
11:15

door
Matthias Eefting

geboren te Velsen
in 1978

PROMOTIECOMMISSIE

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Co-promotores:	Dr. C.J.M. Halkes Dr. L.C. de Wreede
Promotiecommissie:	Prof. dr. J.J. Zwaginga Prof. dr. S. Zweegman (VUMC) Prof. dr. J.J. Cornelissen (Erasmus MC)

The work presented in this thesis was performed at the department of Hematology of the Leiden University Medical Center and was financially supported by the Dutch Cancer Society (KWF Kankerbestrijding).

Voor mijn ouders.

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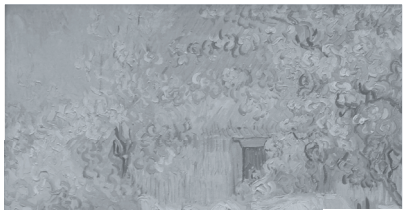

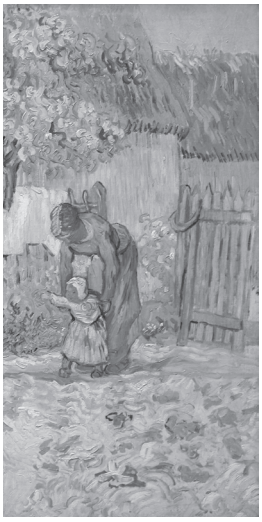

Chapter 1

General introduction

1. Allogeneic stem cell transplantation

Allogeneic stem cell transplantation (alloSCT) is a treatment option with curative potential for many hematologic disorders. It has become the treatment of choice for many patients with high-risk and/or relapsed hematologic malignancies.[1] The primary aim of alloSCT is to transfer stem cells from a donor to a host in order to replace abnormal hematopoiesis, followed by induction of an immune response against patient-derived hematopoiesis or specific hematopoietic cell lineages from which the tumor is derived.[2]

Possible sources of the hematopoietic stem cells are a human leukocyte antigen (HLA-)matched sibling donor (MRD) or unrelated donor (MUD), a first-degree non-HLA-matched family member (haplo-identical), a HLA-mismatched unrelated donor, or umbilical cord stem cells (umbilical cord).[1] The cells can be collected directly from the bone marrow, umbilical cord blood, or from peripheral blood after mobilization of stem cells by cytokines.

After infusion, the donor stem cells will home to the bone marrow and start production of red blood cells, leukocytes and platelets. Only in the rare cases of syngeneic SCT (all host and recipient cells have an identical genetic make-up) no recipient-versus-donor or donor-versus-recipient reactions will occur.[3] In all other recipient-donor combinations, significant immune responses between patient and donor may occur, which heavily impact outcome. An undesired effect of alloSCT is recognition of antigens presented on host tissues by donor T-cells, which may result in a detrimental condition called graft-versus-host disease (GvHD).[4] This complication of alloSCT is associated with high morbidity and mortality.[5,6] However, donor-versus-host responses may also result in immunologic recognition and eradication of residual malignant cells. This unique type of response is called graft-versus-tumor (GvT) or graft-versus-leukemia (GvL) effect.[7]

The general outcome of alloSCT depends on numerous factors.[8] Important disease-related parameters are time between diagnosis and transplant, prior treatment, and remission status at transplant.[9] Important donor/recipient-related factors are age, gender, histocompatibility, and comorbidities.[10] Transplant-related factors affecting outcome are the conditioning regimen, type of immune suppression, stem cell source, and stem cell dose.[11]

2. Alloreactivity after allogeneic stem cell transplantation

The immune responses after alloSCT are primarily mediated by T-cells. The major function of T-cells is to detect and control viral infections. During viral infections, infected cells present viral peptides on their cell surface. These peptides are derived from intracellular (viral) proteins and are presented in HLA molecules on the cell surface (see Figure 1). Under physiological conditions, T-cells detecting self (autologous) peptides are deleted in the thymus by negative selection in order to prevent self-directed immune responses, which may result in auto-immune diseases.[12] A naïve T cell has, unlike

activated or memory T cells, not yet encountered its cognate antigen within the periphery. To provoke an immuneresponse by naïve T-cells, antigens need to be presented by professional antigen-presenting cells (APCs). APCs are mature hematopoietic cells, which play a key role in the induction of immune responses. They contain high levels of HLA-molecules and co-stimulatory molecules on their cell surface. The presented peptides may be processed from endogenous or exogenous proteins. After recognition of HLA-peptide complexes by T-cells, an immune response is elicited to eliminate infected cells, and to stop viral replication. A huge repertoire of T-cells is available to detect all kinds of non-self (viral or other) peptides in the context of (self) HLA molecules.

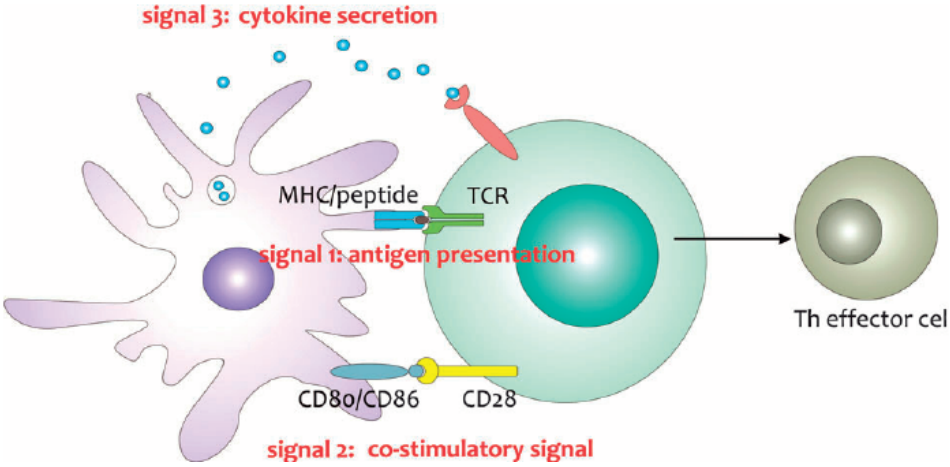

Figure 1. *Initiation of effector T cell responses requires three signals. Stimulation of the TCR by MHC/peptide complexes delivers signal 1, interactions between co-stimulatory ligands on the APC and CD28 on the T cell provide signal 2, and the secretion of inflammatory cytokines that polarise T cell responses delivers signal 3. Reproduced from Chem. Soc. Rev ., 2011 (40): 320–339 with permission of The Royal Society of Chemistry (RSC) on behalf of the European Society for Photobiology, the European Photochemistry Association and the RSC.*

After allosct, host T-cells tolerate all normal cells of host origin and donor T-cells will tolerate all donor-derived cells. After alloSCT T-cells of host origin might not tolerate cells of donor origin and T-cells collected directly from the donor might not tolerate cells of patient origin, respectively.[13] The result is a bidirectional immunologic interaction.[14] In the population, there is large variation in HLA polymorphisms. A donor search has to be performed to find a suitable donor for transplant. Due to small genetic differences between donor and host, patient-derived T-cells may recognize HLA-peptide complexes of donor origin, or vice versa. This may play a role when peptides in non-self HLA molecules are recognized after HLA-mismatched alloSCT. Nowadays, the majority of alloSCT are performed using HLA-matched donors. Despite matching between patient and donor still stong alloimmune responses may occur. This cannot be explained by recognition of differences between HLA-molecules. Peptides that differ between donor and recipient

due to single nucleotide polymorphisms (SNP) can be presented in matched HLA-molecules of donor or recipient, and may be recognized by T-cells from the recipient or donor. These peptides expressed in matched HLA-molecules that can be recognized by T-cells from another individual are called minor Histocompatibility Antigens (MiHA).[15] Difference in MiHA can lead to an immune response from recipient to donor and/or from donor to recipient (see Figure 2).

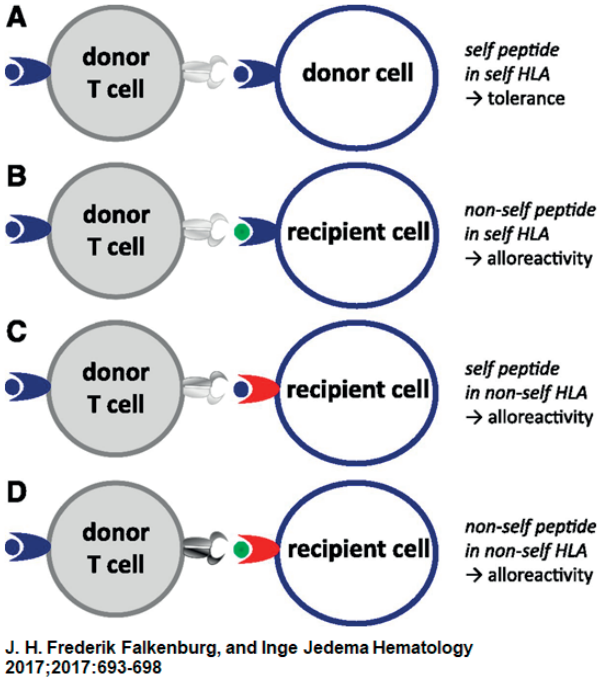

Figure 2. *Peptide/HLA complexes as targets of T-cell mediated alloimmune reactivity. (a) Self peptides expressed in self HLA molecules induce tolerance. (b) Polymorfic nonself peptides recognized in the context of self HLA molecule scan induce alloreactivity; these polymorphic nonself peptides are called minor histocompatibility antigens (MiHA). (c) Monomorphic (self) peptides presented in the context of nonself HLA molecules provoke alloreactivity. (d) Polymorphic nonself peptides in the context of nonself HLA molecule scan also provoke alloreactivity. Reproduced from Hematology Am Soc Hematol Educ Program., 2017 (1): 693-698. with permission of The American Society of Hematology (ASH).*

Donor stem cells and T-cells are administered as a single unmanipulated graft in most transplant centers. This is called T-cell replete alloSCT.[16] After stem cell infusion, donor stem cells will start production of cells of all hematologic lineages, including APCs. Because professional APCs are hematopoietic cells, and endogenous proteins are readily presented on their surface, these cells are preferentially recognized. The level of expression of HLA is especially high on cells of hematopoietic origin. Virtually all immune responses after alloSCT involve antigens from hematopoietic origin and profound

immune responses against cells of hematopoietic origin can be observed.[17] Rejection is a rare immunologic complication of alloSCT. During this event, all donor-derived hematopoietic cells are recognized by host T-cells that were not depleted prior to transplant.[18] This is immediately life-threatening, as all hematopoietic reserve is lost instantly. The risk of graft rejection is higher when the donor source is unrelated to the recipient or in case of a HLA-mismatched setting. To prevent rejection, high intensity conditioning therapy by total body irradiation (TBI), chemotherapy, and/or administration of T-cell depleting antibodies to the recipient is used to deplete host immunity.[19]

HLA is not only expressed on hematopoietic tissues, but also on non-hematopoietic tissues. T-cells of donor origin may recognize MiHAs from non-hematopoietic cells of patient origin in the context of the matched HLA molecules.[20] This may result in a tissue-destructive immune response called GvHD. Like rejection, this disease involves a potentially life-threatening situation. Skin, liver, and intestines are most often involved, especially in acute GvHD, but in essence many other organs may be affected.[5] The number of HLA-mismatches between patient and donor is positively correlated with the severity of GvHD.[21] The nature of the inflammatory GvHD response is characterized by a continuously amplifying cascade: release of pro-inflammatory cytokines, like TNF-α, IL-1, and IFN-γ, results in rapid expansion of alloreactive T-cells with specificity to host tissues that, in turn, secrete additional inflammatory mediators. Cytokines and chemokines influence proliferation and homing of effector cells to GvHD target tissues. The counterbalance of the occurence of GvHD is a decreased likelihood of relapse by GvL leading to superior disease-free survival after transplantation. The immune response against the large variety of expressed antigens is likely to include antigens expressed on hematopoietic cells. As the malignant clone is derived from hematopoietic cells, this may result in GvL. This was noticed already in early transplantation studies when alloSCT appeared more effective in eliminating acute leukemias as compared to syngeneic or autologous transplantation.[3]

3. T-cell depletion

The essential role of donor T-cells after alloSCT in the development of GVHD is supported by the observation that the incidence of severe GvHD after T-cell depletion (TCD) is low.[3,22] Numerous TCD methods exist, and most depend on negative selection of stem cells by immunological techniques.[23] These techniques can be classified as *in-vivo*, when TCD is performed during conditioning therapy by intravenous administration of specific antibodies to the patient, or as *in-vitro*, in case the graft is manipulated prior to infusion.

In-vivo TCD can be used in HLA-mismatched transplant settings to eliminate patient-derived anti-donor HLA-directed T-cells, that may induce rejection. *In-vivo* TCD not only reduces the host immune response, thereby favoring engraftment and limiting rejection, but also causes elimination of circulating donor T-cells. This is the result of ongoing

circulation of the TCD-antibody during and after transplant. The TCD-antibodies will bind to donor T-cells, thereby preventing or reducing GvHD.[23,24] Alemtuzumab and anti-thymocyte globulin (ATG) are frequently used for this purpose.[25,26] Alemtuzumab is a monoclonal antibody that binds to CD52, a protein present on the surface of mature lymphocytes.[27] ATG depletes T-cells in blood and peripheral lymphoid tissues.[28] Both are associated with low rates of severe acute and chronic GvHD.[25] Application of alemtuzumab or ATG is associated with similar overall survival rates, but significant lower rates of GvHD, as compared to T-cell replete alloSCT.[25,26]

In-vitro TCD may be performed by incubation of the graft with antibodies, like alemtuzumab, a strategy which is also called 'alemtuzumab in the bag', or by isolation of stem cells by antibody-loaded magnetic beads (usually CD34+ positive selection).[27] After CD34+ positive selection of stem cells, the average number of T-cells in the graft is lower than after incubation of the graft with antibodies. A lower number of T-cells is associated with delayed immunreconstitution.[25] This is associated with opportunistic infections, like cytomegalovirus (CMV-)reactivation and aspergillus. Both infections require close monitoring and early treatment.[29,30] Effective *in-vitro* TCD obviates the need for additional prophylactic post-transplant immunosuppression. Several studies show very low incidences of severe GvHD and limited non-relapse mortality (NRM) after *in-vitro* TCD.[27,31-33] The low level of severe GvHD after TCD increases the feasibility of alloSCT in elderly and frail patients. TCD is also associated with an increased relapse rate. To (re-) introduce a GvL-effect after TCD-alloSCT, the concept of donor lymphocyte infusions (DLI) which are administered a while after alloSCT has been developed. The aim of DLI is infusion of T-cells from the original donor. Unmodified DLI is obtained by apheresis of leukocytes using peripheral venous access. Timing and dosing of DLI may help to skew the balance from GvHD to more favorable GvL-effect.

4. The balance between graft-versus-leukemia (GvL) and graft-versus-host-disease (GvHD)

To provide space for donor hematopoiesis, patients have to undergo conditioning therapy prior to stem cel infusion. This consists of chemotherapy and/or irradiation and may be myeloablative or non-myeloablative.[34] Myeloablative conditioning for alloSCT usually consists of a combination of cyclophosphamide and TBI and results in (near-) complete destruction of host hematopoietic reserve and immunity. This may be associated with strong anti-leukemia effects, but is not feasible in elderly and frail patients.[35] This group of patients may undergo non-myeloablative conditioning, also known as reduced-intensity conditioning (RIC). Doses of chemotherapy and/or irradiation in RIC are not intended to destroy all hematopoietic cells and may have limited effect on residual disease. As a result, RIC is associated with higher relapse rate than myeloablative conditioning.[35]

After conditioning therapy patient hematopoiesis is replaced by donor hematopoiesis. Early infusion of donor T-cells soon after conditioning therapy, like in T-cell replete alloSCT, is associated with high incidence and severity of GvHD. The conditioning treatment will lead to tissue destruction and inflammatory responses, which will increase the level of HLA-expression and adhesion molecules on non-hematopoietic tissues.[36] T-cell activation also depends on firm adhesion to target cells. APCs express high levels of co-stimulatory adhesion molecules as compared to non-hematopoietic tissues, especially under steady-state conditions.[37] After induction of tissue damage or during inflammatory conditions, release of so-called danger signals results in activation of Toll-like receptors and production of activating cytokines. Following initiation priming by activated APCs, the T-cell response amplifies. Also the levels of co-stimulatory molecules on non-hematopoietic tissues increase and the expression of antigens is upregulated.[36,38-40] This causes targeting of these tissues by allo-reactive T-cells, which will initiate the GvHD-cascade.[39] Although the incidence of GvHD increases with higher conditioning intensity, especially in regimens containing TBI, although the incidence of GvHD after RIC remains high in T-cell replete settings as well.[41,42] Prophylactic immunosuppression is essential to reduce the incidence of GvHD after T-cel replete alloSCT, but is known for side-effects.[43] Despite prophylactic immunosuppression, progressive GVHD needing systemic treatment can develop. After failure of first-line high dose steroids for treatment of GvHD, the outcome is poor: second and thirth-line immunosuppressive therapies have limited efficacy.

Administration of DLI during uncontrolled infections is also associated with development of GvHD.[36] The immune system of the host is heavily suppressed early after transplant. Immunoreconstitution by production of donor immune cells occurs gradually. The result is an increased susceptibility to infectious complications, such as CMV-reactivations. CMV-disease is associated with increased HLA-expression and adhesion molecules on infected tissues, such as intestinal mucosa. This may prime GvHD when DLI is administered during CMV-reactivation.[36]

It will take several weeks to months after conditioning therapy until damaged tissues are fully restored, infections are controlled, and a steady-state situation occurs during which the levels of HLA-expression and co-stimulatory molecules on non-hematopoietic tissues are reduced. The limited rate of GvHD after effective TCD may help to shorten this process. During the replacement of host hematopoiesis by donor hematopoiesis, the level of patient-derived APCs diminishes gradually. APCs are designed to present antigens, which are likely to include broadly expressed endogenous antigens. Later after transplant, the level of antigen-expression by host-derived APCs is likely to be lower than early after transplant. Postponing DLI until infections are cleared and APCs are gradually replaced by cells of donor origin, may help to skew donor to host immune responses from GvHD to GvL by inducing a more favorable composition of HLA- and co-stimulatory

molecules expression profile on hematopoietic versus non-hematopoietic tissues (see Figure 3). A low level of HLA-expression may result in less interaction between T-cells and HLA-peptide complexes. This may result in a lower severity of GvHD. Postponing DLI too long increases the risk of relapse of the primairy disease. This may be explained by the nature of the primary disease, but postponing DLI until all APCs of host origin are fully replaced by donor APCs may also lead to limited expression of patient-derived hematopoiesis-associated antigens, and a weak anti-hematopoiesis immune response.

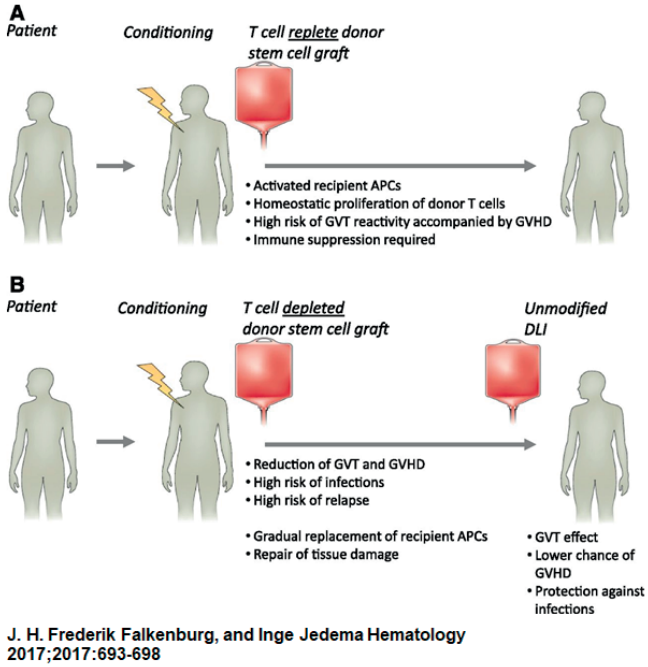

Figure 3. *Balance between GvT and GvHD after T-cell replete or T-cell depleted transplantation. (a) T-cell replete alloSCT: conditioning regimen induced tissue damage and pathogens mediate activation of recipient APC, lymphopenia-associated homeostatic proliferation, and an inflammatory cytokine milieu resulting in highly diverse and high-magnitude alloimmune T-cell responses causing simultaneous GvT and GvHD reactivity, requiring posttransplant immune suppression. (b) T-cell depletion abrogates GvT and GvHD early after transplantation, resulting in an increased risk of relapse and lack of control of pathogens. Postponed donor T-cell infusions (donor lymphocyte infusion; DLI) are needed for GvT reactivity: the risk of GvHD is lower due to tissue repair, less inflammation, and replacement of recipient APC by donor APC. Reproduced from Hematology Am Soc Hematol Educ Program., 2017 (1): 693-698. with permission of The American Society of Hematology (ASH).*

All T-cells in DLI are harvested directly from the donor, and did not undergo negative selection in the thymus of the recipient.[13] One of the benefits of these T-cell is the potential availability of T-cells that recognize MiHAs with high avidity.[44] Usually polyclonal T-cell responses against broadly expressed MiHAs are induced.[45,46] In most circumstances, the immune system of the donor has not been exposed to MiHA of another individual. This

may explain why T-cells that target a specific MiHA, including MiHAs specifically against hematopoietic cells, are usually present within the naïve T-cell repertoire of the donor in low frequencies.[47] Delayed DLI allows higher doses of T-cells as compared to T-cell replete transplantation.[48] Larger quantities of T-cells increase the likelihood of introducing T-cells targeting the patient hematopoietic cells with high avidity. Activation of high avidity T-cells against MiHA's expressed on recipient hematopoietic cells may result in a potent immune response that may eradicate all hematopoiesis of recipient origin, including the malignant cell population.[46,49]

5. Donor lymphocyte infusions (DLI)

DLI may be administered therapeutically, prophylactically, or pre-emptively. The primary aim of therapeutic DLI is to induce durable remissions of any level of measurable residual or relapsed disease. Prophylactic DLI is usually administered at a predefined time-point after alloSCT to prevent relapse in patients with high-risk disease, such as AML with adverse cytogenetic abnormalities.[50] Pre-emptive DLI aims to convert mixed-chimerism to full-donor hematopoiesis.[51] The term pre-emptive and prophylactic DLI are often used interchangeably, as both types of DLI are aimed to prevent relapse. For simplicity, DLI that is given in the absence of disease and aimed to prevent relapse will be called prophylactic DLI in the remainder of this introduction.

The most often observed adverse event after DLI is the occurrence of GvHD.[48] The risk of moderate and severe GvHD is approximately 30-40%, and may display symptoms which resemble both acute and chronic GvHD.[48,52] A longer time period between alloSCT and DLI is associated with lower intensity of GvHD and allows infusion of higher doses of DLI.[52] The risk of GvHD after DLI is increased after reduced-intensity conditioning.[53] GvHD after DLI usually responds well to corticosteroids. The reported NRM rates rates after DLI vary between 9-44%.[53]

Dosing of DLI depends on conditioning therapy, donor type, time after transplant, and clinical setting: lower doses of DLI are administered early after RIC transplantation, from unrelated donors, in a prophylactic setting, and/or after TCD.[48,50,52,54-56] A DLI dose of 1 x 10^5 CD3+ cells/kg may be administered prophylactically from an unrelated donor at 3 months after TCD-alloSCT, or 1 x 10^7 CD3+ cells/kg from an related donor may be administered during relapsed disease at the same timepoint after T-cell replete alloSCT. In the absence of GvHD, DLI may be applied in escalating doses.[57] Escalating doses may comprise up to 2 x 10^8 CD3+ cells/kg.[57,58]

The effectiveness of therapeutic DLI may be increased by adding interferon-α (IFN). IFN accelerates the immune response when combined with low-dose DLI, with acceptable GvHD.[56] IFN stimulates T-cell therapy probably by inducing upregulation of APCs, cytokine production, and activation of T-cells.[59] The effectiveness of therapeutic DLI may also be increased by administation of DLI in escalating doses.[57] The impact of DLI may

be increased by manipulation of the host milieu to promote expansion of donor T-cells. Prior lymphodepletion or radiotherapy are applied for this purpose. Lymphodepletion may stimulate T-cell proliferation by providing lymphoid space, elimination of regulatory T-cells, and reduced competition for growth factors.[58] Local or total-body radiation may stimulate immune reactivation when radiation-damaged cells interact with Toll-like receptors by induction of danger-signals, which may lead to activation of allo-responsive T-cells by production of chemo-attractants and pro-inflammatory cytokines.[60]

6. The role of DLI for acute leukemias and multiple myeloma (MM)

Although alloSCT for acute leukemias may have curative potential, approximately 30% of transplanted patients develop a relapse, which is associated with a very poor prognosis.[55,61] The relapse risk increases when a patient is transplanted in second or thirth remission.[62] Both relapsed AML and ALL conver a poor prognosis, with estimated overall survivals of patients at 5-year after relapse of 13% and 0%, respectively.[63] Hematologic responses of relapsed acute leukemias to DLI alone are rare.[53] Therapeutic DLI can induce complete remissions in 20% to 80% of patients, with the success rate highly dependent on the underlying malignancy.[64] A favorable effect of therapeutic DLI is highly unlikely when relapse occurs shortly after or during severe GvHD. This type of relapse reflects failure of an allogeneic anti-tumor response. The level of disease burden appears to play an important role also. A limited tumor burden may be associated with reduced antigen exposure and delayed T-cell activation, which may impact time to induce an adequate immune response. Observations of remission up to one year after DLI have been described.[48] In some patients, the disease may even progress before remission is induced. A very high blast count is associated with poor responses.[53,65] Salvage re-induction chemotherapy for relapsed leukemia may lead to clinical remissions in a substantial number of patients, but these remissions are usually of short duration.[48] The high proliferation rate of acute leukemia appears to suppress the immune response and shortens the time span during which the T-cells may exert their anti-leukemic activity.[66] In the majority of DLI administrations, clinical immune responses are observed within one or two months after administration. Even this relatively short timespan may be too long to overcome the high proliferation rate of an aggressive malignancy.[63] Preferentially a large number of alloreactive T-cells are administered to increase the magnitude of the immune response as the time-span to induce an immune responses has to be as short as possible. Long-term remissions have been observed primarily in patients who were treated by intensive chemotherapy or radiotherapy combined with DLI.[67] The failure of DLI in ALL may be due to the nature of pre-B-cells which are poor APCs because they induce anergy and lack co-stimulatory molecules.[68] HLA-incompatibility between donor T-cells and tumor cells appears to induce the strongest GvL reactivity, but is obviously associated with a high risk of GvHD.[69]

The poor response of DLI for relapsed acute leukemias led to the development of prophylactic DLI to prevent relapse.[50] This strategy appears to be associated with a beneficial effect on outcome, with controllable toxicity.[54,70] Prophylactic DLI is administered to patients with a high-risk of relapse, for example a patient with poor-risk AML who shows mixed-chimerism without signs of GvHD.[51] Mixed-chimerism, defined as any level of simultaneously detectable patient and donor hematopoiesis, resembles the absence of a strong donor-derived immune response against patient hematopoiesis.[71] The remaining patient hematopoiesis may include a sub-detectable malignant clone, which may explain the increased risk of relapse. In particular increasing mixed-chimerism is associated with high-risk of relapse.[72] The aim of prophylactic DLI is to introduce a sufficient number of T-cells that recognize hematopoietic cells of the donor to eradicate all remaining patient-derived hematopoiesis, including any sub-detectable malignant clone. This is associated with conversion of mixed-chimerism to full-donor hematopoiesis, which is frequently observed after DLI after TCD and/or RIC.[73] Preferentially only limited numbers of alloreactive T-cells are used to minimize the risk of GvHD.

The role of DLI as a treatment option for MM is currently under debate[74]. AlloSCT can result in cure or long-term control of MM because immune-competent donor T-cells can exert efficient graft-versus-myeloma (GvM) effects.[75,76] The beneficial GvM is negatively impacted by GvHD and infectious complications after alloSCT leading to increased NRM as compared to patients who do not receive alloSCT.[77] TCD effectively reduces NRM.[78] Despite initial disease control early after transplant, the majority of patients ultimately relapse.[79] Patients respond to DLI for relapsed myeloma.[80] Conversion to full-donor chimerism and occurrence of GvHD are associated with persisting disease responses.[76,81] The durability of responses is unclear: some may be transient and extramedullary relapses are common.[82,83] Escalating doses of DLI frequently do not result in new long-term remissions, in particular in the extramedullary compartment.[84] Several groups tried to improve the outcome by combining DLI and immune modulatory drugs, such as thalidomide, bortezomib, and lenalidomide.[85-87] This resulted in partial and complete remissions, but cure was achieved in a minority of patients only. The role of cytoreductive therapy prior to DLI in case of residual disease of MM is unclear. Nowadays, checkpoint inhibitors are gradually implemented in treatment strategies for relapsed MM.[88] Whether combining these therapies with DLI will improve the outcome of MM remains to be established.

In general, the effect of DLI on relapse and survival differs between disease-type/stage, conditioning therapy, transplant-type, immunosuppression, time since transplant, infections, development of prior GvHD, dosing, and clinical setting (therapeutic or prophylactic). The effect of DLI on relapse and disease progression may even differ between different body compartments, like bone marrow and extramedullary sites. To study the effect of DLI more sophisticated statistical methods than commonly used are required. This will be the topic of the following paragraph.

7. Analysis of time-dependent events after alloSCT

The impact of dose and time of application of DLI on the clinical outcomes of alloSCT is difficult to determine. Randomized trials have not been performed. Transplant-type also plays a role: it is virtually impossible to dissect the effect of donor T-cells from the effect of conditioning therapy in T-cell replete alloSCT. However, the effect of DLI may be estimated when DLI is administered at predefined circumstances after TCD-alloSCT, which is associated with a lower incidence of severe GvHD. The DLI incidence may be high, and there are less circumstances which limit interpretation of a direct clinical effect. To study the role of DLI for prevention or treatment of relapses, conventional survival analysis does not suffice. The time-dependent nature of DLI and interfering post-transplant events, like development of GvHD, require analysis by means of more sophisticated statistical methods.

Conventional outcome measures of interest after transplantation are OS, progression-free survival (PFS), and cumulative incidence of GvHD.[89] By definition, these outcomes are based on a single start and end point. This is useful to determine the incidences of death, relapse, and development of GvHD since alloSCT. Intermediate events during follow-up may serve as outcomes. When using statistical methods that are based on a single start and end point, it is not possible to estimate the effect of intermediate outcomes as predictors of later (final) outcomes, like the impact of GvHD on relapse rate or survival.

In an attempt to increase insight in morbidity and mortality after alloSCT new composite outcome measures have been introduced, such as GvHD-free-relapse-free-survival.[90] All relevant outcomes with negative impact on morbidity and mortality can be clustered into a single composite end-point. Composite endpoints have the advantage of achieving adequate power in clinical trials by measuring more events, which increases statistical precision and efficiency to detect difference between groups. This may be important in a trial setting, as trials can be smaller and less costly. A disadvantage of using composite endpoints is that outcomes of different importance to the patient are combined. In case of GvHD-free-relapse-free-survival the endpoints are development of GvHD, relapse and death. These outcomes are (potentially) reversible or irreversible: development of GvHD may be successfully treated by immunosuppression, and relapse may reflect a poor survival, but only death is irreversible. Also, the order and composition of failures can not be ascertained: the first failure serves as endpoint and subsequent events are not taken into account.[91] In reality there may be inverse correlation between endpoints: development of GvHD is associated with a decreased relapse rate. Including both events in the composite endpoint excludes analysis of this type of interaction. To extract more insights from clinical trials and observational studies, all relevant events should be analyzed separately. In the case of transplant analysis many events compete with other outcomes. By definition, a patient who suffers a relapse cannot experience NRM. Competing events should be taken into account appropriately by competing risks analysis.[92]

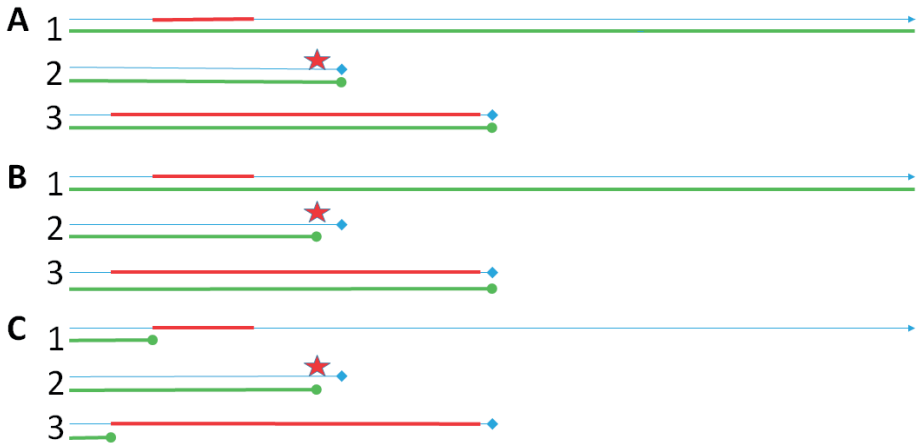

Figure 4. *Conventional outcome measures. Three patients (1, 2 and 3) are observed over time (blue line) until the end of follow up (blue arrow) or death (blue diamond). During the observation period, intermediate events may occur: presence of GvHD (red line) and/or relapse (red star). The green line represents the time period that is included for analysis of the three outcome measures (a, b and c) until censoring (green dot) or end of follow-up: (a) overall survival (OS), (b) progression-free survival (PFS), and (c) GvHD-free-relapse-free-survival (combined-endpoint). All analyses are based on single start and end point.*

Another problem in transplant analysis may arise when patients are assigned to different groups after an intervention, like responders or non-responders, in order to determine survival differences between patients from baseline. This type of analysis is hampered by survival advantage of the responders who (by definition) have to show a response before they can be assigned to this group. In contrast, non-responders may be assigned to their group when they fail to survive until a response would occur. This type of artificial survival advantage is called 'immortal time bias'.[93]

To overcome 'immortal time bias', a landmark approach can be used. Landmark analysis focuses on the survival of patients conditional on being failure-free (or just alive) and on the response status assessed at the landmark time.[94,95] A fixed point in time (landmark) is considered, for instance 1 year after alloSCT, and all patients with event (or censored) prior to this time point are removed from the analysis. It may also be useful to introduce a time-dependent covariate, like administration of DLI, to assess the impact of this event on outcome.[96] A time-dependent covariate reflects the phenomenon that a covariate is not necessarily constant through the whole study. The effect of prophylactic DLI for prevention of relapse can only be determined when the time-dependent nature of this event is taken into account, as there will always be a certain time-span between alloSCT and administration of DLI.

To overcome the statistical problems addressed above, a 'multi-state model' can be constructed (see Figure 5). This model is capable of analyzing the occurrence and effect

of multiple competing time-dependent events, which is essential when analyzing the effect of DLI.[97,98]

A multi-state model permits mapping of all relevant events by combining the sequence of events of main interest and competing risks analysis.[98,99] The building blocks of a multi-state model are one (or several) starting state(s), transient (non-absorbing or intermediate) states and absorbing states.[99] The starting state may be the date of infusion of stem cells. Patients enter a new state when an event of interest occurs. The most simple multi-state model for outcome analysis after alloSCT is the two-state model with one starting state 'alive after alloSCT' and one absorbing state 'death', which is equivalent to a standard model for OS. In slightly more advanced models transient and/or parallel competing states may be introduced.[98] Transient states may be development of GvHD or administration of DLI. In most models, patients can not visit the same state twice, although reversibility can be added into more complex multi-state models.[98] The absorbing states are typically endpoints of the study, such as relapse or death.

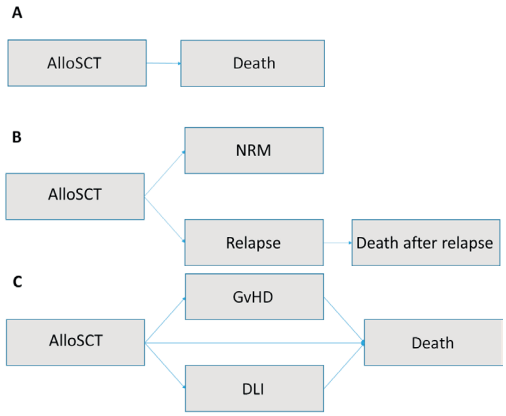

Figure 5. *Multi-state models. (a) Two-state model. Starting state: being alive after alloSCT 'AlloSCT' and absorbing state: Death after alloSCT 'Death'. A Patient remains in the starting state 'AlloSCT' until he or she experiences an event: death. This model is similar to overall survival. (b) Multi-state model with one starting state: day of stem cell infusion 'AlloSCT', two competing parallel states: 'NRM' and 'Relapse'. 'Relapse' is an intermediate state. The patient remains in this state until he or she suffers the event 'Death after relapse'. 'NRM' and 'Death after Relapse' are absorbing states. This model can be used to determine the cumulative incidences of NRM and relapse, but also PFS, or OS, as well as the impact of relapse on outcome (survival). (c) Multi-state with one starting state: 'AlloSCT', two competing intermediate states: occurrence of GvHD 'GvHD' and infusion of donor lymphocytes 'DLI', and one absorbing state: 'Death'. This model can be used to determine the impact of absence of GvHD (a patient remains in the starting state 'AlloSCT' until an event occurs), and the time-dependent competing events GvHD and DLI on outcome (survival).*

From multi-state models, transition intensities and probabilities can be estimated by techniques from survival analysis based on the timing of events and number of patients in a certain state during follow-up. The impact of presence in a state at a predefined time point on outcome can be estimated.[98] The probability of a combined outcome, for

instance alive without GvHD or relapse, can be estimated by calculating the sum of probabilities of all states that match this outcome during follow-up. An important difference with standard composite end-points is that events after the first failure are also taken into account: patients may develop GvHD, but they may also experience disappearance of GvHD after adequate treatment. When using standard composite endpoints, these patients would serve as a failure, but when using a multistate model, these patients could serve as treatment success.

Multi-state models can be highly informative to obtain predictions from detailed observational studies, especially in case of alloSCT, where large randomized studies are scarcely performed.

8. Aims of the study

AlloSCT aims to eradicate hematologic malignancies by transfer of alloreactive T cells from the donor to the recipient. It is a broadly accepted treatment modality for several high-risk and relapsed hematologic malignancies, but is associated with severe morbidity and mortality. The risk of GvHD increases when alloreactive T cells are introduced during inflammatory circumstances, like after conditioning therapy or during uncontrolled infections. Postponing administration of donor T-cells too long is associated with an increased relapse rate. To limit the incidence of severe GvHD and prevent relapse, a treatment strategy was developed to introduce timely DLI after TCD-alloCT. The research described in this thesis aims to study the effect of pre-scheduled DLI after TCD-alloSCT in several clinical situations.

The outcome of adult patients ALL remains unsatisfactory. Despite high complete remission rates after induction chemotherapy, five-year overall survival ranges between 40 and 60% with standard consolidation/intensification and maintenance treatment.[100] Several studies report the beneficial anti-leukemia effect of alloSCT as compared to autologous SCT or chemotherapy maintenance for adult patients with ALL.[101] The outcome of young adult patients with ALL is improved after implementation of pediatric-inspired regimes also.[102] We studied the general outcome of our treatment strategy in patients with ALL in **chapter 2**. A cohort of adult patients with ALL in first complete remission (CR1) who underwent myeloablative TCD-alloSCT followed by early-sequential DLI was analyzed. Patients were eligible for administration of prophylactic DLI between 3-6 months after alloSCT when in continuous CR1 with absence of active GvHD. We aimed to determine the impact of GvHD, the incidence of DLI, the number of relapses and overall survival in this cohort. We also aimed to compare the outcome of patients with a related or unrelated donor. Finally, we aimed to compare the outcomes of patients with Philadelphia Chromosome (Ph-)positive ALL, which is associated with a poor outcome, to patients with Ph-negative ALL.

Approximately 30% of transplanted patients with AML develop a relapse, which is associated with a very poor prognosis.[103,104] The role of DLI for relapsed AML is unclear.[55,61] To improve outcome we developed a DLI-based strategy, which aimed to induce an effective immune response: leukemic cytoreduction was followed by administration of DLI during the lymphopenic phase, with additional interferon-α when no acute GvHD was observed within 3 weeks after DLI. The results of this study are presented in **chapter 3**. In particular, we aimed to estimate the impact of a DLI-induced immune response on the incidence of second relapse, OS and GvHD-related mortality by determining the (time-dependent) impact of absent GvHD, controllable GvHD (grade 1-3), and severe GvHD (grade 4) after DLI.

In the field of alloSCT, the common approach is to focus on outcome analyses on time to relapse and death. When assessing the impact of post-transplant interventions, conventional statistical methods do not apply. In **chapter 4** we aimed to study the role of prophylactic DLI after TCD-alloSCT for AML/high-risk MDS by means of a multi-state model. A cohort of patients with AML or high-risk MDS in CR, who underwent a treatment strategy of myeloablative TCD-alloSCT followed by prophylactic DLI, was analyzed. Patients were eligible for administration of prophylactic DLI between 3-6 months after alloSCT when in continuous CR1 with absence of active GvHD. We constructed a multi-state model to analyze the impact of DLI and GvHD on the probabilities of relapse and non-relapse mortality over time. Being alive without relapse and immunosuppression for GvHD can be considered to be a favorable outcome of alloSCT. Therefore, we aimed to determine the number of patients who experienced these combined criteria over time, which we called 'treatment success'. Using this outcome, we aimed to provide insights in long-term prognosis which is relevant for clinical decision making.

AlloSCT can result in long-term disease control because immune-competent T-cell can exert a GvM-effect, but many patients relapse or NRM. However, important lessons can be learned from the effect of T-cell therapy in the context of alloSCT: alloreactive T-cells may influence the impact of disease progression patterns. In **chapter 5** we aimed to provide insight in the impact of DLI in disease progression patterns. A cohort of patients with MM who underwent TCD-RIC alloSCT with scheduled DLI was analyzed. For comparison, a cohort of patients with MM for whom a donor search was started but no alloSCT was performed was analyzed. We aimed to ascertain the differences between bone marrow progression and focal progression after DLI, which may indicate the strength of disease responses by T-cells in different body compartments.

In chapter 6, the results of the research described in this thesis is summarized and discussed in the light of alternative strategies to prevent or treat relapses after alloSCT. Also, difficulties when investigating the role of post-transplant interventions on relapse and mortality are discussed.

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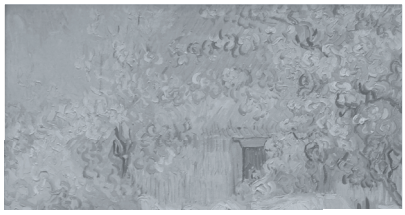

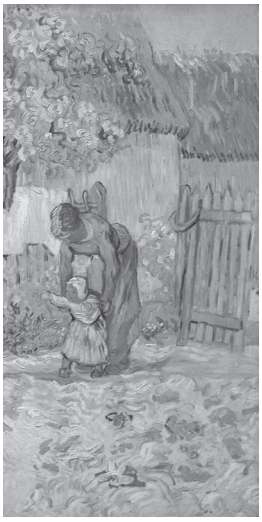

Chapter 2

Myeloablative T cell-depleted alloSCT with early sequential prophylactic donor lymphocyte infusion is an efficient and safe post-remission treatment for adult ALL

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This article has been corrected since Online Publication and a corrigendum has also been published

ABSTRACT

The prognosis of adult patients with ALL remains unsatisfactory. AlloSCT is associated with a beneficial GVL response mediated by donor T cells. However, GVHD results in substantial mortality and long-term morbidity. T-cell depletion (TCD) of the graft reduces the severity of GVHD, but is associated with an increased relapse rate after alloSCT. Therefore, early sequential donor lymphocyte infusion (DLI) is likely to be necessary for a successful GVL reaction. Twenty-five adult ALL patients (10 Ph^+ ALL) were eligible for early DLI after initial disease control with myeloablative TCD-alloSCT in first CR (CR1), if active GVHD was absent at 3–6 months after alloSCT. Patients with a sibling donor or an unrelated donor were scheduled for 3.0×10^6 $CD3^+$ cells/kg or 1.5×10^6 $CD3^+$ cells/kg, respectively, at 6 months after alloSCT. Three patients died before evaluation (one early relapse). Five patients had active GVHD. Fourteen of the remaining seventeen patients received DLI (median time-to-DLI: 185 days). Overall, only 17% required long-term systemic immunosuppression for GVHD. With a median follow-up after TCD-alloSCT of 50 months, 2-year survival probability was 68% (95% confidence interval (CI) 49–87%). In conclusion, myeloablative TCD-alloSCT with early sequential DLI is an efficient and safe post-remission treatment for adult ALL patients in CR1.

Keywords: alloSCT, T-cell depletion, ALL, donor lymphocyte infusion, DLI

INTRODUCTION

The prognosis of adult patients with ALL remains unsatisfactory. Despite high CR rates after induction chemotherapy, 5-year OS ranges between 40 and 60% with standard consolidation/ intensification and maintenance treatment including hematopoietic SCT.[1,2] AlloSCT is associated with a beneficial antileukemia effect as compared with autologous SCT or chemotherapy maintenance for adult patients with ALL.[3–7] This beneficial effect is due to donor T cells mediating a GVL response, thereby preventing a relapse. However, GVHD and infectious complications result in a substantial non-relapse mortality (NRM) and alloSCT-associated morbidity. Therefore, recommendation of alloSCT from non-sibling donors has remained controversial for patients without high-risk ALL in first CR (CR1).[8]

T-cell depletion (TCD) of the stem-cell graft reduces the severity of GVHD, but is associated with an increased relapse rate after alloSCT, especially if patients are transplanted in second CR or with refractory disease.[2,9,10] Therefore, the concept of prophylactic donor lymphocyte infusion (DLI) has been developed.[11] DLI not only decreases relapse rate in ALL patients who are at high risk for relapse, but can also induce molecular remissions in patients with detectable minimal residual disease.[11,12] TCD-alloSCT with postponed administration of DLI is associated with a decreased severity of GVHD.[11,13] We describe here the results of a treatment strategy with myeloablative conditioning before TCD-alloSCT in CR1 for efficient medium-term leukemia control, followed by early sequential prophylactic DLI to establish a GVL response for definitive prevention of relapse. We postulated that this strategy should limit the incidence of early relapses in conjunction with a low incidence of severe GVHD.

MATERIALS AND METHODS

Study population

All patients who underwent myeloablative TCD-alloSCT for ALL in CR1 at Leiden University Medical Center, between January 2003 and June 2011 were included in this study. All patients gave informed consent. High-risk ALL was defined by high leukocyte count at diagnosis (>30 $\times$ 10^9/L in B-ALL and >100 $\times$ 10^9/L in T-ALL), failure to achieve CR after prephase and first induction therapy, and/or unfavorable karyotype at diagnosis, that is, t(9;22), t(4;11) and other 11q23 abnormalities, hypodiploidy or complex abnormalities (≥5, excluding hyperdiploidy). Data were analyzed as of October 2012.

Transplantation procedure and follow-up

The conditioning regimen consisted of CY 60 mg/kg i.v. at days –6 and –5 and TBI in all patients. Patients with a matched sibling donor received 9 Gy TBI on day –1. Patients with a mismatched sibling or an unrelated donor received 9 Gy TBI on day –8 or –7, 30 mg alemtuzumab i.v. divided between days –6 and –5, and cyclosporine 3 mg/kg i.v. (and corresponding oral dose after engraftment) as GVHD prophylaxis from day –1 until day + 60. *In vitro* TCD was performed by adding 20 mg of alemtuzumab to the stem-cell product.[9] $CD34^+$ cell dose was determined by flow cytometry. Starting in 2008, patients with Ph^+ ALL received 400 mg imatinib per day after engraftment until at least 3 months after DLI.

The day of granulocyte engraftment was defined as the first of 3 consecutive days of absolute granulocyte counts $>0.5 \times 10^9$/L. GVHD was graded according to modified Glucksberg and Shulman criteria.[13,14] BM chimerism was determined every 3 months during the first 2 years after alloSCT, and additionally at 6 weeks after DLI. In sex-matched patient– donor pairs, chimerism was determined on total BM leukocytes, and mononuclear cell or granulocyte fractions after ficoll separation, using a short-tandem-repeat-PCR-based protocol.[15] In sex-mismatched patient–donor pairs, FISH analysis was performed on unseparated BM leukocytes until 2007. Thereafter, chimerism analysis was performed analogous to sex-matched pairs. Mixed chimerism was defined as ≥1% patient cells in any of these cell fractions.

Relapse was defined as reappearance of ≥5% blasts in BM by morphology (hematological relapse), and/or by reappearance of a molecular marker, that is BCR-ABL (molecular relapse). NRM was defined as death in continuous CR. OS was defined as time from alloSCT until death from any cause. Study end points were incidence of acute GVHD, NRM, relapse and OS. All time intervals were calculated from the date of alloSCT onwards.

Eligibility for DLI

Eligibility for prophylactic DLI was defined as continuous CR1 with the absence of active GVHD, defined as acute GVHD grade ≥2 or chronic GVHD requiring systemic immunosuppression, between 3 and 6 months after alloSCT. In patients with bacterial infections (not with viral or fungal infections) DLI was postponed. If a patient with active GVHD at evaluation developed mixed chimerism after tapering of immunosuppressive drugs, then DLI could be administered at that time point. Unmanipulated lymphocytes were collected from the original donors by leukapheresis without G-CSF priming. The number of $CD3^+$ cells was analyzed by flow cytometry. Donor cells were administered either on the day of collection, or they were temporarily frozen in the vapor phase of liquid nitrogen. Patients with a matched sibling donor were scheduled for 3.0×10^6 $CD3^+$ cells/kg and patients with a mismatched sibling or an unrelated donor for 1.5×10^6 $CD3^+$ cells/kg, respectively, at 6 months after alloSCT.

Statistical analysis

Probabilities of OS were calculated with 95% confidence intervals (95% CIs) by the Kaplan–Meier method. Survival curves for different groups were compared using a log-rank test. *P*-values of <0.05 were considered as significant. Relapse and NRM were considered as competing events and were analyzed by means of cumulative incidence statistics. Occurrence of any degree of GVHD after alloSCT was also analyzed in a competing risk framework, taking death before occurrence of GVHD as a competing event. Statistical softwares used were SPSS, PASW Statistics 20, release 20.0.0 (IBM, Amsterdam, the Netherlands, 2011) and R2.1.5.0 software, library 'cmprsk' (http://cran.r-project.org).

RESULTS AND DISCUSSION

The study population consisted of 25 patients (median age at alloSCT: 38 years, range 19–54), corresponding to 83% of all adult ALL patients referred to our center in the study period (Table 1). All 5 patients (17%) who underwent alternative treatment are described below. Ten patients (40%) had Ph^+ ALL and eighteen patients (72%) had high-risk ALL (cytogenetic abnormalities were unavailable in one Ph^- patient). Nine Ph^+ patients (90%) received 400 mg imatinib daily from induction to alloSCT. Median time from diagnosis to alloSCT was 202 days (range 99–354 days). Thirteen patients (52%) were transplanted from a sibling donor. Three patients received a BM graft (12%) and 22 patients (88%) received G-CSF mobilized peripheral blood stem cells with a median $CD34^+$ cell dose of 8.0×10^6 $CD34^+$ cells/kg (range $4.2–14.5 \times 10^6$ $CD34^+$ cells/kg). All 25 patients showed granulocyte engraftment after a median of 18.5 days (range 10–36 days) after alloSCT. No rejection of the graft occurred.

Despite TCD, only one patient (Ph^- ALL) suffered an early relapse (before planned DLI) at day + 92 and died at day + 165. Two patients suffered NRM within 3 months after alloSCT from miliary tuberculosis (day + 66) and gancyclovir- and foscarnet-refractory CMV pneumonitis (day + 91), respectively. Five patients were not eligible for early DLI due to active GVHD between 3 and 6 months after alloSCT as pre-defined by the treatment strategy. One of these patients died from NRM due to invasive pulmonary aspergillosis on day + 163. The remaining 17 patients were eligible for DLI. Three of these patients did not receive DLI for undocumented reasons. One of these three patients (Ph^- ALL) suffered a hematological relapse on day + 201 and died on day + 314.

Prophylactic DLI was administered to 14 patients at a median interval of 185 days (range 108–659 days) from alloSCT. One patient died 4 days after DLI from adenovirus infection (diagnosed 71 days before DLI) and could not be evaluated for the development of GVHD after DLI. After DLI, 3 of 13 patients (23%) developed acute GVHD (grades 1 and 2) of whom one died due to infectious complications. Two patients (one Ph^+ ALL and one

Table 1 Baseline characteristics and general outcomes

No.	Diagnosis (high risk)	Age (years)	Patient/ donor sex	Donor type (match)	Acute GVHD first 6 Mo	IS at 6 Mo	Time to DLI (days)	$CD3^+$ cells infused/kg bw	Acute GVHD after DLI	Relapse (days after alloSCT)	Follow-up (Mo)	Current status
1	Ph + ALL (yes)	36	M/F	RD (10/10)	Grade 3	Yes	NA	NA	NA	No	27	CR/alive
2	Ph + ALL (yes)	38	M/M	RD (10/10)	Grade 2	Yes	NA	NA	NA	No		CR/alive
3	Ph + ALL (yes)	48	F/M	RD (A-MM)	No	No	659	1.5 × 10E6	No	No	108	CR/alive
4	Ph + ALL (yes)	54	M/M	RD (10/10)	No	No	180	3.0 × 10E6	Grade 1	No	105	CR/alive
5	Ph + ALL (yes)	45	F/M	RD (10/10)	No	No	140	1.0 × 10E6	No	Yes (533, molecular)	50	CR/alive
6	Ph + ALL (yes)	20	M/M	UD (C-MM)	Grade 3	Yes	NA	NA	NA	No	87	CR/alive
7	Ph + ALL (yes)	50	M/M	UD (10/10)	Grade 2	NA	NA	NA	NA	No	5	NRM
8	Ph + ALL (yes)	28	F/F	UD (A-MM)	Grade 2	No	177	1.5 × 10E6	No	No	48	CR/alive
9	Ph + ALL (yes)	42	F/F	UD (10/10)	Grade 1	No	428	1.5 × 10E6	No	No	40	CR/alive
10	Ph + ALL (yes)	50	M/M	UD (DQ-MM)	No	No	219	0.05 × 10E6[a]	Grade 2	No	19	NRM
11	B-ALL (no)	46	F/M	RD (10/10)	Grade 1	NA	NA	NA	NA	Yes (92)	5	Relapse/dead
12	B-ALL (no)	26	M/M	RD (10/10)	No	No	NA	NA	NA	No	68	CR/alive
13	B-ALL (no)	21	F/F	RD (10/10)	Grade 1	No	108	1.0 × 10E6	No	Yes (188)	7	Relapse/dead
14	B-ALL (no)	38	F/M	RD (10/10)	Grade 2	No	211	1.0 × 10E6	No	No	7	NRM
15	B-ALL (no)	26	F/M	UD (10/10)	Grade 1	NA	NA	NA	NA	No	3	NRM
16	B-ALL (yes)	27	F/F	UD (10/10)	No	No	189	1.5 × 10E6	No	No	86	CR/alive
17	B-ALL (yes)	42	M/M	UD (10/10)	No	No	146	1.5 × 10E6	Grade 1	No	79	CR/alive
18	B-ALL (yes)	29	M/M	UD (DQ-MM)	Grade 1	No	167	1.5 × 10E6	No	No	15	CR/alive
19	T-ALL (yes)	42	F/M	RD (10/10)	Grade 1	No	NA	NA	NA	Yes (201)	10	Relapse/dead
20	T-ALL (no)	29	M/M	RD (10/10)	Grade 1	No	NA	NA	NA	No	33	CR/alive
21	T-ALL (yes)	19	M/M	RD (10/10)	Grade 1	No	NA	NA	NA	No	23	CR/alive
22	T-ALL (yes)	45	M/F	RD (10/10)	Grade 2	No	141	0.3 × 10E6	No	No	28	CR/alive
23	T-ALL (yes)	26	M/M	UD (10/10)	No	NA	NA	NA	NA	No	2	NRM
24	T-ALL (yes)	36	F/F	UD (DR-MM)	Grade 2	No	226	1.5 × 10E6	No	No	87	CR/alive
25	T-ALL (no)	28	F/F	UD (10/10)	Grade 1	No	204	1.5 × 10E6	No	No	78	CR/alive

Abbreviations: bw = body weight; DLI = donor lymphocyte infusion; F = female; IS = systemic immunosuppression; M = male; Mo = months after transplant; NA = non-applicable; No. = patient number; NRM = non-relapse mortality; Ph + ALL = Philadelphia chromosome positive B-cell ALL; RD = related donor; UD = unrelated donor; (X-) MM = (locus-)mismatch. [a] Patient received low-dose DLI due to persisting infectious complications (*Aspergillus* and *Pseudomonas* species), which persisted after DLI; after DLI, this patient developed an immune thrombocytopenia and a pericardial effusion.

Ph$^-$ ALL) showed increasing mixed chimerism after DLI and subsequently relapsed. The Ph$^+$ patient suffered a molecular relapse on day + 533 despite imatinib maintenance and DLI after alloSCT. After combination therapy with DLI, IFN-α and nilotinib,[16] this patient is in continuing second molecular CR and has full donor chimerism at 3 years after relapse. The Ph$^-$ patient had a hematological relapse within 12 weeks after DLI and died from progressive disease. At the end of follow-up, 11 of 14 patients receiving DLI (79%) were alive and in CR.

As expected, the overall incidence of severe GVHD in our study was low. One-year cumulative incidences of grade 2 and grade 3 acute GVHD were 28% (95% CI 10–46%) and 8% (95% CI 0–19%), respectively. Given the detrimental impact of chronic GVHD on quality of life, we determined the percentage of patients with a follow-up of >1 year who required systemic immunosuppression for GVHD at 1 year after alloSCT.[17] This was observed in 3 of 18 patients (17%) only.

This is the first study with long follow-up presenting the outcome of myeloablative TCD-alloSCT with pre-planned sequential DLI in adult ALL patients in CR1. Overall, 14 of 25 study patients (56%) received prophylactic DLI. Median follow-up of surviving patients was 50 months (range 15–108 months). Two-year OS was 68% (95% CI 49–87%). Two-year cumulative incidences of NRM and relapse were 20% (95% CI 4–36%) and 16% (95% CI 1–31%), respectively. There was no significant difference in survival between patients with a sibling donor or an unrelated donor ($P = 0.78$, Figure 1a).

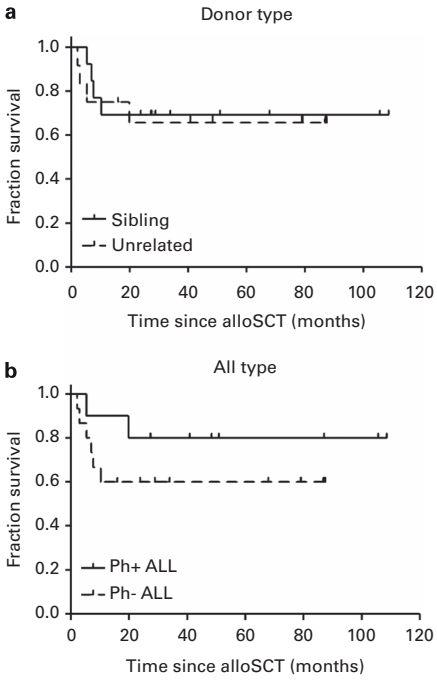

Figure 1 Kaplan–Meier plots of probabilities of OS after alloSCT. (**a**) Outcome of 13 patients with a sibling donor (solid line) and 12 patients with an unrelated donor (dashed line). (**b**) Outcome of 10 Ph + ALL patients (solid line) and 15 Ph ALL patients (dashed line).

To explore whether the favorable outcome could be due to possible systematic selection of favorable patients for transplantation, we studied the reasons for alternative treatment for all five adult ALL patients (aged 18–55 years) who were treated without receiving a myeloablative transplant in CR1 during the same time period at our institution. Reasons for alternative treatment were severe cardiomyopathy (n = 1, this patient underwent non-myeloablative TCD-alloSCT), or non-high-risk disease (n = 4, two of these patients relapsed and underwent alloSCT in CR2). Since the majority of patients included in our study had high-risk ALL, the favorable outcome observed is unlikely due to selection of good-risk patients for TCD-alloSCT.

In our cohort, 2-year OS of Ph^+ patients was at least as good as for Ph^- patients: 80% (95% CI 55–100%) and 60% (95% CI 35–85%), respectively (P = 0.27, Figure 1b). In the pre-imatinib era, a 2-year OS of 44% was reported for myeloablative TCD-alloSCT in children and adolescents with Ph^+ ALL in CR1.[10] While incidence and severity of GVHD was low, the main reason for failure was a high relapse rate of 40%. Concomitant long-term therapy with tyrosine kinase inhibitors may have an important role in prevention of relapse before DLI and during the development of the definitive GVL response.[18–20]

The necessity and optimal duration of imatinib maintenance after alloSCT for Ph^+ ALL cannot be defined by our study. Tyrosine kinase inhibitors are known to exert substantial side effects and may even inhibit donor-derived GVL activity due to immunosuppressive effects.[21,22] In our study, imatinib maintenance was restarted in five Ph^+ patients after alloSCT with a median duration of 450 days (range 12–542 days) after alloSCT. Imatinib was stopped prematurely in two Ph^+ patients because of intolerable nausea (after 12 days) and NRM (fungal death), respectively. In the first patient, imatinib was restarted 2 months later, and stopped at 3 months after DLI.

Finally, sensitive detection of molecular relapse by means of quantitative PCR of the BCR-ABL fusion transcript in Ph^+ ALL allows efficient salvage by switching to a second-generation tyrosine kinase inhibitor in combination with DLI and IFN-α.[16,23,24] This combination aims to induce a rapid GVL reactivity to prevent a virtually incurable hematological relapse and achieve long-term freedom from relapse as indicated by the only informative patient in our cohort.

In conclusion, this single-center experience in a predominantly high-risk adult ALL population indicates effective disease control with low risk of NRM by myeloablative TCD-alloSCT in CR1 with early sequential DLI for preventing relapse. The low overall impact of GVHD and the favorable outcome of Ph^+ patients support a combined strategy of targeted therapies with TCD-alloSCT followed by prophylactic DLI, which should be evaluated in a prospective study. Ultimately, the combination of targeted therapy and post transplant immunological interventions may lead to less intensive transplantation strategies, like reduced-intensity conditioning, to offer alloSCT for elderly and frail patients.[25]

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

This work was supported by research grant 2008-4263 from the Dutch Cancer Society, Amsterdam, The Netherlands.

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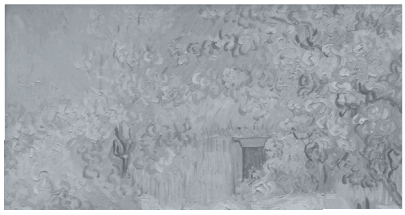

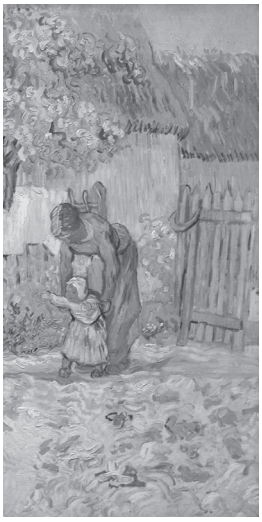

Chapter 3

Intentional donor lymphocyte-induced limited acute graft-versus-host disease is essential for long-term survival of relapsed acute myeloid leukemia after allogeneic stem cell transplantation

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ABSTRACT

The prognosis of patients with relapsed acute myeloid leukemia after allogeneic transplantation is poor. We hypothesized that initial disease control by effective cytoreduction, followed by rapid induction of a profound allo-immune response by donor-lymphocyte infusion during the neutropenic phase, is essential for long-term survival. Additional interferon-α was administered when no acute graft-*versus*-host-disease occurred within 3 weeks after donor-lymphocyte infusion. Overall, 44 patients with relapsed acute myeloid leukemia were assessed; 26 had relapsed after myeloablative conditioning and 18 after reduced-intensity conditioning. Of these 44 patients, seven were not eligible for cytoreductive treatment because of poor performance status (n=3) or severe graft-*versus*-host-disease (n=4) at the time of relapse. Patients with smoldering relapses (n=5) received donor-lymphocyte infusion only. Thirty-two patients received cytoreductive treatment, followed by donor-lymphocyte infusion in 22 patients. Reasons for not receiving donor-lymphocyte infusion were chemotherapy-related death (n=1) and chemotherapy-refractory disease (n=9). The 2-year overall survival rate after donor-lymphocyte infusion was 36% (95% confidence-interval: 16-57%). The impact of acute graft-*versus*-host-disease on survival was calculated with a Cox-regression model including onset of acute graft-*versus*-host-disease as a time-dependent variable. Development of grade 1-3, but not grade 4, acute graft-*versus*-host-disease was associated with superior survival as compared to absence of graft-*versus*-host-disease (hazard ratio 0.22, *P*=0.03). In conclusion, efficient cytoreduction followed by donor-lymphocyte infusion and subsequent interferon-α leading to limited acute graft-*versus*-host-disease represents a potentially curative option for patients with relapsed acute myeloid leukemia after allogeneic transplantation.

INTRODUCTION

Allogeneic stem cell transplantation (SCT) as treatment for patients with acute myeloid leukemia (AML) aims to combine high-dose chemotherapy with immunotherapy by donor T cells mediating a graft-*versus*-leukemia (GvL) response. The curative potential of the donor-derived allo-immune effect is illustrated by the survival advantage of patients with AML after allogeneic SCT as compared to survival after autologous SCT or high-dose chemotherapy.[1,2] The importance of the GvL response is further supported by donor *versus* no donor comparisons in patients who are potential candidates for allogeneic SCT, favoring the outcome of patients with a stem cell donor.[3] However, the T-cell-mediated alloimmune response also leads to the occurrence of graft-*versus*-host disease (GvHD), which negatively influences outcome after allogeneic SCT.[4,5]

GvHD after allogeneic SCT can be reduced by T-cell depletion of the stem cell graft, but this has been associated with an increased incidence of relapses.[6–10] However, a recent study by Pasquini *et al.* showed that the reduction of T cells in the graft does not necessarily have a negative impact on the relapse rate of AML after myeloablative allogeneic SCT as compared to T-cell-replete allogeneic SCT.[11] This finding suggests that even a limited GvL response may contribute to the cure of patients with AML. Furthermore, a reduced GvL response of T-cell-depleted allogeneic SCT may be restored by post-transplant immunological interventions, such as low-dose, prophylactic donor lymphocyte infusions (DLI) to prevent relapse. This postponed infusion of donor T cells after T-cell-depleted allogeneic SCT is associated with a lower incidence of severe GvHD.[12]

Although AML can be cured by allogeneic SCT, approximately 30% of transplanted patients with AML develop a relapse after the transplant, and these relapsed patients have a dismal prognosis.[13–15] Salvage re-induction chemotherapy leads to clinical remissions in a substantial number of patients, but these remissions are usually of short duration. A retrospective analysis by the EBMT Acute Leukemia Working Party indicated that long-term survival of patients with relapsed AML is almost exclusively achieved by induction of complete remission followed by DLI.[16] Apparently, additional control from a GvL immune response by donor lymphocytes is essential to consolidate chemotherapy re-induced remissions. In contrast, Pollyea *et al.* did not observe an advantage for cellular therapy compared to chemotherapy for patients with relapsed AML.[17]

Although low doses of DLI can result in GvL reactivity, the interval between the clinical response and the administration of the T cells may be several months.[18] To achieve an effective GvL response in relapsed AML, a rapid, large amplitude immune response is likely to be required, since the fast proliferation rate of an acute leukemia can lead to a high tumor burden, which will suppress the immune response.[19,20] A rapid and profound immune response is, however, likely to come at the cost of GvHD.

To maximize the effect of combined cytoreductive treatment and DLI administration, we adopted a therapeutic strategy for post-allogeneic SCT relapsed AML based on disease control by salvage re-induction chemotherapy followed by DLI administration in the neutropenic phase. The pro-inflammatory milieu after chemotherapy might favor the induction of the immune response, whereas the expansion of infused donor cells is promoted by lymphopenia-induced homeostatic proliferation. [21,22] If no acute GvHD was observed within 3 weeks after DLI, the immune response was further stimulated by treatment with interferon-α.[23] We hypothesized that the combination of efficient cytoreduction by re-induction therapy for initial disease control, with DLI administered in rapid succession under circumstances favoring the development of an early and profound immune response may be essential to eradicate relapsed leukemia, but likely at the cost of GvHD.[24]

METHODS

Data collection

Between January 2000 and January 2010, 44 patients with relapsed AML after allogeneic SCT were treated. The patients were categorized according to their pre-transplant disease characteristics as having intermediate-risk, poor-risk, or very poor-risk AML (see *Online Supplementary Methods*). The study was approved by Leiden University Medical Center Research Ethics Committee. Informed consent was obtained from the patients prior to data collection. Data were analyzed as of November 2012.

Transplantation protocol

Details about the transplantation protocols, T-cell depletion, donor matching, and diagnosis and treatment of GvHD are provided in the *Online Supplementary Methods* section.

Relapse

Relapse after allogeneic SCT was defined as an increase of morphologically determined blasts in the bone marrow to 5% or more, and/or by the presence of more than 1% blasts in peripheral blood, and/or by the reappearance of molecular and/or cytogenetic markers. Further details and definitions of smoldering relapse and high tumor burden relapse are provided in the *Online Supplementary Methods* section.

Treatment strategies for relapse

Patients with a poor performance status, defined as WHO performance status 3 or higher, and/or with severe GvHD requiring systemic immunosuppression at the time

of relapse were unlikely to benefit from an intensive DLI-based strategy and received palliative treatment only.

Patients with high tumor burden relapse received salvage reinduction therapy prior to infusion of donor lymphocytes. If a patient responded to re-induction therapy, with response being defined as absence of circulating leukemic blasts, unmanipulated donor lymphocytes collected from the original donor were administered 3 weeks after the start of re-induction therapy (during the neutropenic phase). In the case of a smoldering relapse, patients received DLI without prior cytoreductive treatment.

DLI-dosing depended on the time point after allogeneic SCT, administration of pre-emptive DLI prior to relapse and donor type (see *Online Supplementary Methods* section).

The occurrence and impact of GvHD on outcome after relapse were evaluated in all patients receiving DLI for relapse. If no acute GvHD was observed within 3 weeks after DLI, interferon-α was administered subcutaneously at a daily dose of 3 million units until GvHD occurred. Escalating doses of DLI were administered if acute GvHD failed to develop by 3 months after starting treatment.

Statistical analysis

For different analyses, time was measured since relapse, initiation of re-induction therapy or infusion of donor lymphocytes. Definitions of survival are provided in the *Online Supplementary Methods* section. Probabilities of overall survival with associated 95% confidence intervals (95% CI) were calculated by the Kaplan-Meier method; differences between groups were compared using a long-rank test. The cumulative incidence of acute GvHD after DLI was calculated using R2.15.0 software, library 'cmprsk' (*http://www.rproject.org/foundation*).

To investigate the impact of a rapid immune response associated with acute GvHD on long-term survival and disease recurrence after DLI, extended Cox regression models, as proposed by Andersen & Gill, were constructed for overall survival, relapse-free survival and relapse incidence from the starting point of DLI (see the *Online Supplementary Methods* section for details).[25] All *P* values <0.05 were considered statistically significant. The statistical software used was SPSS, PASW Statistics 20, release 20.0.0 (2011).

RESULTS

The baseline characteristics of the entire cohort of 44 patients with relapsed AML after allogeneic SCT are presented in Table 1, and a treatment flowchart of the cohort is shown in Figure 1. Seven of the 44 patients (16%) had poor performance status (n=3) or severe GvHD (n=4) at the time of relapse, and received palliative treatment only. They all died from primary disease.

Table 1 Patients' baseline characteristics.

Variable	Entire cohort (44 patients)
Gender (%)	26 Male (59%), 18 Female (41%)
Median age at alloSCT (range)	53 years (18-68)
Initial diagnosis prior to alloSCT (%)	41 AML (93%), 3 MDS (7%)
AML risk-group at diagnosis (%)	4 Int-risk (10%), 27 Poor-risk (66%), 10 MK (24%)
HCT-CI pre-alloSCT (%)	18 Score 0 (41%), 20 Score 1-2 (45%), 6 Score ≥3 (14%)
Median EBMT risk score prior to alloSCT (range)	3 (2-6)
Conditioning regimen alloSCT (%)	26 Myeloablative (59%), 18 Reduced-intensity (41%)
Stem cell source (%)	6 Bone marrow (14%), 38 G-CSF stimulated peripheral blood (86%)
T cell depletion (%)	41 'Campath in the bag' (93%), 3 CD34-selection (7%)
Donor type (%)	30 Sibling donor (68%; 28 fully matched), 14 Unrelated donor (32%; 11 fully matched)
Median time alloSCT to relapse (range)	191 days (65-1117)
Relapse within 6 months after alloSCT (%)	21 (48%)
Type of relapse (%)	39 High tumor burden (89%), 5 Smoldering (11%)
Median % BM blasts / PB blasts (range) at relapse	40 (0-90) / 0 (0-94)
<30% BM blasts and <10% PB blasts (%) at relapse	15 (34%)
Extramedullary relapse (%)	4 (9%)
Prophylactic DLI prior to relapse (%)	11 (25%)
Maximum grading of acute GvHD prior to relapse (%)	16 No acute GvHD (36%), 24 Grade 1-2 (55%), 4 Grade 3-4 (9%)

*AlloSCT: allogeneic stem cell transplantation; Int-risk: intermediate risk AML; MK: monosomal karyotype AML; HCT-CI: hematopoietic cell transplantation comorbidity index; CR1: first complete remission; CR2: second complete remission; CD34-selection: collection*Stoof *MACS-sorted CD34^{+}cells collection; relapse indicates first relapse after alloSCT; BM: bone marrow; PB: peripheral blood; MDS: myelodysplastic syndrome; G-CSF: granulocyte colony-stimulating factor.*

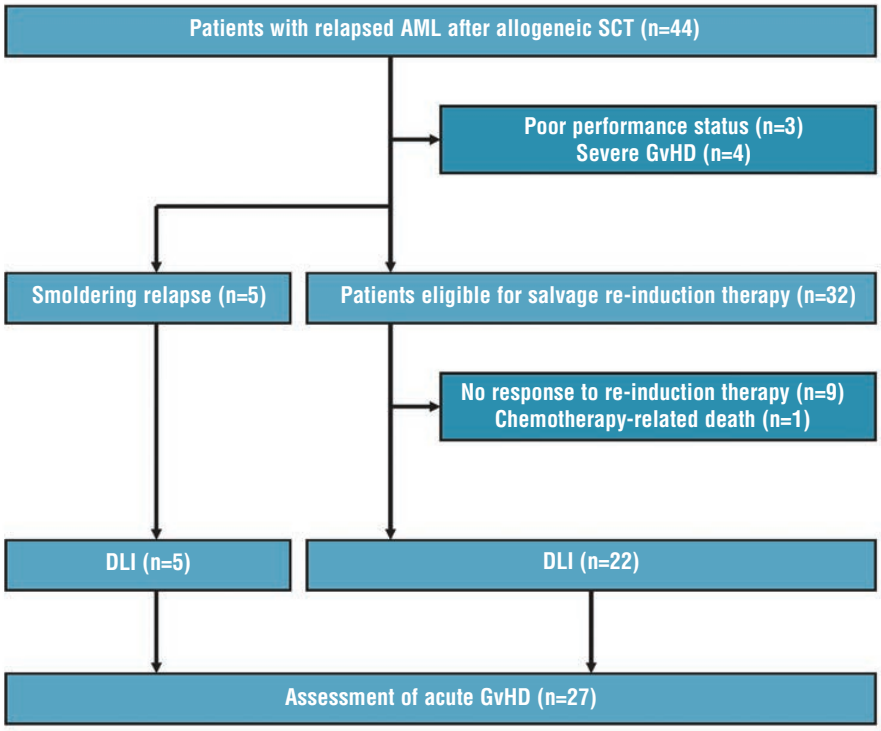

Figure 1 Flowchart of 44 relapsed AML patients.

Five of the remaining 37 patients (14%) met criteria for smoldering relapse (Table 2), and they received DLI without preceding cytoreductive treatment. Four of these patients developed overall grade 2-4 acute GvHD. Two of these patients achieved long-term overall survival, whereas the other two patients died from GvHD-related complications (Figure 2). One patient did not develop any GvHD after DLI and interferon-α and persistence of bone marrow blasts (<10%) was observed. This patient ultimately died from infectious complications.

Table 2 Outcomes of five patients with smoldering relapse (patients are ordered according to grading of acute GvHD after DLI

N. of patients	Sex	Age at transplant (years)	Disease prior to transplant	Conditioning regimen	Donor type	Prophylactic DLI	Acute GvHD prior to relapse	Time transplant to relapse (days)
1	male	63	MDS, IPSS 1.0	RIC	MUD	Day 206; $1.5x10^6$ $CD3^+$cells/kg	grade 1	234
2	male	66	Poor-risk AML	RIC	MUD	Day 134; $0.I5x10^6$ $CD3^+$cells/kg	grade 1	176
3	female	50	Poor-risk AML	RIC	MMUD	No	no	65
4	male	66	Poor-risk AML	RIC	MMRD	Day 196; $2.5x10^6$ $CD3^+$cells/kg	no	329
5	male	36	MK AML	MAC	MRD	No	grade 1	282

Relapse indicates first relapse after allogeneic stem cell transplantation; MK: monosomal karyotype; RIC: reduced-intensity conditioning; MAC: myeloablative conditioning; MRD: 10/10 matched related donor; MMRD: mismatched related donor; MUD: 10/10 matched unrelated donor; MMUD: mismatched unrelated donor; BM: bone marrow; PB: peripheral blood; IFN: interferon-α; CR: complete remission; '-': not available/not evaluated/not applicable.

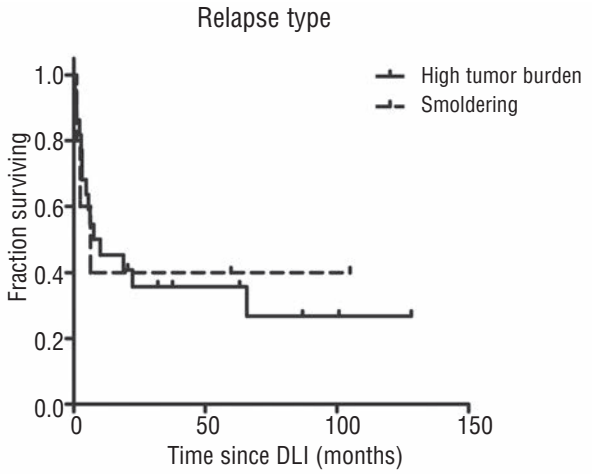

Figure 2 Overall survival. Kaplan-Meier curves showing overall survival after infusion of donor lymphocytes to 22 patients with high tumor burden relapse who received re-induction therapy followed by DLI (solid line) and five patients with smoldering relapse who received DLI without prior cytoreductive treatment (dashed line).

Thirty-two patients with relapsed AML were treated with re-induction chemotherapy. The median time from relapse to start of treatment was 13 days (range, 0-49 days). One patient with a molecular relapse of BCR-ABL p190-positive AML (phenotype positive for CD34, CD133, cyMPO, CD117, HLA-DR, CD13, CD33, CD4 and CD7) received DLI on the day of relapse, followed by targeted therapy with imatinib. Another patient received DLI 4 days after relapse, followed by gemtuzumab-ozogamycin, after which the patient developed acute GvHD. The remaining 30 patients were treated with gemtuzumab-

).

% blasts BM at relapse	DLI dose (x106 $CD3^+$cells /kg)	IFN	Time DLI to acute GvHD (days)	Acute GvHD after DLI	Immuno-suppression for GvHD (days)	Follow-up after DLI (months)	Current status
6	2.5	yes	-	No	0	6	Dead, primary disease
8	1.5	no	39	grade 2	182	60	Alive, CR
5	2.5	no	52	grade 3	207	105	Alive, CR
5	7.5	no	14	grade 3	20	1	Dead, GvHD-related
3 (2 PB)	50	yes	22	grade 4	51	2	Dead, GvHD-related

ozogamycin (n=6), intensive chemotherapy (n=17) or both (n=7) before DLI was planned. One patient died from chemotherapy-related toxicity. Nine patients did not respond to salvage re-induction therapy and died from primary disease. The remaining 20 patients received DLI after re-induction chemotherapy. The median time from the start of chemotherapy to DLI in these 20 patients was 23 days (range, 13-91 days).

The relapse and treatment characteristics of all 22 patients receiving re-induction chemotherapy and DLI are presented in Table 3. At 3 weeks after DLI, four of these 22 patients had developed signs of acute GvHD. Five patients without signs of acute GvHD did not receive interferon-α due to chemotherapy-related toxicity (n=2) or severe infectious complications (n=3). The remaining 13 patients received interferon-α.

Overall survival

Median follow-up from relapse of surviving patients of the entire cohort was 63 months (range, 24-128 months). The estimated 2-year overall survival rate calculated from day of relapse of the entire cohort of 44 patients was 23% (95% CI 10-35%). The estimated 2-year overall survival rate calculated from start of treatment in the 37 patients eligible for re-induction treatment and/or DLI was 27% (95% CI 13-41%), while that in the 32 patients with high tumor burden relapse starting re-induction treatment was 25% (95% CI 10-40%). Finally, the estimated 2-year overall survival rate calculated from DLI was 36% (95% CI 16-57%) in the group of 22 patients who received re-induction therapy followed by DLI (Figure 2).

The overall outcome of AML patients with a monosomal karyotype was particularly poor. The main reason for their poor outcome appeared to be poor primary disease control, and only four of these ten patients ultimately received DLI. The only surviving patient with monosomal karyotype had a molecular relapse of BCR-ABL p190-positive AML which was treated with imatinib and DLI (Table 3).

Table 3A Outcomes of 22 patients with relapsed AML receiving re-induction therapy and DLI (patients are ordered according to grading of acute GvHD after DLI, see Table 3B).

N.	Sex	Age at transplant (years)	Disease prior to transplant	Conditioning regimen	Donor type	Prophylactic DLI	Acute GvHD prior to relapse	Time transplant to relapse (days)	% blasts in BM at relapse
1	M	55	MK AML	MAC	MRD	no	grade 1	148	<1*
2	F	51	int-risk AML	MAC	MMRD	no	grade 2	360	50
3	F	18	poor-risk AML	MAC	MUD	day 121: 0.15x10^6 $CD3^+$cells/kg	no	211	75
4	F	29	poor-risk AML	MAC	MRD	no	no	194	- (94PB)
5	F	59	poor-risk AML	MAC	MRD	no	grade1	284	50
6	M	35	int-risk AMLF	MAC	MRD	no	no	183	70
7	M	32	poor-risk AML	MAC	MUD	no	no	270	40
8	F	27	poor-risk AML	RIC	MRD	no	grade 1	128	40
9	M	64	poor-risk AML	RIC	MUD	day 134: 0.15x10^6 $CD3^+$cells/kg	grade 1	183	90
10	M	37	poor-risk AML	MAC	MRD	no	grade 1	253	90
11	M	54	poor-risk AML	MAC	MRD	day 88: 1.0x10^6 $CD4^+$cells/kg	no	130	45
12	M	53	MDS, IPSS 2.0	RIC	MRD	no	grade 2	370	40
13	M	47	poor-risk AML	RIC	MUD	no	no	514	60
14	M	65	poor-risk AML	RIC	MRD	no	grade 2	213	15
15	M	63	poor-risk AML	RIC	MUD	day 220: 1.5x10^6 $CD3^+$cells/kg	grade 1	259	50
16	F	60	poor-risk AML	RIC	MRD	no	grade 1	158	18
17	M	36	MK AML	MAC	MRD	no	grade 2	216	<1 §
18	F	67	poor-risk AML	RIC	MUD	no	no	108	33
19	M	45	MK AML	MAC	MRD	no	grade 1	328	- (12 PB)
20	F	55	int-risk AML	RIC	MRD	no	grade 1	187	68
21	F	33	poor-risk AML	MAC	MMUD	day 120: 0.52x10^6 $CD3^+$cells/kg	grade 1	426	12
22	F	61	poor-risk AML	RIC	MRD	no	no	88	70

*Relapse indicates first relapse after allogeneic stem cell transplantation; N.: patient number; M: male; F: female; MK: monosomal karyotype; int-risk: intermediate risk; MAC: myeloablative conditioning; RIC: reduced-intensity conditioning; MRD: 10/10 matched related donor; MMRD: mismatched related donor; MUD: 10/10 matched unrelated donor; MMUD: mismatched unrelated donor; BM: bone marrow; PB: peripheral blood; '-': not available/not evaluated/not applicable. * Cytogenetic relapse with clonal evolution. § Molecular relapse BCR-ABL p190.*

Table 3B Outcomes of 22 patients with relapsed AML receiving re-induction therapy and DLI, continued (patients are ordered according to grading of acute GvHD after DLI).

N.	Time relapse to treatment (days)	Re-induction therapy	Time start treatment to DLI (days)	DLI dose (x10^6 $CD3^+$cells/kg)	IFNα	Time DLI to acute GvHD (days)	Acute GvHD after DLI	Immuno-suppression for GvHD (days)	Follow-up after DLI (months)	Current status
1	14	GO	13	5.0	no	-	no	no	3	dead, primary disease
2	7	MITO/ETOP	31	1.5	no	-	no	no	3	dead, primary disease
3	6	GO/MITO/ETOP	22	3.9	no	-	no	no	1	dead, infection
4	2	DAU/ARAC	28	100	yes	-	no	no	5	dead, primary disease
5	4	GO/DAU/ARAC	31	5.0	yes	-	no	no	66	dead, primary disease
6	6	2xGO/CLOF	91	1.5	yes	-	no	no	8	dead, primary disease
7	13	DAU/ARAC	26	1.5	yes	-	no	no	22	dead, primary disease
8	14	ARAC/AMSA	20	5.0	no	-	no	no	6	dead, primary disease
9	12	GO	13	2.5	no	-	no	no	1	dead, infection
10	7	DAU/ARAC	29	15	yes	7	grade 1	no	6	dead,primarydisease
11	19	DAU/ARAC	21	5.0	yes	30	grade 1	yes, 219¥	38	alive,CR
12	8	DAU/ARAC	21	5.0	yes	108	grade 1	yes, 165¶	21	alive,CR
13	29	ARAC/AMSA	26	5.0	no	75	grade 1	yes, 223	10	dead,GvHD-related
14	4§	GO§	0§	10	yes	41	grade 2	yes, >365	128	alive,CR
15	14	DAU/ARAC	24	7.5	yes	38	grade 3	yes, >365	32	alive,CR
16	9	GO/IDA/ARAC	28	5.0	no	27	grade 3	yes, 66	101	alive,CR
17	0*	Imatinib*	0*	3.0	yes	92	grade 3	yes, 154	87	alive,CR
18	32	ARAC/AMSA	21	2.5	no	21	grade 4	yes, 5	1	dead,GvHD-related
19	11	DAU/ARAC	26	100	yes	26	grade 4	yes, 51	3	dead,GvHD-related
20	9	ARAC/AMSA	22	5.0	yes	14	grade 4	yes, >365+MSC	20	dead,GvHD-related
21	14	GO	15	7.8	yes	60	grade 4	yes, 78+MSC	63	alive,CR
22	3	ARAC/AMSA+itMTX/ARAC	20	1.0	no	35	grade 4	yes, 28	2	dead, GvHD-related

*Relapse indicates first relapse after allogeneic stem cell transplantation; N.: patient number; IFNα: interferon-α; GO: gemtuzumab-ozogamycin; MITO: mitoxantrone; ETOP: etoposide; DAU: daunorubicin; ARAC: cytarabine; CLOF: clofarabin; AMSA: amsacrine; IDA: idarubicin; MTX: methotrexate; MSC: mesenchymal stem cell infusion; CR: complete remission; '-': not available/not evaluated/not applicable. § Patient relapsed and received DLI 32 days before GO (date of DLI = start treatment). * Patient received DLI and IFN followed by imatinib for 8 months (date of DLI = start treatment). ¥ Patient developed a pericarditis, which was treated with immunosuppression. ¶ Patient developed chronic GvHD, which was treated with immunosuppression.*

Assessment and impact of acute graft-*versus*-host disease on long-term survival after relapse

All 27 patients who received DLI (see Tables 2 and 3) were evaluated for the presence of acute GvHD after infusion of the lymphocytes. The cumulative incidence of acute GvHD at 3 months after DLI was 56% (95% CI: 36-75%) (Figure 3).

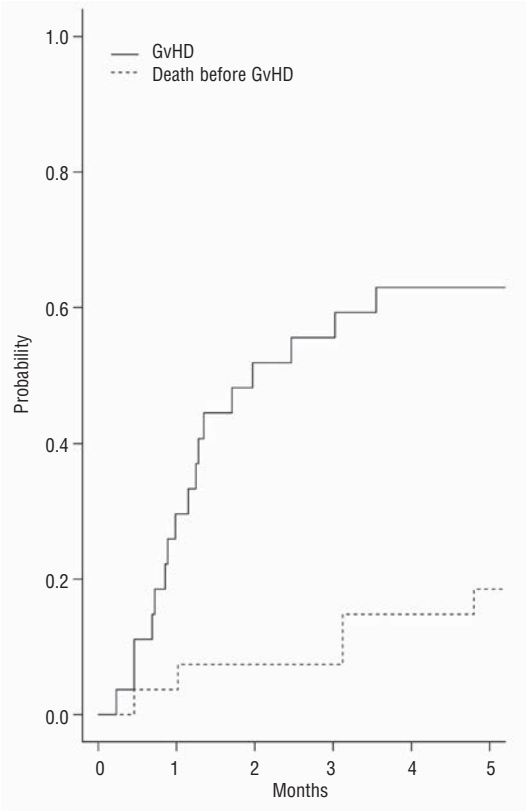

Figure 3 Cumulative incidence of acute GvHD after DLI.

Eleven patients developed grade 3-4 acute GvHD, of whom nine required systemic immunosuppression for GvHD until death or for more than 1 year after relapse. Only one patient with signs of acute GvHD developed a second relapse. No relapses were observed in six patients who developed grade 4 acute GvHD. However GvHD-associated mortality in these patients was high: five patients died due to GvHD-associated complications, including infections and multiple organ failure. In total, seven of 27 patients (26%) receiving DLI died due to GvHD-related complications. We did not observe a clear difference in distribution pattern (i.e. skin or liver involvement) or severity of GvHD between patients who developed GvHD within 3 weeks after DLI and patients who developed GvHD after administration of interferon-α (see *Online Supplementary Table S1*).

The impact of an immune response leading to acute GvHD on long-term survival after relapse was estimated by time-dependent Cox regression models (Table 4). Compared to the absence of acute GvHD, development of grade 1-3 acute GvHD was associated with superior overall survival [hazard ratio (HR)=0.22; 95% CI: 0.06-0.86; *P*=0.03)] and reduced relapse incidence (HR=0.09; 95% CI: 0.01-0.87; *P*=0.04). Development of grade 4 acute GvHD did not result in an improved overall survival (HR=1.61; 95% CI: 0.52-4.97; *P*=0.41). Outcomes of patients with grade 1-2 as compared to grade 3-4 acute GvHD did not differ significantly (*data not shown*).

Table 4 Cox regression models with time-dependent covariates constructed to estimate the impact of development of grade 1-3 or grade 4 acute GvHD compared to absence of acute GvHD on the hazards for overall survival, relapse-free survival and relapse incidence (cause-specific hazard) from the starting point of DLI.

	Overall survival			Relapse-free survival			Relapse incidence		
Grading of acute GvHD	Hazard ratio	95% CI	*P* value	Hazard ratio	95% CI	*P* value	Hazard ratio	95% CI	*P* value
No GvHD	1	-	0.03*	1	-	0.11	1	-	-
Grade 1-3	0.22	0.06-0.86	0.03	0.28	0.07-1.16	0.08	0.09	0.01-0.87	0.04
Grade 4	1.61	0.52-4.97	0.41	1.28	0.41-4.00	0.67	-**	-	-

* P *values indicating overall significance of the variable. ** No relapses were observed in patients developing grade 4 acute GvHD after DLI, therefore these patients were omitted from the model for relapse. Hazard ratios in the model indicate changes in risk for the time interval between onset of acute GvHD until end of follow-up with respect to a baseline patient who has not experienced acute GvHD at the same time.The grade represents the maximum grade of acute GvHD during follow-up. 95% CI indicates 95% confidence interval.*

Finally, to determine the impact of remission status prior to transplant (first *versus* second complete remission), donor type (HLA-matched sibling *versus* other donor), type of conditioning regimen (reduced intensity *versus* myeloablative), and time between transplant and relapse (first relapse within 6 months after SCT *versus* first relapse more than 6 months after SCT) on overall survival after DLI, these factors were compared within the 27 patients who received DLI using a log-rank test. None of these variable differed significantly (*P*-values 0.73, 0.44, 0.13, and 0.89, respectively). Exploratory analysis of lymphocyte recovery after DLI and early chimerism data also did not indicate correlations with GvHD or outcome (*data not shown*).

DISCUSSION

Our study with a median follow-up of more than 5 years illustrates that patients with relapsed AML after allogeneic SCT can be cured when salvage re-induction therapy leading to a low tumor burden is combined with a rapid and profound, but controllable donor T-cell-mediated immune response resulting in limited GvHD. In the absence of GvHD after DLI, no long-term disease control was observed. In contrast, most patients developing GvHD after DLI did not experience a second relapse. These findings illustrate the prominent role for alloreactive donor lymphocytes in inducing a persistent GvL response in relapsed AML after allogeneic SCT, which was not obvious from other studies.[17] However, the benefit of this GvL response was virtually obliterated in patients who developed very severe GvHD, resulting in death due to GvHD-related complications including multiple-organ failure and infections.

Previous studies had already illustrated that reduction of the tumor burden was necessary to gain significant time to allow the immune response to develop.[14,19,26] We hypothesized that tumor reduction was also necessary to limit the inhibitory effect of the malignant cells on the induction of an immune response, to induce immunological danger signals to promote development of a primary immune response, and to induce leukopenia to promote homeostatic proliferation of the antigen-experienced T cells further favoring a GvL response.[20–22,24] Based on these prerequisites, we preferentially used gemtuzumab-ozogamycin as targeted chemotherapy for first-line treatment for patients with relapsed AML. Gemtuzumab-ozogamycin is a CD33 antibody linked to the cytotoxic drug calicheamycin. Gemtuzumab-ozogamycin-induced apoptosis of both CD33-positive and CD33-negative leukemic cells in the bone marrow may lead to presentation of antigens from the leukemic cell debris by local antigen-presenting cells.[27,28] After gemtuzumab-ozogamycin was withdrawn from the market, subsequent patients received myeloablative therapy with cytarabine-based re-induction regimens, which also led to control of leukemia in several patients, but at the cost of more systemic toxicity. Unfortunately, even with high-dose chemotherapy, failure Ferrato obtain sufficient initial disease control was observed in approximately 28% of patients with high tumor burden relapse, advocating treatment with immunological interventions in circumstances of minimal residual disease.

Some patients displayed a relapse of AML with limited leukemic load and an indolent course, i.e. a smoldering relapse.[29,30] These patients could benefit from our DLI-based strategy without prior cytoreductive treatment. This approach resulted in a profound immune response in four out of five patients. Apparently, the immune response can develop with limited tumor burden, under circumstances similar to a molecular relapse of chronic phase chronic myeloid leukemia. In chronic phase chronic myeloid leukemia, GvL reactivity by DLI can develop without chemotherapy or lymphodepletion, although

the interval between administration of the T cells and the clinical response may last several months.[18,31] Previously, we demonstrated that treatment of patients with low-dose interferon-α could shorten the interval between the DLI and onset of the immune response.[23] Therefore, interferon- α was implemented in our strategy to boost the immune response by activating rapid and efficient antigen-presentation. [23,32–34] In contrast to treatment of chronic myeloid leukemia, we used relatively high doses of DLI to induce a rapid immune response with high amplitude, although we anticipated that this would occur at the cost of GvHD.

In the patients with sufficient initial disease control, development of acute GvHD was observed in approximately 56% of patients at 3 months after DLI. Importantly, interferon-α was withheld instantly when the first clinical signs of GvHD were observed. No clear difference in distribution pattern or severity of GvHD was observed between patients who developed GvHD within 3 weeks after DLI and patients who developed GvHD after boosting with interferon-α. Obviously, the adverse impact of severe GvHD on morbidity and mortality limits the window of opportunity for a favorable outcome of patients with relapsed AML. The necessity for systemic immunosuppression to taper the immune response increases susceptibility to opportunistic infections. Nevertheless, our results illustrate that relapse of AML after transplant can be successfully controlled by an alloimmune response.

Since all patients in our study received a T-cell-depleted graft, it may be questioned whether our strategy could be equally successful in patients with relapsed AML after T-cell-replete allogeneic SCT. Patients transplanted with a T-cell-replete allograft are more likely to have experienced an alloimmune response after transplantation, and patients who relapse may, therefore, be selected to be resistant to a cellular immune intervention. However, several patients in our cohort with long-term survival after relapse had already shown limited GvHD or had received prophylactic DLI prior to their relapse. In these patients, re-introduction of DLI-induced GvL with acute GvHD was seen. This observation supports the assumption that our strategy is able to boost a GvL response in patients who apparently have had an immune response prior to relapse with insufficient amplitude, which can be similar in patients without severe GvHD after T-cell-replete allogeneic SCT. On the other hand, we assumed that patients with severe GvHD at the time of relapse were indeed resistant to alloreactivity by donor T cells.

To limit potential selection bias all patients with relapsed AML after allogeneic SCT who were treated at our institution during a 10-year time period were included in our retrospective analysis. Although cure was apparently achieved in a substantial fraction of patients with relapsed AML, overall survival at 2 years after relapse of the entire cohort was only 23%. This outcome was negatively influenced by the seven patients who were not eligible for our treatment strategy and received palliative treatment only. In patients eligible for cytoreductive treatment and DLI, the 2-year overall survival

improved to approximately 36%. On top of this, the likelihood of a favorable outcome was correlated with low tumor burden and development of controllable GvHD after DLI. Prevention of high tumor burden relapses might further improve the outcome of relapsed AML after allogeneic SCT. Early detection of an impending relapse with highly sensitive markers of minimal residual disease, including *WT1* gene expression, may be useful to guide immunological interventions.[35] In addition, the use of new cellular therapies which circumvent the negative impact of GvHD may be attractive, such as infusion of hematopoiesis-restricted or leukemia-specific T cells.[36] Due to the small sample size of the dataset, no additional multivariate analyses could be performed to investigate the impact of conditioning regimen, degree of HLA-matching, or disease stage at transplant.[25] Also at present, no registered targeted therapies are available, but perhaps less toxic re-induction therapy, compared to high-dose chemotherapy, might lead to reduced GvHD after DLI.

In summary, a regimen combining efficient salvage re-induction therapy, followed by infusion of donor T cells in the neutropenic phase after cytoreduction and subsequent interferon-α administration if needed to induce donor-derived anti-tumor immunity may result in cure of patients with relapsed myeloid malignancies after allogeneic SCT. In contrast, the prognosis in the case of absent or excessive acute GvHD remains dismal due to refractory disease or GvHD-related mortality. We conclude that achievement of limited tumor burden in combination with relatively high-intensity immune responses, as reflected by controllable acute GvHD, are essential for the cure of relapsed AML after allogeneic SCT.

ACKNOWLEDGMENTS

This work was supported by research grant 2008-4263 from the Dutch Cancer Society, Amsterdam, the Netherlands.

AUTHORSHIP AND DISCLOSURES

Information on authorship, contributions, and financial & other disclosures was provided by the authors and is available with the online version of this article at www.haematologica.org.

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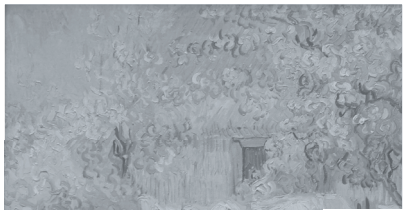
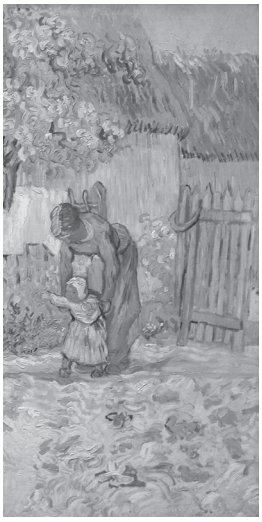




Chapter 3

Supplementary Appendix

SUPPLEMENTARY METHODS SECTION

Between January 2000 and January 2010, 44 patients with relapsed AML after alloSCT were treated. The patients were categorized according to their disease characteristics prior to alloSCT as intermediate, poor-risk, or very poor-risk AML. Intermediate risk (int-risk) AML was defined as <60 years old, white blood cell count (WBC) <100x10^9/L at diagnosis, cytogenetically normal (except for -X, -Y), and transplanted in first complete remission (CR1). Poor-risk AML was defined as ≥60 years old and/or WBC >100x10^9/L at diagnosis, and/or transplanted in second complete remission (CR2) and/or not cytogenetically normal, favorable (t(15;17), t(8;21), or inv(16)) or very poor (monosomal karyotype), and/or FLT3/ITD positive. In addition, acute erythroid leukemia, acute megakaryocytic leukemia and mixed-phenotype acute leukemia were also considered poor-risk. AML with a monosomal karyotype (MK) was defined as very poor-risk.

Analysis of the data was approved by Leiden University Medical Center Research Ethics Committee. Informed consent was obtained prior to data collection. Data was analyzed as of November 2012.

Transplantation protocol

Patients had been transplanted with a myeloablative (MAC) or a reduced-intensity conditioning (RIC) regimen. The MAC regimen consisted of cyclophosphamide 60 mg/kg i.v. for 2 days, and TBI 9 Gy (n=26) or busulphan (Busilvex©) 3.2 mg/kg i.v. for 4 days (n=1). Patients transplanted with an unrelated donor also received alemtuzumab 15 mg i.v. for 2 days, and cyclosporine 3 mg/kg as GvHD-prophylaxis from day -1 until day 60 in the absence of GvHD. The RIC regimen consisted of fludarabine 30 mg/m2 i.v. or 50 mg/m2 orally for 6 days, busulphan 3.2 mg/kg i.v. for 2 days and either alemtuzumab 15 mg i.v. for 2 days (n=10) or horse-derived anti-T lymphocyte globulin (Lymphoglobulin©) 10 mg/kg/day i.v. for 4 days (n=8). After RIC, patients did not receive additional GvHD-prophylaxis. In both MAC and RIC transplanted patients in vitro T-cell depletion (TCD) of the stem cell product was performed by incubation with alemtuzumab (20 mg) for 30 min at room temperature under continuous agitation (n=41), or by $CD34^+$-cell MACS-sorting (n=3).(1-4) Fully matched donors were defined as 10 out of 10 HLA-matched (HLA-A, -B, -C, -DR and -DQ).

From 2002, all patients with AML or MDS were eligible for prophylactic DLI at 6 months after alloSCT when the patient showed mixed-chimerism in the absence of GvHD requiring systemic immunosuppression. From 2007, all patients with high-risk MDS (IPSS >1), poor-risk AML and MK AML were candidate for low dose prophylactic DLI at 3 months after alloSCT, if overall grade 2 (or higher) acute GvHD was absent at that time point.

Relapse

Relapse after alloSCT was defined as an increase of blasts in the bone marrow (BM) to 5% or more by morphology, and/or by the presence of more than 1% blasts in peripheral blood (PB), and/or by the reappearance of molecular and/or cytogenetic markers. Smoldering relapse was defined as a low percentage of blasts by morphology (<10% blasts in BM and <5% blasts in PB) during a prolonged interval of at least 4 weeks evaluated by repeated bone marrow aspiration. Other relapses were defined as high tumor burden relapse. Remission status after DLI was assessed by bone marrow aspiration at 6 week, at 12 weeks after DLI, and at every 3 months hereafter. Second relapse was defined as first relapse after DLI.

Treatment strategies for relapse

Patients with a poor performance status, defined as WHO performance status 3 or higher, and/or with severe GvHD requiring systemic immuno-suppression at the time of relapse were unlikely to benefit from an intensive DLI-based strategy and received palliative treatment only. Patients with high tumor burden relapse received salvage re-induction therapy prior to infusion of donor lymphocytes. In case of a response to re-induction therapy, defined as absence of circulating leukemic blasts, unmanipulated DLI collected from the original donor was administered at 3 weeks after start of re-induction therapy (during the neutropenic phase). In case of a smoldering relapse, patients received DLI without prior cytoreductive treatment. DLI-dosing depended on the time point after alloSCT, administration of pre-emptive DLI prior to relapse and donor type. Median DLI-dose of patients transplanted with a (10 out of 10 HLA-matched) sibling donor was $5.0x10^6$ $CD3^+$-cells/kg (range $1.0\text{-}100x10^6$ CD3+cells/kg) and $2.5x10^6$ $CD3^+$-cells/kg (range $1.5\text{-}7.8x10^6$ CD3+cells/kg) for patients transplanted with an unrelated donor (or a HLA-mismatched related donor), respectively.

The occurrence and impact of GvHD on outcome after relapse was evaluated in all patients receiving DLI for relapse. Assessment and grading of GvHD was performed using modified Glucksberg and Shulman criteria.(5, 6). If no acute GvHD was observed within 3 weeks after DLI, interferon-α was administered subcutaneously in a daily dose of 3 million units until GvHD occurred. Escalating doses of DLI were administered in case of failure to develop acute GvHD at 3 months after start of treatment. Finally, grade 2-4 acute GvHD and severe chronic GvHD were treated with prednisone 1-2 mg/kg/day. Ciclosporine or mofetil mycophenolate were added if prednisone was administered for more than 2 weeks.

Statistical analysis

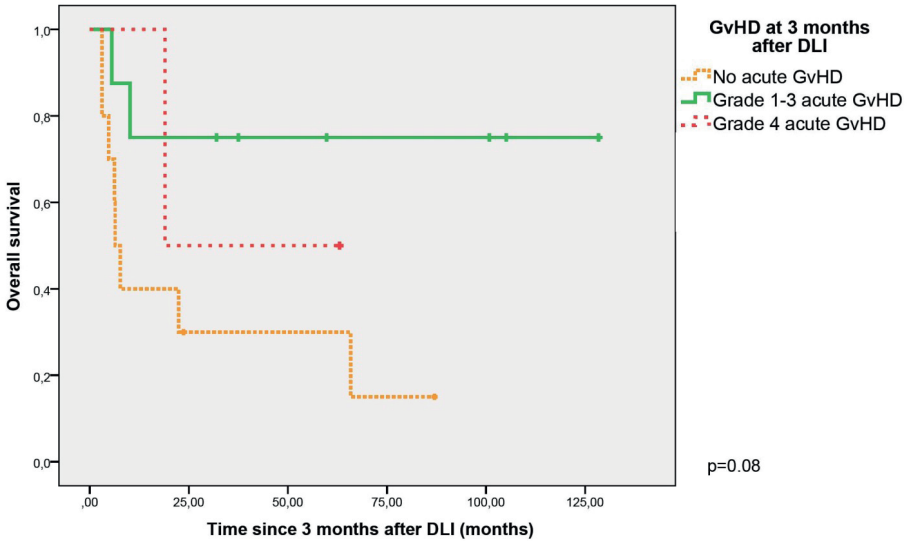
For different analyses, time was measured since relapse, initiation of re-induction therapy or infusion of donor lymphocytes. Non-relapse mortality (NRM) was defined

as death after DLI while in continuous complete remission. Relapse-free survival was defined as survival after DLI without second relapse. Probabilities of overall survival with associated 95% confidence intervals (95% CI) were calculated by the Kaplan-Meier method; differences between groups were compared using long-rank test. The cumulative incidence of acute GvHD after DLI was calculated using R2.15.0 software, library 'cmprsk' (http://www.r-project.org/foundation).

To investigate the impact of a rapid immune response associated with acute GvHD on long-term survival and disease recurrence after DLI, extended Cox regression models as proposed by Andersen&Gill for overall survival, relapse-free survival and relapse incidence from starting point DLI were constructed (see online supplementary method section for details).(7) These models included onset of acute GvHD after infusion of donor lymphocytes as a time-dependent variable, separating between grade 1-3 and grade 4. The small number of events prevented the inclusion of other relevant predictors, for which reason the estimated Hazard Ratios should be interpreted as averages over different patient characteristics. Impact of acute GvHD on survival was also studied by landmark analysis (see supplementary Figure 1).

Supplementary Table 1 Severity and distribution pattern of GvHD after DLI according to administration of interferon-α.

	Interferon-α	No interferon-α
Acute GvHD	(n=15)	(n=12)
None	1	5
Grade 1	3	1
Grade 2	1	1
Grade 3	2	3
Grade 4	4	2
Localization acute GvHD	(n=10)	(n=7)
Skin only	5	1
Intestines only	0	1
Skin and liver	5	3
Skin, liver and intestines	0	2
Chronic GvHD	(n=15)	(n=12)
None	8	2
Limited	1	1
Extensive	4	2
Not applicable (Follow-up ≤ 3 months)	2	7



Supplementary Figure 1 Landmark analysis of the impact of the presence of acute GvHD at 3 months after DLI on overall survival.
Kaplan meier curves starting at 3 months after infusion of donor lymphocytes showing overall survival according to maximum grading of acute GvHD before that time point: 10 patients without signs of GvHD (dashed line), 8 patients with grade 1-3 acute GvHD (solid line) and 2 patients with grade 4 acute GvHD (dotted line).

All P-values of <0.05 were considered significant. Statistical software used was SPSS, PASW Statistics 20, release 20.0.0 (2011).

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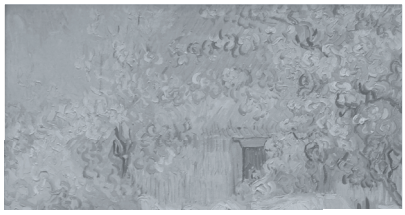

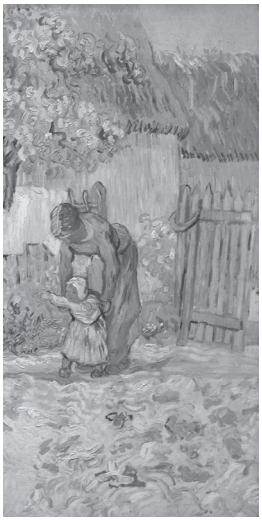

Chapter 4

Multi-state analysis illustrates treatment success after stem cell transplantation for acute myeloid leukemia followed by donor lymphocyte infusion

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ABSTRACT

In the field of hematopoietic stem cell transplantation, the common approach is to focus outcome analyses on time to relapse and death, without assessing the impact of post-transplant interventions. We investigated whether a multi-state model would give insight into the events after transplantation in a cohort of patients who were transplanted using a strategy including scheduled donor lymphocyte infusions. Seventy-eight consecutive patients who underwent myeloablative T-cell depleted allogeneic stem cell transplantation for acute myeloid leukemia or myelodysplastic syndrome were studied. We constructed a multi-state model to analyze the impact of donor lymphocyte infusion and graft-*versus*-host disease on the probabilities of relapse and non-relapse mortality over time. Based on this model we introduced a new measure for outcome after transplantation which we called 'treatment success': being alive without relapse and immunosuppression for graft-*versus*-host disease. All relevant clinical events were implemented into the multi-state model and were denoted treatment success or failure (either transient or permanent). Both relapse and non-relapse mortality were causes of failure of comparable magnitude. Whereas relapse was the dominant cause of failure from the transplantation state, its rate was reduced after graft-*versus*-host disease, and especially after donor lymphocyte infusion. The long-term probability of treatment success was approximately 40%. This probability was increased after donor lymphocyte infusion. Our multi-state model helps to interpret the impact of post-transplantation interventions and clinical events on failure and treatment success, thus extracting more information from observational data.

INTRODUCTION

In the field of allogeneic stem cell transplantation (SCT), outcome analyses are mainly focused on time to relapse and death, measured from the moment of transplantation. [1] This approach, however, does not take into account the increasing availability and potential of post-SCT interventions. These interventions, in particular donor lymphocyte infusions (DLI), are of utmost interest as part of a treatment strategy in which T-cell depleted (TCD-)SCT is followed by T-cell based therapeutic interventions. T-cell depletion of the graft efficiently prevents the development of severe graft-*versus*-host disease (GvHD) directly after transplantation, but also adversely affects post-transplant anti-tumor and anti-infectious immunity.[2–4] Early intervention with DLI after TCD-SCT may prevent the relapse of the malignancy and improve immune reconstitution against pathogens, but is associated with the reintroduction of the risk of GvHD.[5,6] However, a long-term delay of DLI in patients without clinical signs of GvHD may increase relapse risk in these patients. At present, the optimal timing of DLI has not been established.

There is a growing interest in the analysis of treatment outcome measures other than overall survival and relapse-free survival. Although these are crucial endpoints, they do not concentrate on highly relevant intermediate events or interventions essential to evaluate causes of failure or success after transplantation. To assess the dynamic impact of post-transplant clinical events and interventions, the methodology of multi-state models has been developed. [7–9] Multi-state models have several advantages with respect to more widely used standard survival analysis methods.[10] The primary advantage is that sequences of events -such as SCT, DLI, GvHD, death - and competing events - such as relapse and death before relapse - can be modeled simultaneously. Thus, the models can deal with different post-transplant sequences and timing of events. In contrast to composite survival outcomes such as failure-free survival[11] or GvHD-free survival[12] where a patient's subsequent events after the first failure are not considered anymore, multi-state models can be used to assess the role of temporary states such as GvHD in relation to additional interventions after transplantation. These models are essential to evaluate more complex treatment strategies like TCD-SCT with differentially scheduled post-transplant cellular immune interventions.

Even though this methodology has been available for over a decade thanks to the work of Klein and colleagues, the number of clinical questions in the field of hematology addressed by means of these models has been very limited thus far.[8,13–17] This has partly been caused by a lack of familiarity with the method or its potential, and partly by insufficient data quality: a multi-state model requires reliable follow-up information for the events in the model. Another major reason was the lack of easily accessible software. This has changed in the last years, among others by the development of the package 'mstate' in R that enables users to analyze general multi-state models.[7,9]

In the current study we investigated how multi-state models can be applied to complex phenomena including cellular interventions after transplant. We focused on two topics: (1) the impact of DLI and development of GvHD requiring immuno-suppressive treatment (IS) on the failure probabilities of relapse and non-relapse mortality (NRM) over time, and; (2) the probability of treatment success, which is defined as the absence of disease and GvHD requiring IS over time. We demonstrate how a multi-state model helps to interpret the consequences of the therapeutic interventions after SCT in a cohort of patients with acute myeloid leukemia (AML) and myelodysplastic syndrome (MDS) who were transplanted in our center using a TCD-SCT strategy including scheduled post-transplant DLI.

METHODS

Study protocol

Seventy-nine patients who underwent myeloablative allogeneic SCT for AML or MDS in complete remission (CR) in our center between January 2002 and June 2011 were included in this analysis. Two other patients who received $CD4^+$-DLI in the context of an experimental $CD4^+$-DLI study during this time period were not included in the analysis. Until June 2007 only patients with mixed-chimerism but without early relapse of AML or MDS were eligible for prophylactic DLI at 6 months after SCT. If severe GvHD (over-all grade II or higher) was present, DLI infusion was postponed. From June 2007 onwards, patients with poor or very poor-risk AML were eligible for low dose prophylactic DLI at 3 months after SCT (see Table 1 for definition of risk groups). For details on transplantation protocol, engraftment, dosing of DLI and assessment of GvHD and mixed-chimerism, see the *Online Supplementary Appendix*. The study was approved by Leiden University Medical Center Research Ethics Committee. Informed consent was obtained prior to data collection. Data were analyzed as of December 2012.

Definitions of events after SCT

For an accurate and objective determination of the duration of severe GvHD, the time interval between the start date of IS (indicated for GvHD) until cessation of IS (stop IS) was taken. In patients with an unrelated donor receiving prophylactic cyclosporine, the date of the start of additional IS, e.g. prednisone, was taken as the starting point of severe GvHD.

Relapse after SCT was defined as an increase of blasts in the bone marrow (BM) to ≥5% by morphology; and/or by the presence of >1% blasts in peripheral blood (PB); and/or by the reappearance of molecular and/or cytogenetic AML markers. NRM was defined as death in continuous complete remission after SCT.

Table 1 Patient characteristics.

Variable	Categories	Entire cohort (79 patients) n (%)/ Median (range)
Gender	male	41 (52%)
	female	38 (48%)
Age at SCT	years	median 46 years (18-61 years)
Initial diagnosis prior to SCT	AML	70 (89%)
	MDS	9 (11%; median IPSS 2.0 range 1.0-3.0)
AML risk group at diagnosis (n=70)	intermediate	12 (17%)
	poor	45 (64%)
	very poor	13 (19%)
Remission status prior to allo SCT	CR1	66 (84%)
	CR2	13 (16%)
Conditioning regimen	Cyclophosphamide/TBI	77 (97%)
	Busulphan/Cyclophosphamide	2 (3%)
Stem cell source	bone marrow	6 (8%)
	G-CSF stimulated PB	73 (92%)
Donor type	sibling	52 (66%; 51 10/10 HLA-match)
	unrelated	27 (34%; 17 10/10 HLA-match)
Ex vivo T-cell depletion	Alemtuzumab	73 (92%)
	CD34-selection	6 (8%)
CD34 cells	10^6/kg	median 7.0 (1.1-28.3)
Acute GvHD after SCT of patients who engrafted (n=78)	grade 0-1	53 (68%)
	grade 2	18 (23%)
	grade 3-4	7 (9%)
Number of prophylactic DLIs per patient	0	42 (53%)
	1	16 (20%)
	2	9 (11%)
	3	9 (11%)
	4	3 (4%)
Time to first DLI (n=37)	days	median 216 (91-820)
GvHD after first DLI (n=37)	no	24 (65%)
	yes	13 (35%; 6 required IS)

SCT: allogeneic stem cell transplantation; IPSS: international prognostic scoring system; CR1: first complete remission; CR2: second complete remission; TBI: total body irradiation; G-CSF: granulocyte-colony stimulating factor; CD34-selection: MACS-sorted $CD34^+$cell collection; DLI: donor lymphocyte infusion; IS: systemic immunosuppression. Intermediate-risk AML was defined as the combination of WBC <$100x10^9$/L at diagnosis, absence of cytogenetic abnormalities (except for -X, -Y), achievement of morphological complete remission after the first remission induction course and transplantation in first complete remission (CR1). Poor-risk AML was defined as either WBC >$100x10^9$/L at diagnosis, and/or presence of cytogenetic abnormalities (excluding the favorable cytogenetic abnormalities t(15;17), t(8;21) and inv(16)), and/or FLT3/ITD positivity, and/or absence of morphological complete remission after the first remission induction course and/or transplantation not in CR1.Very poor-risk AML was defined as monosomal karyotype, and/or 3q26 rearrangement, and/or EVI-1 expression at diagnosis. One patient (AML, poor-risk) was not included in the multi-state analysis because of graft rejection.

Statistical analysis

For all analyses, time was measured from the date of SCT. Probabilities of overall survival with associated 95% confidence intervals (95% CI) were calculated by the Kaplan-Meier method. Overall survival was estimated with SPSS/PASW Statistics 20, release 20.0.0 (2011). The cumulative incidence of prophylactic DLI was estimated in a competing risks framework, considering relapse and NRM before prophylactic DLI as competing events. The cumulative incidence of DLI was estimated by means of the 'cmprsk' library in R.

All other outcomes were analyzed by means of a multi-state model (see the *Online Supplementary Appendix* for an explanation of the methodology). The quantities of interest in the multi-state model were estimated in R, version 3.0.1, with library 'mstate'.[7,9]

RESULTS

Patient cohort

Characteristics of patients and transplantation procedures are presented in Table 1. A rejection was observed in one patient (day 43). The median follow-up of surviving patients of the entire cohort was 63 months (range 20-128 months). The cumulative incidence of DLI was 21% at 6 months and 29% at 9 months (see *Online Supplementary Figure S2*). At the end of follow-up, 37 patients (47%) had received prophylactic DLI (median 2 prophylactic DLIs per patient; range 1-4). Although very poor-risk patients were scheduled for early DLI, in practice the timing of DLI was not very different for those patients compared to the other patients (median time to DLI for the 5 very poor-risk patients: 6.9 months (range 3.2-9.9 months) for the other patients: 7.7 months (3.0-27.0 months)). Forty-two patients (53% of 79 patients) did not receive DLI (very poor-risk AML n=8, poor-risk AML n=24, intermediate-risk AML n=7, MDS n=3). Reasons for not receiving DLI were rejection (n=1), NRM (n=13), early relapse (n=9), severe GvHD (n=8), full-donor hematopoiesis (n=9, including one patient with poor-risk AML who relapsed at day 270), and logistical problems (n=2; these patients with very poor-risk AML who were transplanted before June 2007 showed mixed-chimerism at 6 months after SCT but relapsed before DLI was initiated (at days 216 and 275, respectively)).

The estimated 2-year overall survival calculated from the start day of SCT of the entire cohort of 79 patients was 58% (95% CI 48-68%). The two-year overall survival of patients with intermediate-risk AML, poor-/very poor-risk AML, and MDS calculated from the start day of SCT were 67% (95% CI 40%-94%), 55% (95% CI 42-68%), and 67% (95% CI 35%-98%), respectively (see Figure 2).

The multi-state model

A multi-state model was constructed to model the occurrence and impact of relevant events after SCT (see Figure 1). Since no patient received prophylactic DLI before IS had been tapered, the transition between 'start IS' and 'DLI' was omitted from the model. Only one patient in the entire cohort experienced a rejection (at day 43), which excluded transition to the state DLI, and therefore both this patient and the state 'Rejection' were not included in the model. Outcomes of the other 78 patients were analyzed in the multi-state model. None of them were lost to follow-up. The number of events in the dataset and the number of patients in each state at the end of their follow-up time are presented in the *Online Supplementary Figure S1*.

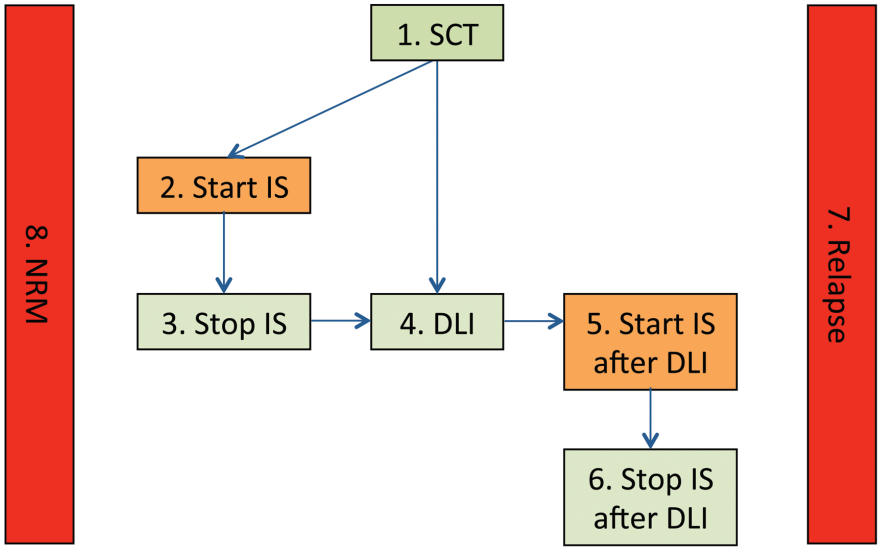

Figure 1 Multi-state model. The name of each state reflects the event the occurrence of which makes the patient enter the state; he/she remains in this state until a next event occurs or until the end of follow-up. Patients entered a new state on the day when such an event took place and could not visit the same state twice. The starting state of all patients is 1. SCT. The intermediate states are depicted by numbers 2 to 6. Each arrow indicates a possible transition to an intermediate state in the model. Transition between severe GvHD (Start IS) and initiation of DLI was only possible after cessation of systemic immunosuppression. The absorbing states (in red) are Relapse and NRM. States 1, 3, 4, 6 (in green) indicate treatment success; states 2, 5, 7, 8 (in orange and red) indicate treatment failure. Transition to the absorbing states was possible from any other state in the model; for simplicity these transitions are omitted from the figure.

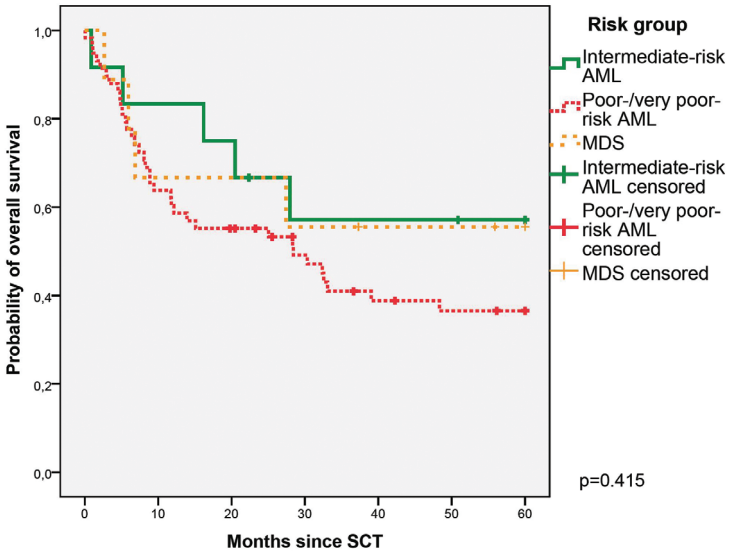

Figure 2 Overall survival curves. Kaplan Meier curves illustrating probabilities of overall survival of 12 patients with intermediate-risk AML (solid green line), 58 patients with poor-/very poor-risk AML (dashed red line), and 9 patients with MDS (dotted orange line).

Outcomes of the multi-state model

All transition probabilities from SCT during the first 60 months of the follow-up are presented in the *Online Supplementary Figure S3*. The proportions of patients suffering relapse and NRM are comparable in size. From 11 months after SCT, a substantial proportion of patients (ca. 30%) remained in the 'DLI' state, implying they had received DLI, were still alive without relapse and did not require IS for GvHD after DLI. Correspondingly, we observed that only a minority of patients suffered from severe GvHD after DLI (see 'Start IS after DLI', *Online Supplementary Figure S3*) and that the probability to be in the 'Start IS' or 'Start IS after DLI' state was zero after 33 months, indicating that IS could be tapered successfully in all patients with an episode of severe GvHD.

It cannot be excluded that patients likely to develop GvHD post-DLI failed before DLI could have been administered. However, several factors are likely to contribute to the reduced development of GvHD after DLI. The timing of DLI is one factor. DLI was not given to patients with active GvHD, but postponed in these patients (n=9). After DLI, new episodes of IS-requiring GvHD were hardly observed (1 out of 9 patients (11%) with previous IS-requiring GvHD, 5 out of 28 (18%) without previous IS-requiring GvHD). In conclusion, postponing DLI appears to alleviate the induction of severe GvHD, even in patients with GvHD prior to infusion of donor lymphocytes. Thus, the selection of patients who were unlikely to develop GvHD does not appear to have played a major role.

Finally, the small probability of remaining in the state SCT in the long run indicates that follow-up without experiencing a further event was highly uncommon, which is in line with the strategy of scheduled prophylactic post-transplant DLI. The wide confidence intervals reflect the small size of the dataset and show that all estimates should be interpreted with caution.

Figure 3 gives an overview of the outcomes of the multi-state model, by combining all individual transition probabilities as represented in the *Online Supplementary Figure S3* in one figure. The area below the relapse curve of this figure represents relapse-free survival and indicates how the patients alive and relapse-free were distributed over the different states at different moments during follow-up.

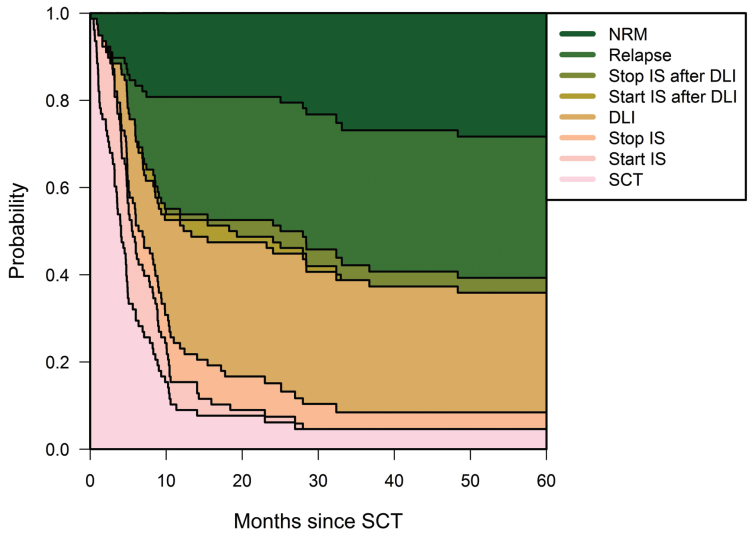

Figure 3 Transition probabilities to all states from SCT. Transition probabilities derived from the multi-state model of Figure 1. At each point in time, the distance between 2 adjacent curves represents the probability of being in the corresponding state, given that the patient was in the 'SCT' state at time 0. *Online Supplementary Figure S3* shows these same transition probabilities in separate panels. As before, names of states represent the events with which patients enter them. Since all patients started in the 'SCT' state, the probability of being in that state was 1 at time 0 and decreased afterward, because patients could only leave this state. For all other states, the probability to have entered this state at time 0 was 0. For the 'Relapse' and 'NRM' states, which are absorbing states, the probabilities can only increase over time since patients cannot leave these states anymore. For the 5 intermediate states, probabilities increase and decrease over time. The figure shows that already at 1 year post-SCT only a very small minority of patients are still in the SCT state. The probability to receive IS is always low, both before and after DLI, and transient in all cases. For relapse-free patients, from ca. 1 year onward, the dominant state is being alive after DLI.

Predictions from 7.5 months after SCT

The multi-state model enables the update of predictions of subsequent failures when information about post-SCT events accumulates over time. This approach can be considered as an extension of landmark modeling in which both start and end time, and starting and

end state are variable. Figure 4 shows transition probabilities for patients in different starting states (1 to 4) at 7.5 months after SCT. The time-point 7.5 months after SCT was chosen as the start time of these analyses because prophylactic DLI was scheduled at 6 months after SCT, after which an effective immune response is generally seen 5-6 weeks later, adding up to 7.5 months.[18] According to the model, patients who had not experienced severe GvHD and had not yet received prophylactic DLI within 7.5 months after SCT (in state 'SCT' at 7.5 months) had a predicted probability of relapse of 0.26 (95% CI: 0.07-0.45) within the first 2 years after transplantation. In contrast, patients in the state 'DLI' at the same time point had a predicted probability of relapse of 0.07 (95% CI: 0.00-0.21) within 2 years after transplantation. These results suggest that the risk of relapse, which is the major reason for failure from the 'SCT' state, is reduced after GvHD, and especially after DLI administration. In patients without any event, and also in patients in the 'DLI' state at 7.5 months after SCT, NRM did not play a prominent role during later follow-up.

These differences in outcomes cannot be explained by relevant differences in the baseline risk characteristics of the patients in the respective states at 7.5 months, since (very) poor-risk patients were not underrepresented in the 'DLI' state compared to the other states ('SCT' state: 55% poor AML, 15% very poor AML; 'Start IS' state: 45% poor, 9% very poor; 'DLI' state: 58% poor, 17% very poor).

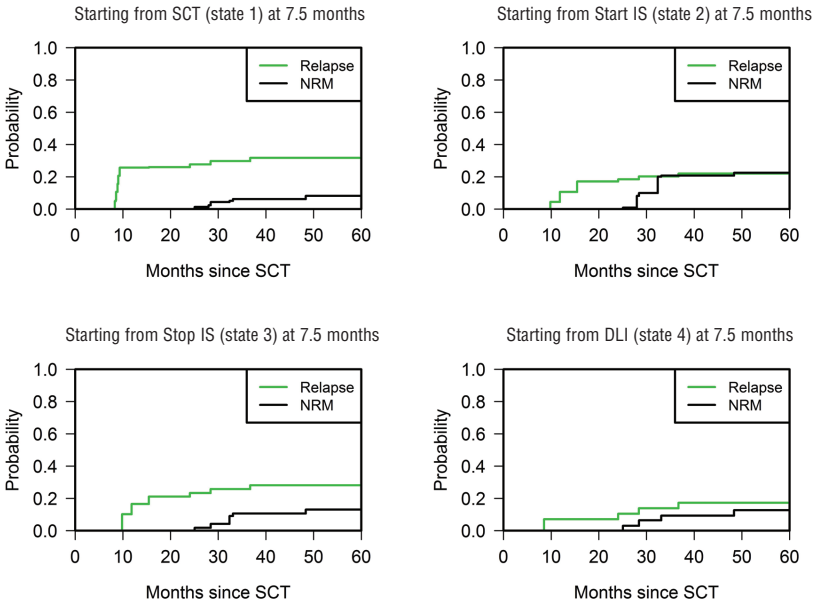

Figure 4 Predictions from 7.5 months after SCT. Estimated probabilities of relapse (green line) and NRM (black line) over time for patients present at 7.5 months since transplantation in states 'SCT', 'Start IS', 'Stop IS', and 'DLI', respectively. Results from 'Start IS after DLI' and 'Stop IS after DLI' are not shown because of the small sample size.

Treatment success

The same multi-state model can be used to summarize the outcomes of the transplantation procedure by focusing on the probability of treatment success at different time points after transplantation (see Figure 5). In general, the goal of our treatment strategy was to administer DLI safely to all patients at risk for relapse. Therefore, at each time point, treatment success included patients who experienced relapse-free survival, and did not suffer from severe GvHD at that time point, i.e. patients remaining in the state 'SCT', or present in the states 'Stop IS', 'DLI', or 'Stop IS after DLI'. The probability of treat-

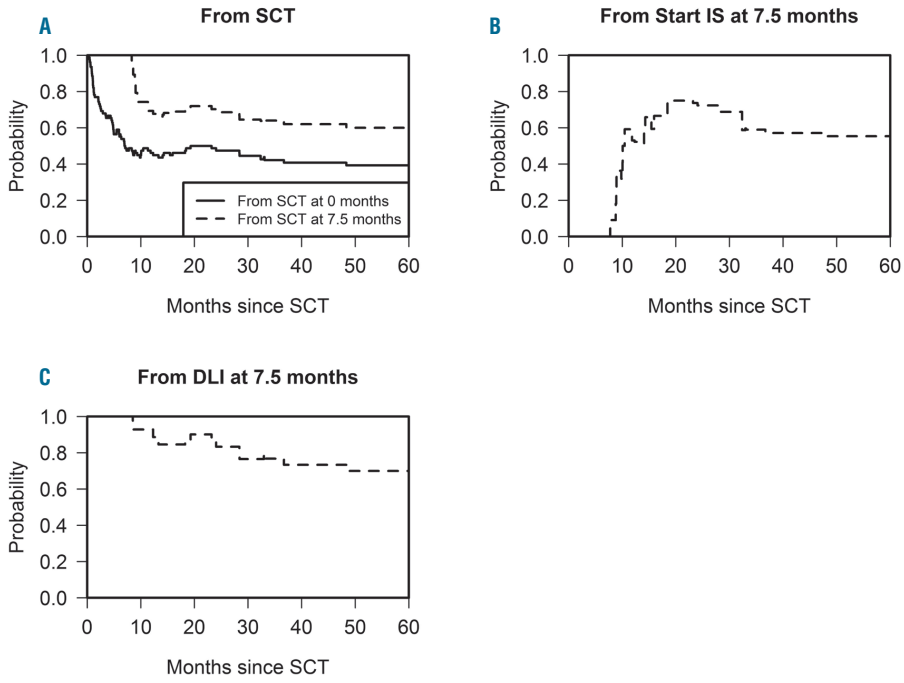

Figure 5 Probability of treatment success over time. Treatment success as calculated in the multi-state model of Figure 1 is defined as the probability of being alive without disease or severe GvHD, i.e., the sum of the probabilities of being in either of the states 1, 3, 4 and 6. (A) probability of treatment success for a patient in the 'SCT' state at 0 and 7.5 months after SCT, respectively. (B) probability of treatment success for a patient in the 'Start IS' state at 7.5 months after SCT. (C) probability of treatment success for a patient in the 'DLI' state at 7.5 months after SCT. Outcomes from 'Start IS' and 'DLI' at 0 months have been omitted since these do not represent a clinically meaningful situation. The curves from 'SCT' and 'DLI' start at probability 1 since a patient in one of these states by definition experiences treatment success at the moment when the clock starts; on the contrary, the curve from 'Start IS' begins at 0 because a patient in that state first has to make a transition to a beneficial state before being considered a treatment success. The steep rise in this curve can be interpreted as a sign of successful quick cessation of IS in the majority of patients who require IS for the treatment of early GvHD. The treatment success probabilities increase and decrease over time depending on the proportions of patients entering and leaving the beneficial states.

ment success over time was calculated by adding up the estimated time-dependent probabilities of being in these states. The long-term estimated probability of treatment success from start was approximately 0.43 (Figure 5A). If a patient survived the first 7.5 months after SCT without subsequent events, this success probability substantially improved to approximately 0.60. Patients who required IS for GvHD at 7.5 months after SCT (Figure 5B) showed a similar success probability of approximately 0.55 at 60 months after SCT. By definition, these patients were not considered to experience treatment success at 7.5 months after SCT, but a part of them acquired treatment success after cessation of IS possibly followed by DLI, corresponding to an increasing curve. Patients who required IS after DLI or failing from NRM or relapse led the curve to decline again.

Finally, a comparison with the prospects for patients in the 'DLI' state at the same point in time (Figure 5C) suggests that their probability of treatment success was higher: 0.70 at 60 months after SCT.

DISCUSSION

This detailed study of a group of AML/MDS patients with a long, complex follow-up illustrates the potential of a multi-state model to assess the risk of relapse and NRM for patients in a TCD-SCT population for which an infusion of donor lymphocytes was an essential intervention within the strategy. The model separates fatal and nonfatal events, thus enabling the summing up of several favorable episodes to estimate the probability of treatment success. The model also enables one to give long-term prognoses adjusted during follow-up which is relevant for clinical decision making.

We previously reported the feasibility of a TCD-SCT with sequential DLI strategy in a population of patients with acute lymphoblastic leukemia.[19] In the present study, the majority of patients had poor or very poor-risk AML prior to SCT, and thus the estimated 2-year overall survival of 58% of the entire cohort appears favorable. A substantial proportion of patients receiving DLI did not experience an adverse event during later follow-up. This implies that few patients relapsed after DLI, and also that the development of severe GvHD after DLI was rare. These results cannot be explained by a selection for DLI of patients with favorable-risk characteristics at SCT as a comparison of the patients in different states at 7.5 months shows. Predictions from 7.5 months after SCT show that patients who had not experienced any event (i.e., no severe GvHD, DLI or relapse) so far had the highest probability of relapse, whereas NRM did not play a prominent role in these patients. According to these predictions, patients in the 'DLI' state at 7.5 months after SCT had a relatively low relapse risk and NRM, leading to the highest probability of treatment success. Finally, most patients never needed IS, and IS was stopped during later follow-up in the majority of patients who required IS for GvHD at 7.5 months after

SCT. The results show the favorable outcomes of a strategy of TCD-SCT with DLI. The vast majority of patients alive and relapse-free were also free from IS. After 1 year, by far the largest part of this group consisted of patients who had received a DLI.

Although the decision to administer DLI may be driven by many clinical events, and although the variable timing of the DLI itself is associated with different disease histories hampering causal interpretation, the results show that ultimately being in the DLI state is beneficial. From these different observations it can be interpreted that the prevention of severe GvHD by T-cell depletion contributes to the reduction of NRM, and that the absence of significant GvHD leads to an increased relapse rate that may be decreased by DLI. Apparently, the low probability of relapse after DLI was not outbalanced by the induction of severe GvHD, which is supported by several other studies.[20–22] In conclusion, treatment of patients with absent or limited GvHD after TCD-SCT can safely be consolidated by DLI, which appears to reduce the relapse rate.

The observation that the relapse risk was decreased after DLI without a high probability of severe GvHD can be explained by a sufficiently long time interval between the myeloablative conditioning regimen and infusion of DLI, allowing the recipient to recover from conditioning-induced inflammation, thus reducing pro-inflammatory conditions at the time of DLI, without fully extinguishing the beneficial effect of the anti-recipient hematopoietic cell directed T-cells.[6,21,23–25] Timely infusion of donor lymphocytes will remain an important factor, because the delay of DLI will increase the time to a favorable immune response and is therefore associated with an increased relapse risk.[26–28]

Our treatment strategy was not part of a randomized trial, but we aimed to administer DLI to all patients with a high-risk of relapse, e.g. patients with high-risk AML or patients with persisting mixed-chimerism, and to all high-risk patients from June 2007 onwards, whose risk may be modulated by DLI.[5,22,23,29] The different DLI strategies for different risk groups make the role of timing of DLI even more difficult to assess and complicates the comparison of outcomes of patients with or without DLI. These issues can only be addressed properly in prospective randomized trials.[30,31] However, in current transplant practice, randomized trials are performed infrequently due to both the relatively small numbers of patients affected and to patient and physician treatment preferences.[32] This lack of randomized trials necessitates alternative analysis methods, in which as many insights as possible are extracted from observational data.[17] For instance, a comparison of treatment strategies could be performed by analyzing the data from different centers by the same model.

Multi-state models such as the example presented herein offer several advantages over traditional statistical approaches. The models are more flexible than Cox models with time-dependent covariates since there is no proportional hazards assumption considering hazard ratios as constant over time. They are more comprehensive than landmark models where the particular choice of the landmark time point leads to

restrictions. Moreover, landmark analyses do not take into account the probability of arriving in the state from where the prediction is made and are not suitable for analyzing sequences of events after the landmark time point. In addition, multi-state models do not only offer insight into the incidence of certain events as yielded by competing risks methods, but also into the probability of remaining in a certain condition over time, thus offering the option to consider new treatment outcomes. With our study, we illustrated the feasibility of assessing outcomes of a treatment strategy by means of a multi-state model.[33] Multi-state models have been advocated before as a means to model alternative outcomes after SCT, e.g. current leukemia-free survival, yet these models focused on fewer events and on outcomes since SCT, not using the full potential of updated prediction.[8,14,15,34] Other similar models can be constructed to assess several post-SCT interventions or events, such as the timing of CMV reactivation in relationship to the development of GvHD, also in the context of T-cell replete transplantations. Multi-state models can also help to extend and refine new outcomes such as GvHD-free, relapse-free survival after transplant, or failure-free survival after the initial systemic treatment of chronic graft-*versus*-host disease.[11,35]

In conclusion, our treatment strategy to infuse donor lymphocytes in patients without severe GvHD after myeloablative TCD-SCT did not lead to an increase of severe GvHD after DLI, and appeared to decrease relapse risk. Therefore, the probability of treatment success of these patients was relatively high. In the absence of randomized trials, large cohorts with detailed follow-up are required to further specify the role of prophylactic DLI in patients at risk for relapse. In general, multi-state models can increase insight into complex clinical situations by predicting outcomes of patients with different disease states which may change over time. The current study shows the potential of multi-state models in the analysis of new clinically meaningful outcomes.

FUNDING

This work was supported by research grant 2008-4263 from the Dutch Cancer Society, Amsterdam, the Netherlands.

ACKNOWLEDGMENTS

The authors would like to thank Illyea Hawke (DKMS, German Bone Marrow Donor Center, Dresden, Germany) for correcting the language of the manuscript.

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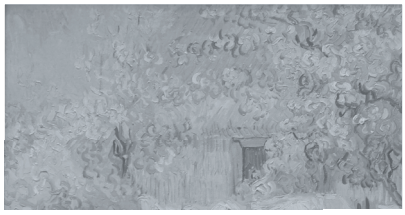

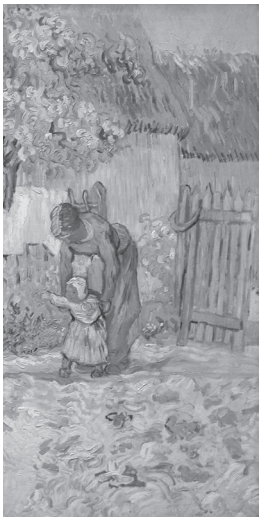

Chapter 4

Supplementary Appendix

APPENDIX

Transplantation protocol, engraftment, and assessment of GvHD

Patients were transplanted with a myeloablative conditioning regimen, consisting of cyclophosphamide 60 mg/kg i.v. for 2 days, and TBI 9 Gy (n=77) or busulphan (Busilvex©) 3.2 mg/kg i.v. for 4 days (n=2). In addition to this standard conditioning, all patients transplanted with a donor other than a fully matched sibling donor received pre-transplant alemtuzumab 15 mg i.v. for 2 days, and cyclosporine 3 mg/kg as GvHD-prophylaxis from day -1 until day 60 in the absence of GvHD. In all patients *in vitro* T-cell depletion (TCD) of the stem cell product was performed by incubation of the graft with alemtuzumab (20 mg) for 30 min at room temperature under continuous agitation, also known as 'Campath in the bag' (n=73) or by $CD34^+$-cell MACS-sorting (n=6).[1] The day of granulocyte engraftment was defined as the first of 3 consecutive days of absolute granulocyte counts > 0.5×10^9/L. Assessment and grading of GvHD was performed using modified Glucksberg and Shulman criteria.[2,3]

Prophylactic DLI

Until June 2007 only patients with mixed-chimerism but without early relapse of AML or MDS were eligible for prophylactic DLI at 6 months after SCT. Mixed-chimerism was defined as ≥1% patient hematopoiesis in any cell fraction (leukocytes, mononuclear cells and/or granulocytes) measured by PCR or FISH. If severe GvHD (overall grade II or higher) was present, DLI infusion was postponed. DLI-dosing depended on the time-point after SCT and on donor type. Median first DLI-dose of patients transplanted with a sibling donor was 3.0×10^6 $CD3^+$-cells/kg (range $1.0\text{-}3.0 \times 10^6$ CD3+cells/kg) and 1.5×10^6 $CD3^+$-cells/kg (range $1.5\text{-}2.4 \times 10^6$ CD3+cells/kg) for patients transplanted with an unrelated donor, respectively. From June 2007 onwards, all patients (n=40) except intermediate risk AML (n=8) were eligible for low dose prophylactic DLI at 3 months after SCT. The starting dose of DLI consisted of a predefined dose of 0.3×10^6 $CD3^+$-cells/kg or 0.15×10^6 $CD3^+$-cells/kg for patients with a sibling donor or unrelated donor, respectively. Escalating doses of DLI were performed in case of persisting mixed-chimerism at 3 months after DLI (see Table 1 for details).

Multi-state models

A multi-state model consists of different states (indicated by boxes) and transitions (indicated by arrows) (see Figures 1 and A1 for an illustration of the model used in this study). Patients experience a transition (i.e., event) when they pass from one clinical situation (state) to the next, and they remain in this state until the next transition takes place or until they are censored at the end of their follow-up. The model is used to estimate transition probabilities between states, which indicate the time-dependent

probabilities of being in a certain state at a certain moment, based on the state (same or other) where the patient stayed before. It can be perceived as an extension of standard survival analysis, which can be modeled with a two-state model: state 1 representing SCT, state 2 death, and the transition probability in the time interval 0 to *t* between these 2 states being equal to 1-survival (*t*). Transition probabilities are either represented by means of graphs (analogous to survival curves) or as numbers at a fixed point in time. As in standard survival analysis, the building blocks of the transition probabilities are the hazards, indicating the instantaneous probability of experiencing an event of a certain type conditional on being at risk for this event.

In the model presented here, it is assumed that Markovianicity holds, which implies that the future only depends on the past through the present, in other words, if the state of a patient at a specific point in time is known, it no longer matters through which route and when she/he arrived there. Transition hazards were estimated by means of Nelson-Aalen estimators, and transition probabilities by the Aalen-Johansen estimator. Standard errors of the transition probabilities were calculated by means of the Greenwood estimator.[4] They were used to estimate 95% point-wise linear confidence intervals. Only results up to 60 months after SCT are shown because of the increased imprecision afterwards due to the smaller number of patients still at risk.

All patients started at 'SCT' (state 1). We considered Relapse and NRM (states 7 and 8) as primary failures, which are the absorbing states in the model, meaning no further transition is possible once either of them is reached. The following intermediate states were defined: 'Start IS', 'Stop IS', 'DLI', 'Start IS after DLI' and 'Stop IS after DLI' (states 2 to 6, respectively). All patients experiencing (severe) GvHD requiring IS entered the state 'Start IS' at the day IS for GvHD was started. After cessation of IS, these patients entered the state 'Stop IS'. All patients receiving prophylactic DLI entered the state 'DLI'. Patients who experienced GvHD requiring IS after receiving prophylactic DLI entered the state 'Start IS after DLI'. After cessation of IS after prophylactic DLI, patients entered the state 'Stop IS after DLI'.

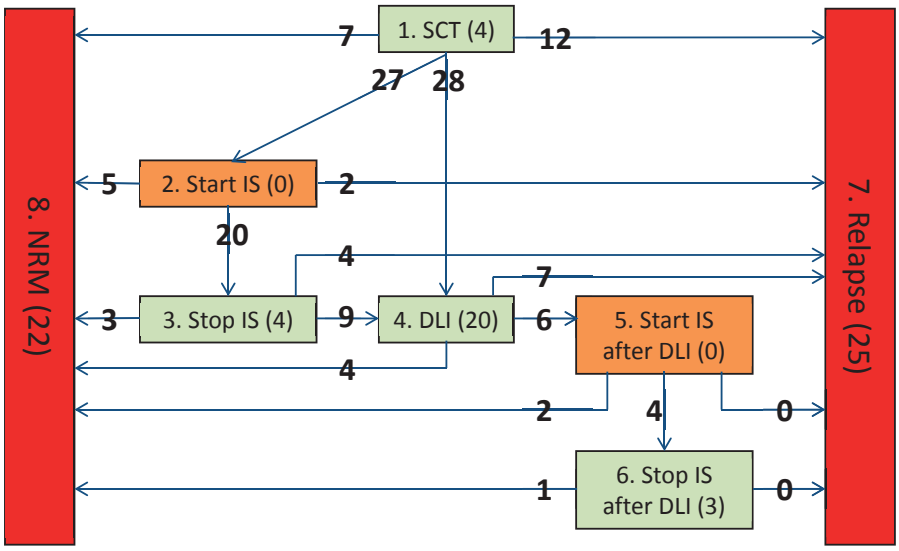

Figure A1 Multi-state model with transition counts.
In contrast to Figure 1, transitions to the absorbing states 'NRM' and 'Relapse' are also depicted by arrows. The number between brackets within each state indicates the observed number of patients in that state at the end of their follow-up. The number on each arrow indicates the observed number of transitions in our dataset (consisting of 78 patients).

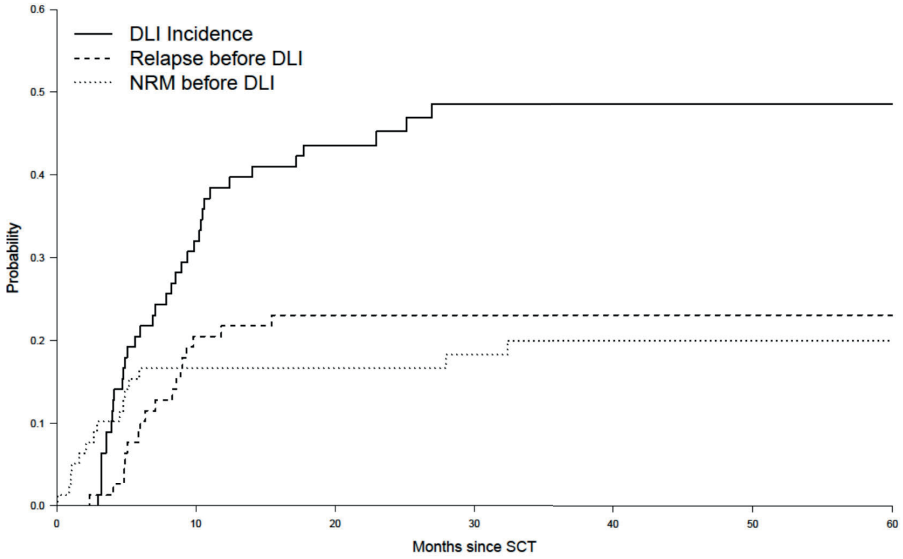

Figure A2 Cumulative incidence curves of prophylactic DLI and its competing risks relapse before DLI and NRM before DLI.

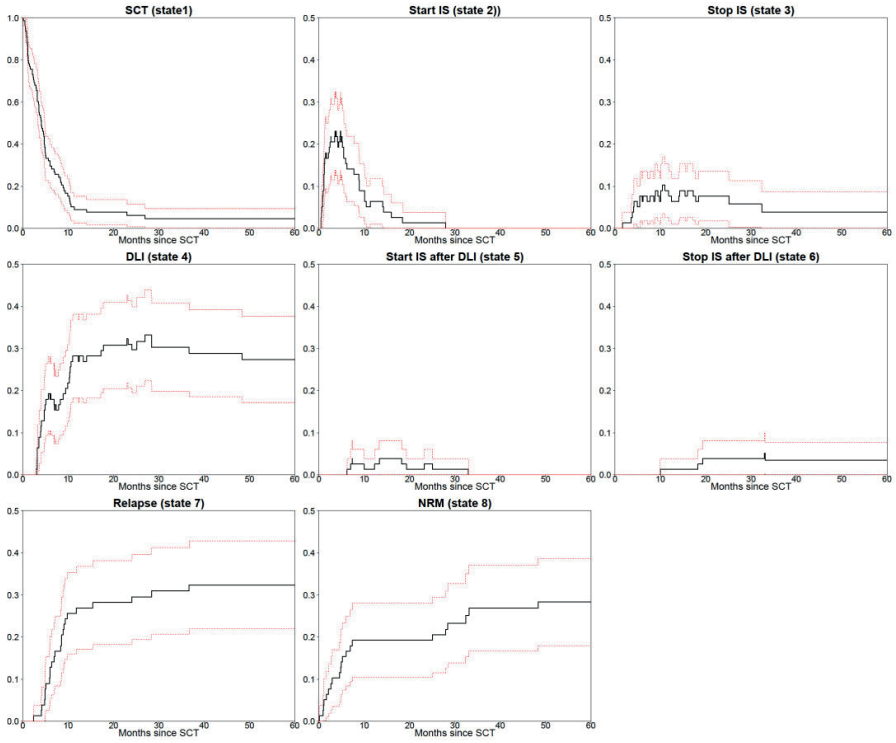

Figure A3 Transition probabilities to all states from SCT.
Transition probabilities (black) and associated 95% point-wise linear Confidence Intervals (red) derived from the multi-state model of Figure 1. At each point in time, the black curve indicates the probability of a patient being in the respective state, given that he/she was in the SCT state at time 0. Since all patients started in the 'SCT' state, the probability of being in that state was 1 at time 0 and decreased afterward, because patients could only leave this state. For all other states, the probability to have entered this state at time 0 was 0. For the 'Relapse' and 'NRM' states, which are absorbing states, the probabilities can only increase over time since patients cannot leave these states anymore. For the 5 intermediate states, probabilities increase and decrease over time. Note that the scale of the y-axis of the first figure (SCT) differs from that of the other figures.

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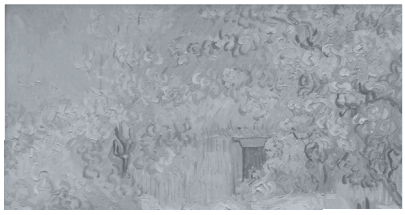

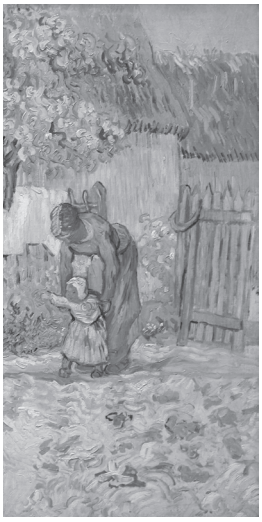

Chapter 5

Donor T-cell responses and disease progression patterns of multiple myeloma

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ABSTRACT

Donor T-cells transferred after allogeneic stem cell transplantation (alloSCT) can result in long-term disease control in myeloma by the graft-versus-myeloma (GvM) effect. However, T-cell therapy may show differential effectiveness against bone marrow (BM) infiltration and focal myeloma lesions resulting in different control and progression patterns. Outcomes of 43 myeloma patients who underwent T-cell-depleted alloSCT with scheduled donor lymphocyte infusion (DLI) were analyzed with respect to diffuse BM infiltration and focal progression. For comparison, 12 patients for whom a donor search was started but no alloSCT was performed, were analyzed. After DLI, complete disappearance of myeloma cells in BM occurred in 86% of evaluable patients. The probabilities of BM progression-free survival (PFS) at 2 years after start of donor search, alloSCT and DLI, were 17% (95% confidence interval 0– 38%), 51% (36–66%), and 62% (44–80%) respectively. In contrast, the probabilities of focal PFS at 2 years after start of donor search, alloSCT and DLI, were 17% (0–38%), 30% (17–44%) and 28% (11–44%), respectively. Donor-derived T-cell responses effectively reduce BM infiltration, but not focal progression in myeloma, illustrating potent immunological responses in BM with only limited effect of T-cells on focal lesions.

INTRODUCTION

Multiple myeloma (MM) is a neoplasia of plasma cells characterized by diffuse bone marrow (BM) plasmacytosis and focal lesions like plasmacytomas and lytic bone lesions. Current treatment strategies for fit younger patients aim to obtain long-term remissions and consist of combinations of immunomodulation, chemotherapy and autologous stem cell transplantation.[1] However, most patients eventually relapse, and these patients require other treatment strategies. The role of allogeneic stem cell transplantation (alloSCT) as treatment option for relapsed MM is currently under debate.[2–8] However, important lessons can be learned from the effect of T-cell therapy in the context of alloSCT. This may help to further develop T-cell therapy exploiting chimeric antigen receptor (CAR) cells, which is increasingly being performed.[9]

AlloSCT can result in long-term control of the disease because immune-competent donor T-cells can exert efficient graft-versus-myeloma (GvM) effects.[10–12] Conventional outcome parameters of interest after transplantation are overall survival (OS) and progression-free survival (PFS). However, these outcomes offer little insight into the kinetics of the disease and the impact of different components of the treatment. The complexity of the course of the disease with occurrence of GvM effects, graft-versus-host disease (GvHD) or non-relapse mortality, requires a richer methodology capable of analysing multiple distinct event types. A multi-state model permits mapping of all relevant events,[13] and from this several competing risks models can be derived to analyse the events of main interest.[14–18] At present, little is known about the clinical kinetics of allo-reactive T-cells in controlling myeloma progression or inducing tumor regression. Efficient GvM responses require targeting of malignant cells by antigen-specific T-cells in all sites of myeloma infiltration. Homing of T-cells to BM has been found to occur constitutively, whereas homing to other tissues may need specific ligands on T-cells, or an inflammatory environment.[19–21] Consequently, the strength of immune responses in BM and focal lesions may differ and result in differential progression patterns of myeloma after alloSCT and donor lymphocyte infusion (DLI) depending on the presence or absence of donor T-cell-mediated GvM reactivity.[22–24]

Precise analyses of T-cell-mediated GvM responses is impaired in T-cell replete alloSCT settings by simultaneous effects of pre-transplant treatment, conditioning regimens, concurrent immune responses, and immune suppression. T-cell depletion (TCD) of the stem cell graft has been developed to prevent development of severe GvHD.[25–27] In general, effective TCD obviates the need for prophylactic immunosuppression.[25,27] However, TCD also adversely affects post-transplant anti-tumor immunity.[28] To overcome this effect, our center has pioneered routine administration of DLI at pre-scheduled time points while maintaining a low risk of GvHD induced by the conditioning regimen.[11,29–32] TCD-alloSCT with sequential DLI, i.e., systematic introduction of post-transplant immune

therapy, has the benefit of offering a unique opportunity to assess the specific influence of donor lymphocytes on myeloma progression patterns since chemotherapy and the immune intervention are given at different points in time.

We hypothesized that alloSCT, and especially donor lymphocytes, modulate patterns of disease progression in MM. To test this hypothesis, BM and focal progression were assessed as separate events in a cohort of patients who underwent TCD-alloSCT with scheduled DLI and compared with outcomes of a cohort who did not undergo alloSCT, representing the course of the disease without TCD-alloSCT.

MATERIALS AND METHODS

Patient population

We analyzed data of all 43 MM patients who underwent *in vivo* and *ex vivo* T-cell depleted reduced-intensity conditioning (RIC) alloSCT at Leiden University Medical Center (LUMC) between May 2002 and July 2010 (alloSCT cohort). We also analyzed data of all 12 MM patients for whom a donor search was started at the LUMC during the same time period, but no alloSCT was performed (donor-search-no-alloSCT cohort; see Supplementary Material). The study was approved by Leiden University Medical Center Research Ethics Committee. Informed consent was obtained prior to data collection. Data was analyzed as of March 2015.

Definitions

Focal progression was defined as an increase in size or new-onset of soft tissue plasmacytomas and/or bone lesions as described by the criteria of the international myeloma working group.[33] New-onset BM infiltration (i.e., BM progression) was defined as 10% diffuse myeloma infiltration after a previously negative BM sample (measured by morphology, immunophe-notyping and/or trephine biopsy). In all other cases, BM progression was defined as 5% increase of myeloma cells in the BM as compared with the previous BM sample. After alloSCT, BM progression was considered as primary failure. Therefore, a lower threshold was chosen to detect new-onset BM involvement as early as possible: any percentage of myeloma cells in the BM with loss of donor chimerism (90% donor chimerism). Progression mortality was defined as death after signs of progressive disease. Non-progression mortality after alloSCT (or DLI) was defined as death after alloSCT (or DLI) without signs of progressive disease between alloSCT (or DLI) and death, respectively.

AlloSCT protocol

Patients with relapsed MM were eligible for alloSCT if the disease was responsive to drug therapy and a HLA-matched (9/10 or 10/10) related or unrelated donor was available. Details about the RIC alloSCT protocol and T-cell depletion are provided in the Supplementary File. Due to *ex vivo* TCD patients did not require additional GvHD prophylaxis. For follow-up after alloSCT, see Supplementary Material.

Administration of DLI

After engraftment, all patients without GvHD requiring systemic immunosuppression were eligible for DLI from 6 months after alloSCT in case of mixed-chimerism or disease progression. DLI-dosing depended on the time interval from alloSCT and on donor type (see Supplementary Material for details).

Statistical analysis

Time was measured from the start of donor search (donor-search-no-alloSCT cohort), infusion of donor stem cells, or DLI (alloSCT cohort). Probabilities of overall survival with associated 95% confidence intervals (95% CI) were calculated by the Kaplan–Meier method and compared by the log-rank test. Survival curves for different groups were compared using a log-rank test. Statistical software used was SPSS, PASW Statistics 20, release 20.0.0 (2011) and R 3.1.0 (http://www.r-project.org/foundation), packages 'prodlim', 'survival', 'cmprsk' and 'mstate'.[18] Code is available from the corresponding author upon reasonable request.

Multi-state model and competing risks analysis

Multi-state models were developed as schematic representations of all events relevant to the different analyses of outcomes after alloSCT and start of donor search, respectively (Figure 1). A detailed explanation of the model is provided in the Supplementary Material. These models were the basis for the estimation of the outcomes of interest by means of different competing risks analyses. Two separate competing risks models were constructed for estimating cumulative incidences of focal and BM progression from alloSCT taking place either before or after DLI with death without focal and BM progression, respectively, as a competing event. Statistical tests were developed to compare the cumulative incidences of focal and BM progression within one group at different points in time (see Supplementary Material for details).

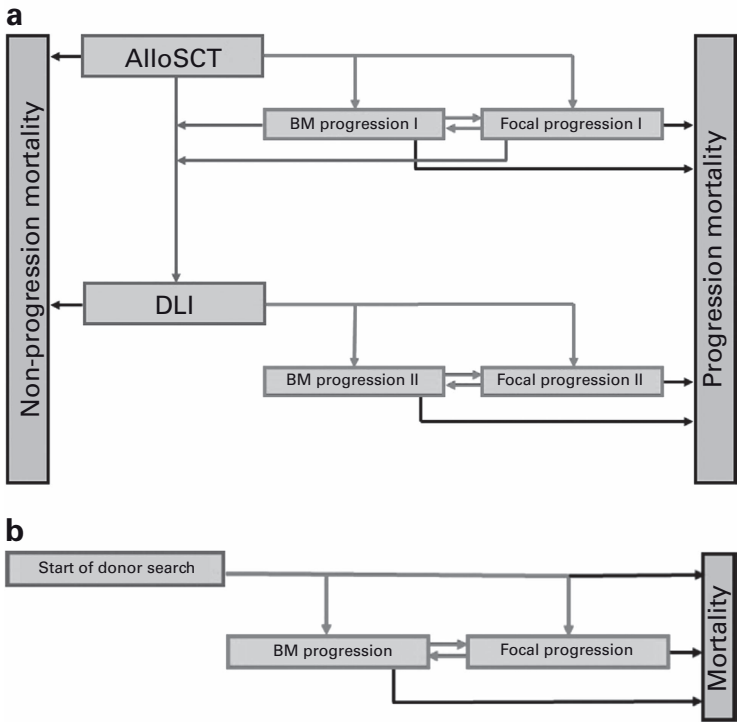

Figure 1 Analysis of progression patterns. (**a**) A model for the analysis of progression patterns after AlloSCT and after DLI. The starting state of all patients in this model was the day of infusion of the donor stem cell graft (alloSCT). All events after alloSCT were recorded at first occurrence only, therefore each patient could only make each transition once and could not return to a state previously visited. The events after alloSCT were development of focal progression (focal progression I), diffuse bone marrow infiltration (BM progression I), as well as the first donor lymphocyte infusion (DLI). Focal and BM progression after DLI were indicated by the states Focal progression II and BM progression II, respectively. The arrows between the two progression states indicate that patients could show BM after focal progression and *vice versa*. Events which are considered to be associated with a favorable outcome are depicted by a green box, myeloma-related events by a yellow box. Transitions to favorable and unfavorable events are depicted by green and red arrows, respectively. The endpoints non-progression mortality and progression mortality are depicted by a gray box. (**b**) A model for the analysis of progression patterns after start of donor search in the donor-search-no-alloSCT cohort. The starting state of all patients in this model was the day of start of donor search. The events after start of search were development of focal progression (focal progression), and BM progression (BM progression). The clinical endpoint Mortality is depicted by a gray box. No distinction was made between death with and without progression. A full colour version of this figure is available at the *Bone Marrow Transplantation* journal online.

RESULTS

Donor-search-no-alloSCT cohort

Baseline characteristics of the donor-search-no-alloSCT cohort are presented in Table 1. Reasons for not performing an alloSCT were: no suitable donor found (n = 6), patient decision (n = 3), poor WHO status (n = 2) and rapidly progressive disease (n = 1). The cumulative incidences of BM and focal progression at 2 years after start of donor search were 67% (95% CI 40–93%), and 50% (95% CI 22–78%), respectively (Table 2; Figure 2a). The probability of BM PFS, and focal PFS at 2 years after start of donor search were both 17% (95% CI 0–38%). Estimated 2-year overall survival since donor search was 42% (95% CI 14–70%). In conclusion, BM progression was observed frequently in patients for whom alloSCT was scheduled, but not performed.

Table 1 Baseline characteristics

Variable	Categories	N (%)/ Median (range)	
		AlloSCT cohort (n = 43)	Donor-search-no-alloSCT cohort (n = 12)
M-protein	IgG	25 (58%)	7 (58%)
	IgA	3 (7%)	3 (25%)
	IgD	3 (7%)	0
	LCD	12 (28%)	2 (17%)
Sex	Male	24 (56%)	8 (67%)
	Female	19 (44%)	4 (33%)
Age at alloSCT/start donor search	Years (range)	53 (36–69)	55 (38–67)
Time diagnosis to alloSCT/start donor search	Years (range)	2.3 (0.6–17.9)	2.9 (0.4–6.5)
Focal disease before alloSCT/start donor search	Yes	39 (91%)	12 (100%)
	No	4 (9%)	
Treatment lines prior to alloSCT/start donor search	Median (range)	2 (1–5)	2 (1–4)
Tandem autoSCT/alloSCT scheduled	Yes	13 (30%)	4 (33%)
	No	30 (70%)	8 (67%)
Thalidomide/bortezomib/lenalidomide before alloSCT/ start donor search	Yes	32 (74%)	9 (75%)
	No	11 (26%)	3 (25%)
AutoSCT before alloSCT/start donor search	Yes	42 (97%)	10 (84%)
	No	1 (3%)	2 (16%)

Table 1 Baseline characteristics (*continued*)

Variable	Categories	N (%)/ Median (range)	
		AlloSCT cohort (n = 43)	Donor-search-no-alloSCT cohort (n = 12)
In vivo T-cell depletion	Horse-derived ATG	25 (58%)	NA
	Alemtuzumab	16 (37%)	NA
	Alemtuzumab + rabbit-derived ATG	2 (5%)	NA
Donor type	Sibling donor	21 (49%)	NA
	Unrelated donor	22 (51%)	NA
Stem cell source	Peripheral blood-collected stem cells	43 (100%)	NA
In vitro T-cell depletion	Alemtuzumab 'in the bag'	43 (100%)	NA

Abbreviations: AlloSCT =allogeneic stem cell transplantation; ATG =anti-T lymphocyte globulin; autoSCT =autologous stem cell transplantation; LCD= light chain disease; NA= not applicable.

Table 2 Outcomes for two cohorts of patients

Cohort		Donor-search-no-alloSCT cohort	AlloSCT cohort	
	Starting time	start donor search	alloSCT	DLI
Outcome	*Time since start*			
BM progression	12 months	58% (30–86%)	26% (13–39%)	14% (1–26%)
	24 months	67% (40–93%)	30% (17–44%)	17% (3–31%)
	60 months	—	35% (21–49%)	21% (6–35%)
BM PFS	12 months	25% (0–50%)	60% (46–75%)	79% (65–94%)
	24 months	17% (0–38%)	51% (36–66%)	62% (44–80%)
	60 months	—	33% (19–47%)	48% (30–66%)
Focal progression	12 months	33% (7–60%)	23% (11–36%)	48% (30–66%)
	24 months	50% (22–78%)	47% (32–61%)	62% (44–80%)
	60 months	—	56% (41–71%)	69% (52–86%)
Focal PFS	12 months	42% (14–70%)	63% (48–77%)	45% (27–63%)
	24 months	17% (0–38%)	30% (17–44%)	28% (11–44%)
	60 months	—	21% (9–33%)	21% (6–35%)

Abbreviations: alloSCT = allogeneic stem cell transplantation; BM = bone marrow; DLI = donor lymphocyte infusion. BM progression was analyzed in a competing risks model in which death before BM progression was considered as a competing event. Therefore, at each time point the sum of the probability to be alive without BM progression (BM PFS) and the cumulative incidences of BM progression and death before BM progression must add up to 100%. At the start time point, the two cumulative incidences are 0. Analogous statements hold for focal progression. Outcomes are given at 12, 24 and 60 months after the respective start points. Outcomes at 60 months after start of donor search are not shown because of the small number of patients at risk beyond 24 months.

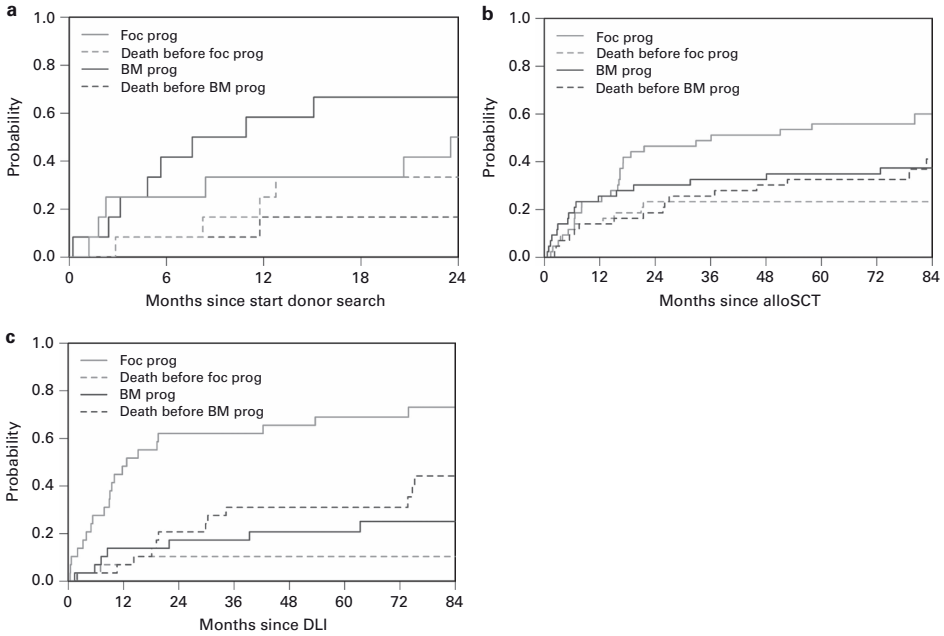

Figure 2 Competing risks analyses. (**a**) Competing risks analysis for the donor-search-no-alloSCT cohort. Cumulative incidences of focal and BM progression since start of donor search (red and blue solid lines, respectively). Death before focal and BM progression, respectively, was taken as competing event (red and blue dashed lines, respectively). (**b**) Cumulative incidences of focal and BM progression since alloSCT (red solid line and blue solid line, respectively). Death before focal and BM progression, respectively, was taken as competing event. (**c**) Cumulative incidences of focal and BM progression after DLI (red and blue solid lines, respectively). Death before focal and BM progression, respectively, was taken as a competing event. A full colour version of this figure is available at the *Bone Marrow Transplantation* journal online.

Outcomes after alloSCT

Baseline characteristics of the alloSCT cohort are presented in Table 1. Patients of the two cohorts had similar risk characteristics, and prior treatment lines, but patients of the donor-search-no-allo were slightly older at the time of search. After alloSCT, one patient did not engraft and rejection was observed in one patient. Overall, six of 41 patients (15%) with persisting engraftment required systemic immunosuppression for treatment of GvHD after engraftment.

Median follow-up of patients alive at last follow-up was 96 months (range 65–117 months). Estimated overall survival since alloSCT of the entire cohort was 60% (95% CI 46–75%, Figure 3) at 2 years and 32% (95% CI 16–47%) at 7 years (see Supplementary Material for details).

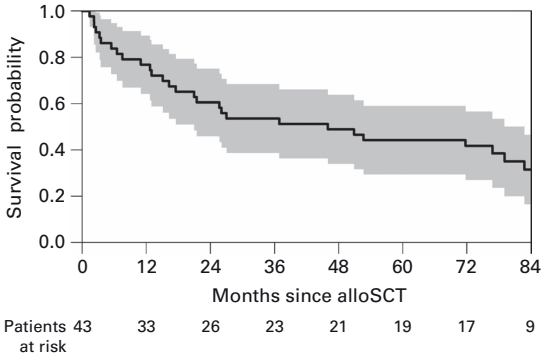

Figure 3 Kaplan–Meier curve. Overall survival since allogeneic stem cell transplantation (alloSCT) of all patients.

Overall survival of patients transplanted with sibling donors or unrelated donor were similar (P = 0.67). In addition, overall survival of patients who underwent up-front tandem auto-/alloSCT did not differ significantly from patients who underwent alloSCT after relapse (P = 0.38).

DLI

In total, 29 patients received DLI (n = 15 sibling donor, n=14unrelated donor). See Supplementary Files for details of not receiving DLI. Median time to first DLI was 258 days (range 132– 560 days). At 12 and 24 months after alloSCT the probability of having received DLI was 56% (95% CI 41–71%), and 67% (95% CI 53–82%), respectively (see Figure 4). Overall, 11 of 29 patients (38%) required systemic immunosuppression for tapering of GvHD after DLI, of whom two patients died due to GvHD-related complications (see Supplementary Material for details).

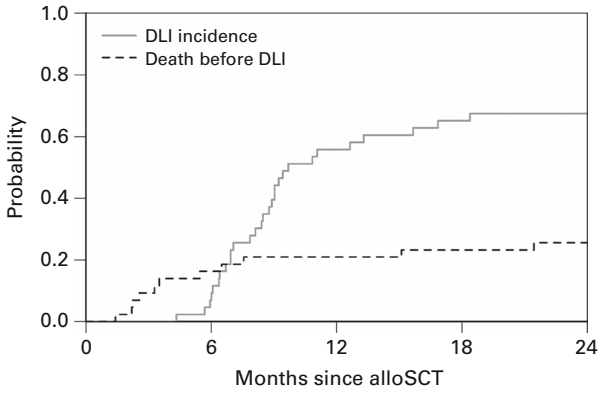

Figure 4 Incidence of DLI. Cumulative administration of DLI (green solid line) since allogeneic stem cell transplantation (alloSCT), with death before DLI (dashed line) as a competing event. A full colour version of this figure is available at the *Bone Marrow Transplantation* journal online.

BM and focal progression after alloSCT

Cumulative incidences of BM progression at 24 and 60 months post-transplant were 30% (95% CI 17–44%) and 35% (95% CI 21–49), respectively (Table 2; Figure 2b). Cumulative incidences of focal progression at 24 and 60 months post transplant were 47% (95% CI 32–61%) and 56% (95% CI 41–71%), respectively. When testing whether both types of progression occurred with the same frequency, it was found that the significance of the difference tended to increase over time (e.g., a borderline significant difference at 2 years after alloSCT ($P = 0.08$)).

The probabilities of BM PFS at 24 and 60 months post transplant were 51% (95% CI 36–66%), and 33% (95% CI 19–47%) respectively. The probabilities of focal PFS at 24 and 60 months post transplant were 30% (95% CI 17–44%), and 21% (95% CI 9–33%) respectively.

Disappearance of BM disease after DLI

In seven patients, at least one DLI was administered for BM progression. After DLI, six of these seven patients (86%) converted to full-donor hematopoiesis with disappearance of myeloma cells in BM. One patient failed to show a response and died.

Cumulative incidences of BM progression at 24 and 60 months post-DLI were 17% (95% CI 3–31%) and 21% (95% CI 6–35%), respectively (Table 2; Figure 2c). Cumulative incidences of focal progression at 24 and 60 months post-DLI were 62% (95% CI 44–80%) and 69% (95% CI 52–86%), respectively. A test comparing the incidence of focal progression to that of BM progression, both after DLI, yielded significant differences from 4 months after DLI onward (e.g., *P*o0.001 at 24 months).

The probabilities of BM PFS at 24 and 60 months post-DLI were 62% (95% CI 44–80%), and 48% (95% CI 30–66%) respectively. The probabilities of focal PFS at 24 and 60 months post DLI were 28% (95% CI 11–44%), and 21% (95% CI 6–35%), respectively.

Finally, similar focal progression patterns were observed in patients without GvHD after DLI as compared with patients with severe GvHD after DLI (see Supplementary File for details).

DISCUSSION

Here we present the first systematic analysis of differential progression patterns in patients with MM following RIC alloSCT and DLI. Due to close follow-up, detailed long-term follow-up data were available after alloSCT and DLI, which facilitated assessment of BM and focal progression as separate outcomes.

Firstly, TCD effectively reduced occurrence of severe GvHD early after alloSCT. Therefore, DLI could be administered in most patients. DLI resulted in long-term use of

systemic immunosuppression in only 7% of patients, and only two patients died from non-progression mortality after DLI (median follow-up 7 years). Secondly, DLI induced BM remission in almost all patients with BM progression prior to DLI. Furthermore, BM progression occurred in fewer than 20% of patients at 2 years after DLI. In contrast, during follow-up focal progression was observed in the majority of patients (62% at 2 years after DLI). Therefore, DLI appears to be highly effective in controlling myeloma infiltration in BM, but lack of focal disease control restricts cure to a minority of patients. The estimated OS at 2-year was 60% and 32% at 7 years, corresponding to previous reports.[4,25]

This study was not designed as a randomized trial. As controls, we therefore selected a cohort of patients with a similar intention-to-treat as our alloSCT cohort, i.e., patients who were referred to our hospital for a donor search, but did not undergo alloSCT. The low number of patients in this group reflects that that it was possible to give the intended treatment to the large majority of eligible patients. The main reason for not performing an alloSCT was lack of a suitable donor. Myeloma progression patterns were as expected with active BM disease in most patients.[34] The probability of BM PFS at 2 years after start of donor search was low (17%) as compared with 2 years after alloSCT (51%) and especially after DLI (62%). In contrast to the alloSCT cohort, in the control cohort BM examinations were performed at the discretion of the treating physician, and we chose a higher threshold to detect clinically relevant disease only, which may have even contributed to potential underreporting of BM progression. Thus, BM progression occurs frequently in patients for whom alloSCT was scheduled, but not performed.

Incidence of focal progression was more similar for both groups (50% versus 47% at 2 years for the donor-search and alloSCT cohort, respectively). However, the probability of focal PFS was higher in the alloSCT cohort (30% versus 17% at 2 years), implying that among patients alive, relatively fewer had a focal progression in this cohort, suggesting restricted efficacy of alloSCT against focal progression. The large and significant difference between focal and BM progression after DLI supports the interpretation that absence of BM progression results in a longer survival, thereby giving the opportunity to develop focal progression.

Myeloma cells are responsive to immunotherapy, which is supported by the frequent responses after DLI in patients with residual BM disease in our cohort.[30] Correspondingly, conversion to full-donor chimerism following DLI is associated with disease response in patients with MM.[12] Several groups tried to enhance this effect by combining DLI with immune modulatory drugs, such as thalidomide, bortezomib, and lenalidomide.[35–37] This resulted in partial and complete remissions, but cure was only achieved in a minority of patients. Also, repeated DLI does not result in new long-term remissions, especially not in the extramedullary compartment.[24,38]

In patients responding to DLI, polyclonal immune responses targeting allo-antigens have been demonstrated.[39] In particular, if hematopoiesis associated polymorphic antigens including minor histocompatibility antigens are targeted, GvM responses will occur.[39] In general, memory T-cells show preferential homing towards the BM, and therefore in the BM environment strong immune responses are induced.[40]

Our study demonstrates that despite effective immune responses after DLI, leading to disease control in the BM, the majority of patients ultimately showed progressive focal disease. Correspondingly, due to a significant incidence of extramedullary disease, repeated chimerism measurements have limited impact on the management of MM after T-cell repleted alloSCT.[41] In general, the strength of an allo-immune response appears to correlate with occurrence of GvHD.[42] However, a strong immune response does not prevent disease progression and is not associated with prolonged disease remission.[43] These observations are especially important in a context where MM is used as a model for the process of metastasis,[44] because they pinpoint the biological difference of immunological behavior of donor T-cells inside and outside the BM.

The question remains why the sensitivity of myeloma cells for immunotherapy seems to be restricted to BM.[24] Several factors may have a role. For instance, the tumor cells in focal lesions may selectively downregulate expression of tumor antigens. This type of immune evasion may be overcome by induction of a local danger signal, for instance by a combination of radiotherapy and administration of interferon, which stimulates T-cell responses.[45,46] Secondly, tumor-infiltrating donor T-cells may be capable of recognizing myeloma cells in focal lesions, but their function can be inhibited by myeloma cells and their microenvironment.[47] For the communication of myeloma cells and with their microenvironment, immune checkpoint pathways have an important role.[48] Therapeutic blockade of these pathways has become a paradigm-shifting treatment in solid tumors and more recently in hematologic malignancies, including myeloma.[49] For instance, ipilimumab can restore anti-tumor reactivity through a graft-versus-tumor effect, including relapsed extramedullary leukemia.[50] Combining DLI with immune checkpoint pathway inhibitors is an interesting strategy for future studies targeting both BM and focal progression of myeloma.

In conclusion, our study shows that T-cell therapy in the context of alloSCT modifies the natural course of myeloma and specifically appears to be highly effective in controlling BM infiltration. However, only a minority of patients will be cured by the potent GvM effect from DLI due to the limited effect on focal lesions. However, the benefit of long-term absence of diffuse BM infiltration after DLI may form the basis for clinical trials where T-cell therapy is augmented by treatment modalities which preferentially target focal myeloma progression.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

This work was supported by research grant 2008-4263 from the Dutch Cancer Society, Amsterdam, The Netherlands. We thank Illyea Hawke (DKMS, Germany) for correcting the language of the manuscript.

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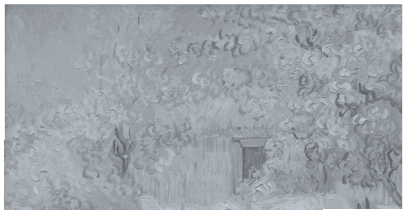

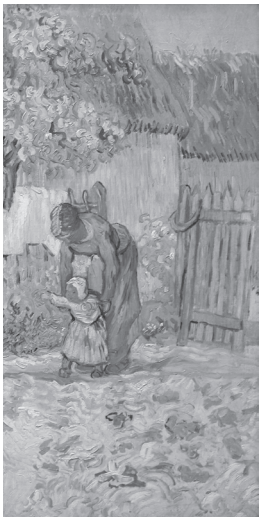

Chapter 5

Supplementary material

METHODS

Donor-search-no-alloSCT cohort

This cohort was analysed to investigate the course of the disease in a patient group similar to the alloSCT cohort who did not receive an allogeneic intervention. We assumed that the outcomes of the alloSCT cohort would have been largely similar to those of this group if the former had not been transplanted. In this cohort, BM evaluations and radiological evaluations were performed at the discretion of the treating physician. The first occurrences of BM and focal progression after start of donor search were analysed separately.

Radiology

MRI and PET-CT were only scarcely performed at LUMC between May 2002 and July 2010.

AlloSCT protocol

Patients with relapsed MM were eligible for alloSCT if the tumor was responsive to drug therapy and a HLA-matched (9/10 or 10/10) related or unrelated donor was available. From 2002, the RIC regimen consisted of fludarabine 30 mg/m^2 i.v. or 50 mg/m^2 orally for 6 days, busulfan 3.2 mg/kg i.v. for 2 days, and horse-derived anti-T lymphocyte globulin (Genzyme) 10 mg/kg/day i.v. for 4 days (n=25). After anti-T-cell globulin was withdrawn from the market in 2008, subsequent patients received alemtuzumab 15 mg i.v. for 2 days (n=16). From January 2010, patients with an unrelated donor received alemtuzumab 15 mg i.v. for 2 days with rabbit-derived anti-T lymphocyte globulin (Sanofi) 1mg/kg/day i.v. for 1 day (n=2). In vitro T-cell depletion (TCD) of the stem cell product was performed for all patients by incubation with 20 mg alemtuzumab for 30 min at room temperature under continuous agitation.

Due to ex-vivo TCD by alemtuzumab patients did not require additional GvHD-prophylaxis.

Follow-up after alloSCT

Routine follow-up was performed every 3 months during the first 2 years after alloSCT and additionally at 6 weeks after DLI, and included measurements of M-protein levels in the serum and a BM aspirate with morphological examination, immunophenotyping by flow cytometry, and molecular measurement of chimerism with a sensitivity of 1%. Radiological examinations of the skeleton were performed regularly at a minimum of one-year intervals in the absence of clinical disease progression. Upon clinical or laboratory signs of disease progression, additional BM examination and radiological assessment were performed.

Engraftment, rejection, GvHD, and chimerism measurements

The day of granulocyte engraftment was defined as the first of 3 consecutive days of absolute granulocyte counts > 0.5 x 10^9/L. Rejection was defined as a sudden onset of pancytopenia with loss of donor chimerism. Assessment and grading of GvHD was performed using modified Glucksberg and Shulman criteria. In addition, GvHD was categorized as GvHD not requiring systemic immunosuppresion, and GvHD requiring systemic immunosuppression. BM chimerism was determined every 3 months during the first 2 years after alloSCT, and additionally at 6 weeks after DLI. In sex-matched patient-donor pairs, chimerism was determined on total BM leukocytes, and mononuclear cell or granulocyte fractions after ficoll separation, using a short-tandem-repeat-PCR based protocol. In sex-mismatched patient-donor pairs FISH analysis was performed on unseparated BM leukocytes until 2007. Thereafter, chimerism analysis was performed analogous to sex-matched pairs. Mixed-chimerism was defined as ≥1% patient cells in any cell fraction.

Administration of DLI

The first pre-emptive DLI-dose was 3.0 x 10^6 $CD3^+$ cells/kg and 1.5 x 10^6 $CD3^+$ cells/kg for patients with a sibling and an unrelated donor, respectively. In the case of active disease, the first DLI-dose was 5.0 x 10^6 $CD3^+$ cells/kg and 2.5 x 10^6 $CD3^+$ cells/kg for patients with a sibling and an unrelated donor, respectively. Escalating doses of DLI were administered in case of failure to convert to full donor hematopoiesis and/or with disease progression.

Treatment strategies for progressive disease

Treatment of progressive disease depended on myeloma treatment prior to disease progression, time after transplant, presence of GvHD requiring immunosuppression, disease burden, and disease localization. In general, single focal lesions were treated with local radiotherapy, followed by DLI. Limited BM disease, defined as <30% malignant plasma cells by BM morphology, was treated with DLI without prior cytoreductive treatment. If no acute GvHD was observed within 3 weeks after DLI, interferon-α was administered subcutaneously in a daily dose of 3 million units until GvHD occurred. Escalating doses of DLI were administered in case of failure to develop acute GvHD at 3 months after start of treatment. In general, multiple focal lesions and/or severe BM disease were treated with 3 to 6 cycles of bortezomib 1.3 mg/m^2 at days +1, +4, +8 and +11, followed by DLI and interferon-α as described above. Other regimens, including lenalidomide or melphalan-containing schedules, were prescribed in case of absent disease response to bortezomib. Finally, patients with symptomatic bone disease received bisphosphonates as supportive treatment.

The multi-state models

All patients in the AlloSCT cohort started in the state 'AlloSCT' at the day of infusion of stem-cell graft. The states (named after the events that made the patients enter the state) that could be reached directly from 'alloSCT' were: 'Non-progression mortality', development of focal progression ('Focal progression I'), BM progression ('BM progression I'), and administration of first DLI ('DLI'). Patients who received more than one DLI before a new progression was observed remained in the DLI state, since these subsequent DLIs were considered part of the same intervention strategy. After DLI, transitions into the states 'Focal progression II', and 'BM progression II' were added to the model to assess the impact of DLI on subsequent outcomes. Only the first occurrences of focal and BM progression after alloSCT, and again after DLI, were included in the model. Final endpoints of the analyses were 'Progression mortality' and 'Non-progression mortality'. 'Non-progression mortality' indicates death directly taking place after alloSCT or DLI without signs of progression, in analogy to the common term 'non-relapse mortality'. Transition into 'Progression mortality' was only possible via any of the progressive disease states, whereas a direct transition into 'Non-progression mortality' was possible from the states 'AlloSCT' and 'DLI' only.

All patients of the donor-search-no-AlloSCT cohort started in the state 'Start donor search' at the day of start of donor search.

Competing risks and multi-state models

The cumulative incidence of DLI was estimated in a competing risks framework, considering death before DLI as a competing even.

Focal progression-free survival indicates the probability to be alive without focal progression, and is calculated as 1 minus cumulative incidence of focal progression minus cumulative incidence of death without focal progression. BM progression-free survival is defined analogously. Similar models as those describing outcomes after alloSCT were constructed to estimate disease progression after start of donor search and post-DLI.

Confidence intervals for the cumulative incidences of BM disease/focal progression and for the probabilities to be alive without BM/focal progression (BM/focal progression-free survival) were calculated by analysing the data in a multi-state framework, with states alive progression-free, progression and death before progression, by means of the 'mstate' package in R.(de Wreede, *et al* 2010, de Wreede, *et al* 2011)

We developed tests for comparing two different competing risks outcomes that are not each other's competing risk. The tests can be used in other frameworks as well, but will be illustrated here for the case where the cumulative incidence of BM progression (with competing event death before BM progression) is compared to that of focal progression (with competing event death before focal progression). A multi-state model was created in which both competing events of interest were incorporated (see Figure

1s). The Cumulative Incidence of focal progression can be expressed as the sum of the transition probabilities from state 1 to states 2, 4, 6 and 8 and analogously for the Cumulative Incidence of BM progression: from state 1 to states 3, 4, 7 and 8. For testing the hypothesis of no difference between those two cumulative incidences at each point in time, the estimated difference between them (transition probabilities from state 1 to 2 and 6 minus transition probabilities from state 1 to 3 and 7) and the variances of and covariances between its different elements have to be evaluated in order to build a local test statistic that follows a Chi-square distribution with 1 degree of freedom (see Andersen *et al* 1993 for the underlying methods). All necessary elements were calculated in 'mstate'.

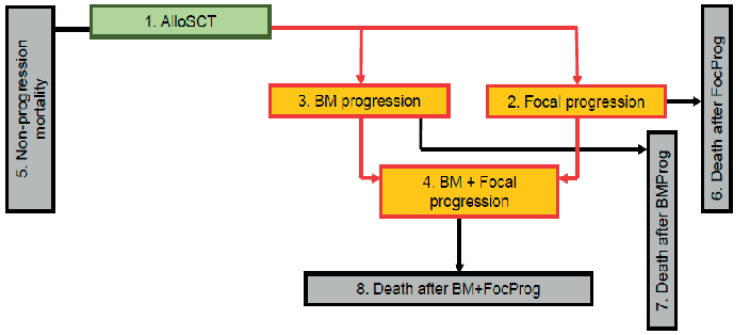

Figure 1s

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RESULTS

Outcomes after alloSCT

After alloSCT, one patient did not engraft and subsequently died from a fungal infection (day +43). The remaining 42 patients showed a median time to granulocyte engraftment of 14 days (range 1-25 days). Rejection was observed in one patient (day +37).

Development of acute GvHD during the first 6 months after alloSCT was assessed in all 41 patients with persisting engraftment. Twenty-eight patients (68%) did not show signs of acute GvHD, eight patients (20%) experienced grade 1-2 acute GvHD, and five patients (12%) experienced grade 3-4 acute GvHD.

During follow-up, nine patients died from non-progression mortality: five GvHD-related complications (including two patients who developed GVHD after DLI), three infections, and a (patient-derived) acute lymphoblastic leukemia shortly after alloSCT in one patient. Twenty-one patients died after progression.

Reasons for not receiving DLI

Fourteen of 43 patients (33%) did not receive DLI during follow-up. Seven of these 14 patients died due to non-progression mortality. Four patients died of progressive disease before DLI could be administered. Three patients did not experience any event after alloSCT, i.e., neither disease progression, nor DLI, nor death (see below).

Median number of DLIs

The median number of DLIs per patient was 3 (range 1-6). Median first DLI-dose was 4.9 x 10^6 $CD3^+$-cells/kg (range 1.0-7.1 x 10^6 CD3+cells/kg) and 2.0 x 10^6 $CD3^+$-cells/kg (range 1.5-5.0 x 10^6 CD3+cells/kg) for patients with a sibling and an unrelated donor, respectively.

Severe GvHD after DLI

Within the first 3 months after DLI, 17 patients (59%) did not develop acute GvHD, 8 patients (28%) developed grade 1-2 acute GvHD, and 4 patients (14%) developed grade 3-4 acute GvHD. Overall, 11 of 29 patients (38%) required systemic immunosuppression for tapering of GvHD after DLI, of whom two patients died due to GvHD-related complications. Another patient died of progressive disease, but also required systemic immunosuppression at time of death. In seven of the remaining eight patients, systemic immunosuppression could be stopped within one year after DLI.

Patients without event after alloSCT and post-DLI

Follow-up times since alloSCT of patients without event after alloSCT were 65, 74, and 78 months (n=2 sibling donor, n=1 unrelated donor; n=2 male patient/female donor). All

three patients developed chronic GvHD after alloSCT (two patients required treatment with systemic immunosuppression).

Five patients (17%) did not experience an event post-DLI, i.e., no disease progression, or death. Follow-up times since alloSCT of these five patients were 75, 96, 98, 103, and 108 months, respectively (n=2 sibling donor, n=3 unrelated donor; n=1 male patient/female donor). Post-DLI, three of these five patients developed GvHD requiring treatment with systemic immunosuppression (including two patients who developed isolated allo-immune phenomena as symptom of GvHD only: pure red cell aplasia and ITP in one patient, and agranulocytosis in another). The remaining two patients did not develop signs of GvHD post-DLI.

Severe GvHD after DLI does not prohibit focal progression

To assess whether a strong immune response prevents focal progression, we analysed the relationship between GvHD after first DLI and focal progression in the 29 patients who received at least one DLI. These patients were divided into three groups: no signs of GvHD after DLI (no GvHD), GvHD after DLI but no systemic immunosuppression required (limited GvHD), GvHD after DLI and systemic immunosuppression required (severe GvHD).

Thirteen patients did not develop GvHD after DLI: one showed no progression at all until end of follow-up, one died due to early BM progression, and eleven (85%) showed focal progression during follow-up. A group of five patients developed limited GvHD after DLI: one showed no progression until end of follow-up, and four (80%) showed focal progression after DLI. Finally, eleven patients developed severe GvHD after DLI: five showed no progression after DLI (of whom two suffered non-progression mortality), and six (55%) showed focal progression during follow-up. Figure 2s illustrates a typical example of a patient with persisting BM remission but ongoing focal progression despite numerous treatment modalities and occurrence of (severe) GvHD.

In conclusion, the incidence of focal progression appeared only moderately reduced, but at the cost of non-progression mortality, in patients with severe GvHD after DLI.

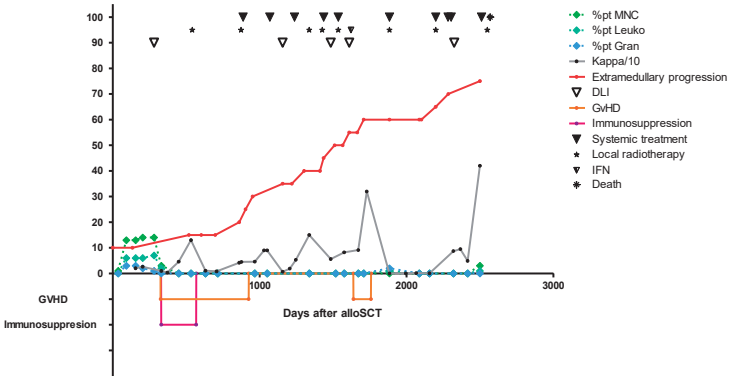
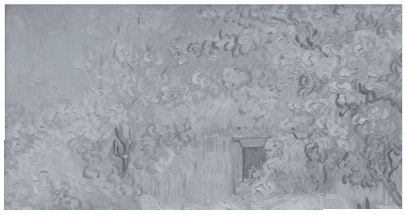
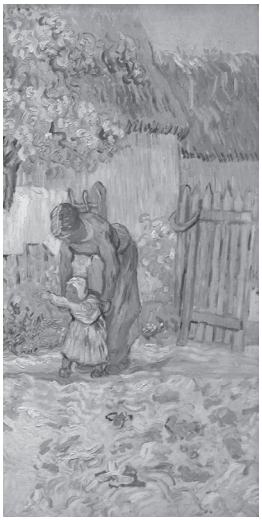


Figure 2s Clinical outcomes of an individual patient.
The x-axis shows days after allogeneic transplantation. The y-axis shows all measurements, observations, and interventions. For instance, the red solid line indicates occurrence of focal progression (the line increases with 5 points at every progression). The dashed lines indicate percentages of patient fraction in mononuclear cells (MNC, green), leukocytes (Leuko, turquoise), and granulocytes (Gran, blue) respectively. The solid grey line indicates absolute M-protein levels (kappa g/L times 0.1 for illustrative purposes). The solid orange and purple lines indicate presence of GvHD and treatment with systemic immunosuppression for GvHD, respectively. Symbols indicate different events: introduction of DLI (open triangle), systemic anti-myeloma treatment (closed triangle), local radiotherapy (star), administration of interferon-alpha (black-and-white triangle) and time of death (asterisk). After DLI, the patient fraction of MNC (and other cell fractions) decreases to zero percent until the end of follow-up, indicating persisting BM remission. However, despite development of severe GvHD and numerous treatment modalities hereafter, the solid red line--indicating focal progression--continues to increase, illustrating lack of myeloma control of focal disease.





Chapter 6

Summary and general discussion

1. Summary

Transfer of donor T-cells to patients after allogeneic stem cel transplantation (alloSCT) has demonstrated its clinical potential in several studies and led to persisting remissions in patients with high-risk and/or relapsed hematologic diseases. Many factors influence outcome. These may be disease-related, like remission status at the time of transplant or T-cell infusion. Donor-recipient-related factors that influence outcome include genetic disparity (HLA-matching), age, (ongoing) viral infections at time of T cell infusion, and stem cell source. The potential of donor T-cells to recognize and eradicate the malignant cell population also plays an important role. Together, these factors influence risk of relapse, morbidity and mortality of T cell infusion.

T-cells play a key role in the immune responses after transplant. T-cells are designed to control viral infections by recognition of viral peptides. These peptides are presented to T-cells in the context of HLA-molecules on the cell surface of infected cells or professional APCs. In essence, a huge repertoire of T-cells recognizing broadly diverse (viral) peptides is available. To prevent development of auto-immune diseases T-cells recognizing self-peptides are deleted by thymic selection. Administration of donor T-cells that are harvested directly from the donor is called donor lymphocyte infusion (DLI). These T-cells may recognize patient-derived peptides in the context of (matched) self-HLA. Professional APCs are cells of hematologic origin, that express high levels of HLA and co-stimulatory molecules and play an essential role in the initiation of T cell responses. They are able to present endogenous and exogenous peptides. Since many peptides are coexpressed by APC and other cells of hematopoietic origin, hematopoietic cells are preferentially recognized after donor T cell infusion. Recognition of patient-hematopoiesis including the malignant clone after alloSCT and DLI may result in a beneficial GvL-response. Recognition of all other host tissues may result in detrimental GvHD.

The balance between GvL and GvHD is influenced by many factors like the quantity and specificity of donor T-cells, the number and origin of APCs involved in the immune response, but also the inflammatory circumstances during which the T-cells are administered. Several of these factors may be manipulated to skew the balance from GvHD to GvL.

Effective T-cell depletion (TCD) prevents induction of GvHD and allows restoration of damaged tissues after conditioning treatment. At Leiden University Medical Center, *ex vivo* TCD by incubation of the stem cell graft with 20 mg of alemtuzumab for 30 minutes is applied to prevent GVHD. In most patients no prophylactic immunosuppression is required. Infections have to be controlled to limit development of an inflammatory environment. A longer time interval between infusion of stem cells and DLI is associated with a reduced chance of GvHD, and allows infusion of larger quantities of T-cells. This increases the chance to infuse sufficient numbers of T-cells with high avidity to the malignant clone. Postponing administration of T-cells too long increases the relapse

rate. Each type of hematologic malignancy is associated with a different response to therapeutic DLI. Overt relapse of the underlying disease after transplantation reduces the responsiveness to DLI. Timely infusion of prophylactic DLI might improve outcome in a high-risk population by preventing relapse.

In this thesis, we aimed to study the effect of pre-scheduled DLI (in predefined doses) after TCD-alloSCT in several clinical situations. The therapeutic potential of DLI for relapse AML and myeloma progression was assessed.

In **chapter 2**, the safety and effectiveness of our strategy was studied in a cohort of adult patients with ALL. A cohort of 25 adult patients with ALL in first complete remission (CR1) underwent myeloablative TCD-alloSCT with early sequential prophylactic DLI. The majority of patients were diagnosed with high-risk ALL. Patients were eligible for administration of prophylactic DLI between 3-6 months after alloSCT when in continuous CR1 and absence of active GvHD. Patients with a matched sibling donor were scheduled for 3.0 x 10^6 $CD3^+$ cells/kg and patients with a mismatched sibling or an unrelated donor for 1.5 x 10^6 $CD3^+$ cells/kg, respectively, at 6 months after alloSCT. Prophylactic DLI was administered to 14 patients at a median of 185 days from alloSCT. Overall, only 17% of patients with a follow-up of more than 1 year required long-term systemic immunosuppression for GvHD. Three patients experienced a relapse of whom one patient with Philadelphia Chromosome (Ph-)positive ALL obtained CR2 after combination therapy with DLI, interferon-α and nilotinib. The median follow-up was more than 50 months. The 2-year survival probability was 68% (95% confidence interval (CI) 49-87%). There was no significant difference in survival between patients with a sibling donor or an unrelated donor. In our cohort, 2-year OS of Ph-positive ALL (which is considered tob e a very high-risk leukemia) was at least as good as for Ph-negative patients: 80% (95% CI 55-100%) and 60% (95% CI 35-85%), respectively (p=0.27). In conclusion, our single-center study in a predominantly high-risk adult ALL population indicates effective disease control with low low risk of NRM by myeloablative TCD-alloSCT in CR1 with early sequential DLI to prevent relapse.

In **chapter 3** we aimed to investigate the role of DLI for relapsed AML. The outcome of relapsed AML after alloSCT had remained poor, and DLI monotherapy was previously found not to result in long lasting remissions. To improve outcome we developed a strategy which aimed to induce an effective immune response by first applying leukemic cytoreduction followed by DLI during the lymphopenic phase, and additional treatment with interferon-α when no acute GvHD was observed within 3 weeks after DLI. The (time-dependent) impact of absence of GvHD, controllable GvHD (grade 1-3), and severe GvHD (grade 4) after DLI on the incidence of second relapse, OS and GvHD-related mortality were estimated in a cohort of 44 patients with relapsed AML. Initiation of cytoreductive therapy was not feasible in seven patients due to poor performance status (n=3) or severe GvHD (n=4). Reasons for not receiving DLI were chemotherapy-

related death (n=1) and chemotherapy-refractory disease (n=9). The 2-year OS after DLI was 36% (95% CI 16-57%). Development of grade 1-3, but not grade 4 GvHD was associated with superior survival as compared to absence of GvHD (hazard ratio 0.22, p=0.03). In conclusion, DLI plays an essential role in obtaining long-term remissions for relapsed AML. A significant number of patients who underwent effective cytoreduction followed by a rapid but controllable GvHD-response could be rescued by this treatment strategy.

In **chapter 4** we aimed to assess the safety and effectiveness of prophylactic DLI after myeloablative TCD-alloSCT for AML/high-risk MDS. The common approach in the field of alloSCT is to focus on outcome analyses from time of transplant to relapse or death. However, when assessing the impact of post-transplant interventions, conventional statistical methods do not suffice. We investigated whether a multi-state model would give insight into the events after transplantation. The study was performed in a cohort of patients in continuous CR1 in the absence of active GvHD requiring systemic immunosuppression who were planned to receive prophylactic DLI between 3-6 months after alloSCT to pevent relapse of the underlying disease. The aim of this study was to analyze the impact of DLI and consecutive GvHD on the probabilities of relapse and non-relapse mortality over time. We built a multi-state model assigning the day of stem cell infusion as starting state: 'SCT'. Patients who required immunosuppression (IS) for GvHD after transplant entered the state 'Start IS' and 'Stop IS' when immunosuppresion was started and stopped, respectively. Patients entered the state 'DLI' at the day DLI was administered. Transition into this state was possible from the states 'SCT' and 'Stop IS'. Hereafter, they could enter the states 'start IS after DLI' and 'stop IS after DLI', if immunosuppression for GvHD after DLI was started and stopped, respectively. The transition of patients into the different states over time was used to determine the impact of each event on NRM and relapse. Based on this model, an outcome measure was introduced, which we called 'treatment success', defined as being alive without relapse and immunosuppression for GvHD. We determined that both relapse and NRM were causes of failure of comparable magnitude in the entire cohort. Our results suggested that the risk of relapse, which was the major reason of failure in patients remaining in the state 'SCT', was reduced after GvHD, and especially after DLI. Only 11% of patients required systemic immunosuppression for GvHD. The long-term probability of 'treatment success' in the entire cohort was approximately 40%, and this probability was increased after DLI. Presence in the DLI state was associated with a probability of 70% of 'treatment succes' at 5 years after transplant. Our multi-state model illustrates that administration of prophylactic DLI appears to favorably affect outcome.

In **chapter** 5 we studied the impact of alloSCT and especially DLI on disease progression patterns of MM. AlloSCT can result in long-term disease control because immune-competent T-cells can exert a graft versus myeloma-effect, but still many patients relapse after alloSCT, and late NRM may occur. Extramedullary relapses are observed

frequently. We aimed to visualize the differences in distribution of bone marrow (BM) progression and new or progressive radiologic lesions, which we called focal progression, after transplant and especially DLI by means of multi-state modelling. A study cohort of 43 patients with MM who underwent TCD-RIC alloSCT with scheduled DLI was analyzed. For comparison, a cohort of 12 patients with MM for whom a donor search was started but no alloSCT was performed was analyzed(donor search cohort). The incidences of BM and focal progression after alloSCT in the study cohort and after the start of donor search in the donor search cohort were calculated as separate events. After DLI, the incidences of BM and focal progression were also calculated separately. By using a multi-state model we estimated the BM progression-free and focal progression-free survival, and determined the differences between the incidences of BM and focal progression. DLI appeared highly effective in clearing BM disease: 86% of evaluable patients showed complete disappearance of myeloma cells in BM. This observation corresponded with the higher probabilities of BM progression-free survival at 2 years after alloSCT (51%) and DLI (62%), as compared to non-transplanted patients (17%). The effect of alloSCT and alloSCT with DLI on focal progression was limited. The probabilities of focal progression-free survival at 2 years after alloSCT, DLI, and start of donor search were 30%, and 28%, and 17% respectively. In conclusion, our study showed that donor-derived T-cell responses effectively reduce BM infiltration in myeloma patients, but not focal progression, illustrating potent immunological responses in BM with only limited effect of T-cells on focal lesions.

2. General discussion

Conditioning therapy as well as timing and dosing of T-cell administration play an important role when determining the impact of the immune response after alloSCT. Myeloablative conditioning (MAC) has stronger anti-tumor effects as compared to reduced-intensity conditioning (RIC) and is associated with a lower relapse risk.[1] The impact of the conditioning therapy on the patient influences optimal timing and dosing of T-cell administration. Total body irradiation (TBI) and high-dose chemotherapy will result in severe tissue damage and thus promote pro-inflammatory circumstances. During T-cell replete alloSCT, the donor T-cells are administered directly after the conditioning regimen. The introduction of a high number of T-cells during these pro-inflammatory circumstances will result in a high incidence of severe GvHD. Therefore, only low quantities of T-cells can be administered in a T-cell replete setting if a low risk of acute GVHD is aimed. Prophylactic immunosuppression is essential under these circumstances and tapering of systemic immunosuppression is one of the few methods to guide the immune response. It is difficult to dissect the effect of donor T-cells from the effect of conditioning therapy and immunosuppression in this type of transplant. Delayed infusion of T-cells is called donor lymphocyte infusion (DLI). The high incidence of GvHD after T-cell

replete alloSCT restricts the use of DLI and makes DLI often redundant. Several methods are being applied to reduce the number of alloreactive T-cells in the graft. T-cells may be depleted *ex vivo* or *in vivo* for instance by antibody treatment or by post-transplant chemotherapy (T-cell depletion; TCD). Effective TCD results in limited post-transplant GvHD and creates an optimal setting to study the impact of DLI after alloSCT. Due to a low incidence of GvHD after TCD transplantation, DLI incidence may be high allowing interpretation of a direct clinical effect of DLI.

All studies in this thesis were performed in patients who underwent *ex vivo* TCD-alloSCT. TCD effectively reduced the incidence of severe GvHD after alloSCT.[2] In the vast majority of patients no immunosuppression was required to treat GvHD. A drawback of TCD without prescheduled DLI is delayed immunoreconstitution and increased relapse rate as compared to T-cell replete alloSCT.[3,4] To improve outcome of TCD-alloSCT the administration of donor T-cells was scheduled at predefined timepoints after alloSCT. We intented to infuse T-cells not too early to prevent development of severe GvHD, but also not too late in order to prevent unnecessary relapses. We demonstrated the safety and efficacy of pre-scheduled DLI after TCD-alloSCT with myeloablative conditioning (MAC) in a predominantly high-risk ALL population (**chapter 2**). We also demonstrated that DLI can induce long-term remission of relapsed AML after alloSCT if an immune response leading to manageable GvHD is induced (**chapter 3**). Furthermore, prophylactic DLI appears to be associated with a favorable outcome after MAC TCD-alloSCT for AML and high risk MDS (**chapter 4**). Finally, the effect of DLI appears to be stronger on diffuse BM disease than focal disease in patients with multiple myeloma (MM, **chapter 5**).

Prophylactic DLI – skewing from GvHD to GvL

The aim of prophylactic DLI is to selectively target and eradicate residual hematopoietic cells of the recipient, including the underlying malignancy.[5] The induction of an immune response depends on presentation of antigens by antigen-presenting cells (APCs).[6] Host-derived APCs will present self-peptides in the context of self-HLA. These may include peptides from hematopoietic and non-hematopoietic tissues. The conditioning regimen will result in tissue damage and a pro-inflammatory milieu. During inflammatory circumstances, the likelihood of cross-presentation of non-hematopoietic peptides is increased and infusion of T-cells will be associated with a high risk of severe GvHD.[7,8] Both amplitude (strength) and diversity of a GvHD-associated immune respons are high.[9] As compared to RIC, MAC is associated with more tissue damage, but probably also with lower quantities of residual host-derived hematopoiesis. This is supported by the observation of a higher rates of mixed-chimerism after RIC as compared to MAC.[10] Over time, tissue damage will be restored and host-derived APCs will be replaced by donor-derived APCs. Depending on the conditioning therapy, there will be high variation in the number and origin of APCs at different timepoints after alloSCT. Waiting until

all APCs are of donor origin decreases the occurrence of severe GvHD as well as the strength of the immune response.[5] However, the low risk of GVHD later after transplant makes it possible to administer higher doses of DLI, which increases the likelihood of including T-cells targeting hematopoietic tissues, which may result in a favorable GvL-response.[11]

Postponing DLI too long may result in an increased risk of relapse. The risk of relapse depends on the underlying disease. AML with adverse cytogenetic abnormalities, i.e. monosomal karyotype, is associated with a high risk of early relapse.[12] These relapses are often refractory to further treatment which results in a dismal prognosis.[13] Therefore, prophylactic DLI for a patient with high-risk AML without development of GVHD after alloSCT should not be postponed too long.

The indications for prophylactic DLI in the studies described in **chapters 2 and 4** were similar. In **chapter 2**, DLI was administered at approximately 6 months after MAC TCD-alloSCT for predominantly high-risk ALL. This strategy appeared successful: the relapse rate and overall rate of severe GvHD in this cohort were low. The overall survival was promising. However, an important limitation of this single-institution study was the small cohort size, which is associated with a relatively large convidence interval. In **chapter 4**, prophylactic DLI was administered between 3 to 6 months after alloSCT for AML or high-risk MDS in all patients without active GvHD or an early relapse. Presence and persistence in the DLI state was considered 'treatment success': indicating long-term survival without relapse or severe GvHD. The primary complication of DLI was the occurrence of GvHD, but only 11% of patients required immunosuppression for GvHD after DLI and virtually nobody required treatment for GvHD at one year after DLI.[16] Our study showed that the balance between the risk of relapse and the risk of severe GvHD can be effectively manipulated by timely administration of DLI during well-controlled circumstances, and supports the use of prophylactic DLI after TCD-alloSCT.

Manipulation of prophylactic DLI to potentiate early administration after alloSCT may favor the outcome of alloSCT. CD4-positive T-cells target antigens in HLA class II molecules, which are primarily expressed on cells from hematopoietic origin.[5] Administration of purified CD4-DLI instead of unmanipulated DLI during steady-state conditions early after transplant may help to skew from GvHD to GvL. This strategy is currently explored in a clinical randomized trial at our center. A downside of any manipulation of DLI is that it may als result in lower quantities of T-cells targeting the malignant clone.[17] Strategies that focus on sequential administration of purified CD4-DLI and unmanipulated DLI may overcome this limitation.

The best strategy to prevent overt relapse is treatment of an impending relapse by monitoring minimal residual disease. Detection of BCR-ABL by quantitative PCR after alloSCT for Ph+ALL should drive urgent administration of DLI.[15] This may be combined with a second or later generation TKI and IFN.[38] This strategy was successful to induce

long-term remission in a patient described in **chapter 2** and is supported by other studies.[25,38] In case of AML, a potential candidate of measuring minimal residual disease (MRD) is a quantitative real-time PCR assay to detect NPM1 type A mutations in NPM1 positive AML.[39] We studied the effect of DLI in a cohort of 13 NPM1A positive AML patients (not published). Quantitative monitoring of NPM1A proved more sensitive than chimerism analysis to predict overt relapse after alloSCT. Limitations of this strategy are the unknown duration of monitoring and the costs of highly frequent monitoring.

Therapeutic DLI – impact of disease state and GvHD

Unmanipulated DLI has shown effect in many patients with relapsed hematologic disease after alloSCT.[18,19] The efficacy of DLI is reduced with increased disease burden and higher proliferative rate of the underlying malignancy.[20] The outcome after DLI monotherapy for relapse of indolent diseases, like chronic leukemias or MM appears favorable as compared to acute leukemias.[16] As demonstrated in **chapter 5**, DLI appeared highly effective in clearing myeloma BM disease: more than 80% of evaluable patients showed complete disappearance of myeloma cells from the bone marrow compartment. In most patients with indolent diseases the effect of DLI monotherapy can be awaited before cytoreductive therapy has to be provided. However, especially in case of high disease burden, the disease response requires close monitoring and timely adaption of this strategy if necessary.

Relapse after or during severe GvHD is considered as primary failure of alloSCT.[5] This may reflect the occurrence of immune escape mechanisms underlying relapse.[21] Correspondingly, the effect of DLI is negligible in patients who delevop a relapse during or after prior severe GvHD.[20,22] Conversely, the response rate appears to be increased in patients who develop GvHD after DLI.[23] GvHD is associated with high diversity and amplitude of the immune response.[9] DLI monotherapy for relapsed acute leukemias is insufficient.[19,20] This led to the hypothesis in **chapter 3** that treatment of relapsed hematologic diseases with high proliferative rates after alloSCT requires rapid induction of a strong immune response after reaching minimal residual disease. The diversity of the immune response should be as large as possible to overcome the immune escape mechanisms of a relapsed malignancy. Thus, high doses of T-cells are likely to be required and development of limited GvHD may favor outcome.[16] In **chapter 3**, we combined several approaches to induce a strong and diverse immune response: effective cytoreduction, administration of high-doses of DLI during lymphodepletion and induction of inflammatory circumstances by IFN if necessary.[25] Lymphodepletion appears to stimulate expansion of infused lymphocytes by lymphopenia-induced homeostatic proliferation.[26] All patients with long survival after treatment for relapsed AML showed a similar pattern: their leukemias were halted with effective cytoreductive treatment to limit disease burden, and GvHD was rapidly induced by DLI with additional IFN if

necessary.[25] Using this strategy, we were able to rescue patients who did not experience GvHD prior to relapse, but also patients with limited GvHD prior to relapse. Our strategy was effective in several patients, but most patients without DLI-induced GvHD experienced second relapses, illustrating that development of new treatment options for relapsed AML after alloSCT are necessary. A promising cellular treatment option for relapsed disease after alloSCT may be the administration of in vitro selected cytotoxic T-lymphocytes (CTLs) with relative tumor specificity.[27] This strategy is already used in the setting of post-transplant lymphoproliferative disease (PTLD).[27] PTLD is a EBV-associated condition resulting in the occurrence of lymphomas in immunocompromised patients. CTLs targeting EBV-associated antigens can result in clearance of this disease.[29] The number of potential tumor-associated antigens that can be targeted is limited. Furthermore, downregulation of the tumor-specific antigen, presence of immunosuppressive cells, or secretion of inhibitory factors from the tumor microenvironment will limit its efficacy.

Another treatment option for relapse after alloSCT is the use of T-cells that express a chimeric antigen receptor (CAR T-cells).[30] The CAR is integrated into T-cells through lentiviral or retroviral transduction. The construct consist of 3 different domains: an extracellular monoclonal antibody-derived fragment that allows specific binding of the tumor-associated antigen; a transmembrane domain; and an intracellular T-cell signaling system. The latter triggers T-cell activation and killing. Unfortunately no effective CAR T-cell therapy is available to treat relapsed myeloid diseases.[31] This treatment option is primarily limited to B-cell malignancies. A benefit of CAR T-cells as compared to antigen specific T cells targeting the tumor by their T cell receptor, is that tumor lysis by CAR T-cells occurs independent of antigen processing or presentation by HLA molecules .[28,31] CAR T-cells can achieve high response rates and long-term antitumor activity by ongoing circulation of antigen-specific cells. The risk of GvHD is negligible.[31] However, the magnitude of immune responses of CAR T-cells is high. In this respect, it mimics the GvHD cascade: the immune response can lead to cytokine release syndrome (CRS), which is a potentially lethal complication of CAR T-cell therapy. CRS is associated with high-grade fever, hypotension, respiratory failure, and capillary leak syndrome. CRS usually responds well to corticosteroids and interleukin-6 blockade, which appears to play a prominent role in the pathophysiology of this adverse event.[32] Another major side effect is (transient) neurotoxicity, which frequently occurs and includes seizures and coma.

As compared to the targeting of multiple epitopes by DLI, an important limitation of any treatment modality targeting a single epitope, like CAR T-cells and CTLs, is antigen escape due to downregulation of the targeted antigen on the malignant cells.[5] This will result in relapse. To overcome this limitation alternative strategies like targeting of multiple epitopes are currently investigated. Finally, generation of CAR T-cells in autologous

setting is not always successful. Off-the-shelf thirth-party CAR T-cells, or alloSCT donor-generated CAR T-cells may help to overcome this limitation.

Therapeutic DLI – responses in different body compartments

In **chapter 5**, we showed that donor-derived T-cell responses effectively reduce BM infiltration, but not focal progression in myeloma. The limited effect of DLI on focal lesions cannot be explained by the absence of alloreactive T-cells as long-term BM remission was observed in the majority of patients.[33] Focal lesions could disappear when local radiotherapy (to introduce a local danger signal) was followed by DLI leading to local GvHD. Unfortunately, in general these responses proved transient and new lesions continued to appear. Thus, the efficacy of DLI appears to be lower when treating extramedullary disease as compared to bone marrow disease. This may implicate other T-cell based therapies, i.e. CAR T-cells, as well. The appearance of new focal lesions can be explained by the induction of immune escape mechanisms and associated T-cell exhaustion by the tumor microenvironment.[21] It has been shown that upregulation of checkpoint receptors such as CTLA-4 and PD-1 on donor-derived lymphocytes and high expression of cognate ligands on residual tumor cells or the microenvironment play a dominant role in hematologic relapse.[34,35] Therefore, checkpoint inhibitors blocking these proteins for treatment of relapse after alloSCT are increasingly used in clinical trials.[36] The use of checkpoint inhibition after alloSCT raises concern for difficult to control immune activation, i.e. GvHD. This was already shown in murine models studying PD-1 and CTLA-4 blockade. A study by Haverkos et al. demonstrated that PD-1 blockade for relapsed lymphoma after alloSCT was associated with treatment-associated GvHD in more than half of cases, but also resulted in a promising overall response rate of 77%.[37]

Statistical aspects of DLI analysis

The precise magnitude and impact of the allogeneic immune response after alloSCT on outcome is difficult to determine. Donor source (related, unrelated, matched or mismatched), graft source (BM or peripheral blood), and conditioning type each influence outcome. Patients may be cured by the conditioning regimen, but more likely through the GvL-effect of alloreactive donor T-cells. DLI after TCD-alloSCT provides an ideal opportunity to estimate the clinical impact of donor T-cells. The GvL-effect is often, but not always, accompanied by the occurrence of GvHD.[5] Limited (manageable) GvHD may decrease relapse risk and favor outcome, but severe (steroid-refractory) GvHD is associated with NRM.

By definition, DLI is administered at different timepoints after alloSCT. In addition, the immune response after DLI may take several weeks to occur. To determine the role of DLI after alloSCT, methods for analysis considering its time-dependent nature should be applied.[42] Examples are the introduction of a landmark timepoint and/or the application of

time-dependent covariates.[43] In **chapter 3**, the occurrence of different grades of acute GvHD were implemented as time-dependent covariates. This led to the observation that the occurrence of manageable GvHD (grades 1-3 acute GvHD) was associated with long-term disease free survival of relapsed AML after alloSCT.

Important adverse events after alloSCT are graft failure, rejection, GvHD, opportunistic infections, and relapse.[44] Many events compete with other events.[45] The competing event of relapse is NRM. DLI should not be installed during GvHD, therefore DLI and GvHD are competing events. Competing events have to be taken into account by estimating cumulative incidences of events after alloSCT.[46]

To determine the impact of GvHD on outcome, the precise start and end of GvHD should be measured. From a clinical perspective, the start of GvHD can be determined easily, but determining the precise end of GvHD can be challenging. Also the duration of 'clinically relevant' GvHD can be matter of debate. Grading of GvHD does not always suffice: the start of a certain grade of GvHD may be relatively easily determined, but measuring the precise duration of this grade of GvHD is virtually impossible. In the studies described in this thesis, the duration of severe GvHD was defined as the interval between start of immunosuppression for GvHD until cessation of immunosuppression, which we interpreted as 'clinically relevant GvHD according to the treating physician'. By defining these easy to determine start and stop points of clinically relevant GvHD, we created several building blocks of a multi-state model.[47-49] GvHD can occur after alloSCT, but also after DLI. Both impact outcome differently.[50] Therefore, the start and end of GvHD after alloSCT and after DLI should be implemented in a multi-state model as separate events.

In **chapter 4**, we aimed to determine the impact of prophylactic DLI on a new outcome measure, which we called 'treatment success'. This was defined as being alive, without relapse and without immunosuppression for GvHD. A patient would experience a failure when immunosuppression was started, but would gain success when GvHD disappeared and immune suppression could be stopped. Conventional statistical methods, which are based on a single start and end point, including composite end-point analysis like GvHD-free-relapse-free-survival, fail when studying such outcomes.[51] They only register the first occurring event. In case of GvHD-free-relapse-free-survival: GvHD and/or relapse and/or NRM. Also, the impact of post-transplant/time-dependent events/interventions, i.e. DLI, on outcome cannot be estimated when using such methods. To overcome the statistical problems addressed above, a 'multi-state model' was constructed. This model proved capable of analyzing the occurrence and effect of multiple competing time-dependent events; in our case the occurrence of GvHD and the introduction of DLI on the outcomes relapse and NRM.[52] Presence in the DLI state was associated with the highest rates of 'treatment success'. This observation supports the importance of timely administration of prophylactic DLI in all patients without GvHD after MAC TCD-alloSCT.

In the multi-state model in **chapter 4**, patients could pass a sequence of states before administration of DLI, namely start of GVHD followed by stop of GvHD, but they could also enter the DLI state directly from the state 'SCT'. The Markov assumption (which implies that after transition to a certain state, all memory of the previous states is lost) may obscure the effect of previous GvHD after DLI. This limitation of our model could have been overcome when a larger dataset had been available and an additional event 'DLI after prior GvHD' had been implemented into the model. The small dataset required a relatively simple multi-state model and did not allow estimation of the impact of covariates.

In **chapter 5**, we aimed to determine separate disease progression patterns in myeloma patients: bone marrow (BM) and focal progression, as measured from start of donor search, alloSCT and DLI.[55] To visualize these separate events we calculated cumulative incidence estimates of each type of progression, each with its corresponding competing event: death without BM-progression and death without focal progression, respectively. We used multi-state-modelling to illustrate these progression patterns and to compare the differences between these patterns.[47,48] This would be impossible using conventional Kaplan-Meier analysis.

Multi-state models can be highly informative to obtain predictions from detailed observational studies, especially in case of alloSCT, when large randomized studies are scarcely performed.[54]

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NEDERLANDSE SAMENVATTING

1. Introductie

Het aanmaken van bloedcellen vanuit stamcellen wordt hematopoiese genoemd. Dit vindt hoofdzakelijk in het beenmerg plaats. De eindproducten hiervan zijn rode bloedcellen, witte bloedcellen en bloedplaatjes. De rode bloedcellen zijn betrokken bij het zuurstoftransport door het lichaam, de witte bloedcellen hebben een belangrijke rol bij afweer tegen infecties en de bloedplaatjes spelen een rol bij de bloedstolling.

Zodra er een stoornis optreedt in de hematopoiese, ontstaat er een hematologische ziekte. Bij de behandeling van hematologische ziektebeelden kan een stamceltransplantatie met stamcellen van een donor de patiënt genezen. Het doel hierbij is om het beenmerg en overige bloedcellen te vervangen door hematopoiese van de donor. Bij een goedaardige hematologische ziekte kan het herstellen van de hematopoiese voldoende zijn om de patiënt te behandelen, maar bij een kwaadaardige ziekte is het essentieel dat alle afwijkende cellen opgeruimd worden, zodat deze geen weefsels kunnen aantasten.

Een stamceltransplantatie met eigen of donor stamcellen worden respectievelijk een autologe of een allogene stamceltransplantatie genoemd. Een allogene stamceltransplantatie (alloSCT) is een frequent toegepaste behandeling bij patiënten met hoog-risico hematologische ziekten, zoals acute myeloïde leukemie (AML) of acute lymfatische leukemie (ALL) met ongunstige cytogenetische kenmerken. Ook wordt een alloSCT bij patiënten met meerdere recidieven van een laaggradig lymfoom toegepast. Bij multipel myeloom (MM) is de rol van alloSCT op dit moment onduidelijk, omdat deze behandeling ondanks een bewezen effect op de ziekte gepaard gaat met een relatief hoge kans op niet-ziekte gerelateerde mortaliteit (NRM).

De uitkomst na transplantatie hangt af van veel factoren, zoals eerdere behandeling voor de onderliggende ziekte, de leeftijd van de patiënt, genetische verschillen tussen patiënt en donor en type transplantatie.

Voor het toedienen van het transplantaat zal de patiënt een conditioneringsbehandeling ondergaan. Deze bestaat uit chemotherapie en/of radiotherapie. Afhankelijk van de zwaarte van de conditionering wordt dit myeloablatieve of reduced-intensity conditionering (RIC) genoemd. Myeloablatieve conditionering heeft als voordeel boven RIC dat het ook een sterk effect op de onderliggende ziekte heeft en hierdoor geassocieerd is met een lagere recidiefkans. Oudere patiënten en patiënten met comorbiditeit verdragen over het algemeen geen myeloablatieve conditionering omdat dit met veel schade aan andere weefsels gepaard kan gaan. Na de conditionering ontvangt de patiënt het transplantaat. In de meeste centra bestaat het transplantaat uit stamcellen met donor T-cellen. De stamcellen produceren de hematopoietische cellen. De immuunresponsen na transplantatie worden vooral door T-cellen geïnduceerd.

De belangrijkste functie van T-cellen is het detecteren en opruimen van (virale) infecties. Tijdens een infectie presenteren geïnfecteerde cellen of professionele antigeen presenterende cellen (APCs) delen van (virale) eiwitten (peptides) in HLA-moleculen op hun celmembraan. Vervolgens worden de T-cellen geactiveerd en start de immuunrespons. APCs zijn hematopoietische cellen, zoals monocyten en dendritische cellen, en deze cellen bevatten naast HLA- ook grote hoeveelheden co-stimulatoire moleculen op hun oppervlak. Hierdoor zijn ze in staat sterke immuunresponsen te induceren. Ze kunnen zowel lichaamseigen (endogene) als lichaamsvreemde (exogene) peptides in hun HLA presenteren.

Over het algemeen worden lichaamseigen peptides niet door lichaamseigen T-cellen herkend. Dit komt doordat T-cellen die lichaamseigen peptides herkennen tijdens hun ontwikkeling in de thymus uitgeschakeld worden. Hiermee worden auto-immuunziekten voorkomen. Echter, na alloSCT kunnen T-cellen van de donor de cellen van de patiënt als lichaamsvreemd herkennen. Andersom kan ook: T-cellen van de ontvanger herkennen bloedcellen van de donor als lichaamsvreemd. Dit kan komen door genetische verschillen tussen patiënt en donor die leiden tot verschillen in HLA-moleculen en/of tot verschillen in de gepresenteerde peptiden. Peptiden die in lichaamseigen HLA-moleculen gepresenteerd en als vreemd herkend worden door T-cellen van een ander individu worden minor histocompatibility antigens (MiHAs) genoemd. Omdat APCs hematopoietische cellen zijn en deze cellen endogene peptiden kunnen presenteren, kunnen er sterke immuunresponsen tegen hematopoietische cellen optreden. Als T-cellen van de ontvanger de hematopoietische cellen van de donor als vreemd herkennen, kan dit leiden tot rejectie: de volledige hematopoiese van de donor wordt afgestoten. Dit kan direct levensbedreigend zijn als er geen rest (eigen-)hematopoiese overblijft. Deze kans is groter bij (HLA-)mismatch transplantaties en moet met onderdrukking van de eigen afweer voorkomen worden. Als T-cellen van de donor de cellen van de ontvanger als lichaamsvreemd herkennen, kan er *graft-versus-host-disease* (GvHD oftewel omgekeerde afstoting) plaatsvinden. Dit is een levensbedreigende situatie waarbij er een zichzelf versterkende immuunrespons optreedt en waarbij met name darm, lever, en huidcellen aangevallen kunnen worden. In principe kan ieder weefsel als lichaamsvreemd worden herkend.

De kans op ernstige GvHD is groter als de donor T-cellen vroeg na conditionering of tijdens een ontstekingsreactie, zoals een infectie, worden toegediend. Weefselschade door de conditionering of ontstekingsreactie kan er toe leiden dat meer HLA-moleculen en co-stimulatoire eiwitten op de oppervlakte van lichaamseigen (niet-hematopoietische) cellen tot expressie worden gebracht. Ook zullen er op dat moment relatief veel APCs van de ontvanger aanwezig zijn die lichaamseigen peptides in hun HLA zullen presenteren. Als deze HLA-peptide complexen door donor T-cellen herkend worden zal een immuunrespons aangezwengeld worden. De toename van HLA-expressie en

presententatie van lichaamseigen peptides werken een immuunrespons tegen deze weefsels (GvHD) in de hand.

Naast het aanvallen van lichaamseigen cellen gaat een GvHD-respons ook vaak gepaard met een sterke immuunrespons tegen de hematopoietische cellen van de ontvanger. De hematopoietische cellen van de ontvanger en de maligne cellen van hematopoietische origine vertonen over het algemeen veel overeenkomsten in eiwitexpressie. APCs zijn ook hematopoietische cellen en presenteren veel peptides die van hematopoietische cellen afkomstig zijn. Daarom zal een immuunrespons van donor T-cellen met name tegen hematopoietische cellen gericht zijn. Als de anti-hematopoietische reactie ook leidt tot het opruimen van de maligne kloon wordt dit *graft-versus-leukemie* (GvL-) of *graft-versus-tumor* (GvT-) effect genoemd. De uitkomsten van alloSCT kunnen verbeteren als het beleid zich richt op het verminderen van GvHD zonder dat dit ten koste gaat van het gewenste GvL-effect.

De belangrijke rol van T-cellen bij de immuunrespons na transplantatie blijkt uit de lage incidentie van ernstige GvHD na T-cel depletie (TCD). TCD kan zowel *in vivo* als *in vitro* plaatsvinden. Bij *in vivo* TCD worden antistoffen tegen T-cellen, zoals anti-thymocyten globuline (ATG) of alemtuzumab (anti-CD52 antistof) aan de patiënt toegediend. Dit heeft als voordeel dat zowel de T-cellen van de ontvanger als circulerende T-cellen uit het transplantaat worden gedood. Dit voorkomt o.a. rejectie bij HLA-mismatch transplantatie en leidt tot minder ernstige GvHD. Bij *in vitro* TCD wordt de antistof (bijvoorbeeld alemtuzumab) aan het transplantaat toegediend of worden de stamcellen met behulp van magnetische beads geïsoleerd. Ook dit leidt tot een lage incidentie van ernstige GvHD. Nadeel van TCD is de verhoogde infectiegevoeligheid en de verhoogde kans op een recidief van de ziekte. Om dit laatste tegen te gaan is het concept van donor lymfocyten infusies (DLI) ontwikkeld.

Het later toedienen van T-cellen van de donor maakt het mogelijk om de ontvanger eerst te laten herstellen van de conditioneringsbehandeling en geeft de mogelijkheid tot het behandelen van infecties. Ook zullen de APCs van de ontvanger geleidelijk vervangen zijn door de APCs van de donor. Dit vermindert de kans op ernstige GvHD en maakt het mogelijk hogere doses T-cellen toe te dienen. Dit kunnen ook T-cellen betreffen die sterk binden aan de hematopoietische cellen van de ontvanger en met name aan de maligne kloon om deze vervolgens op te ruimen.

DLI kan profylactisch toegediend worden. Dit heeft bijvoorbeeld als doel om een recidief van AML te voorkomen. Als er sprake is van recidief of persisterende ziekte wordt DLI therapeutisch ingezet. Het effect van therapeutische DLI is klein als er tussen de transplantatie en het recidief sprake was van ernstige GvHD. De sterke immuunrespons die tot GvHD heeft geleid heeft blijkbaar niet geleid tot het uitroeien van de maligne kloon. Dit toont het falen van een allogene immuunrespons. Ook bij een agressieve ziekte met hoge tumorload is het effect beperkter. Zo is het effect van DLI als monotherapie

bij recidief AML of ALL minimaal. De beste resultaten worden gezien als therapeutische DLI wordt gecombineerd met chemotherapie. Blijkbaar onderdrukt de snelle proliferatie van de leukemie de immuunrespons en is een combinatie van effectieve cytoreductie en een snelle immuunrespons nodig om de leukemie te bestrijden. Om de effectiviteit van therapeutische DLI te verbeteren wordt ook wel interferon-alfa toegediend. Verder kan DLI in de lymfopene fase na chemotherapie (of na radiotherapie) worden toegediend om zo de snelheid van de T-cel proliferatie te vergroten.

Om de klinische toepasbaarheid van DLI te verbeteren, is het belangrijk om te weten onder welke omstandigheden het gunstige effect van DLI het grootst is. Het effect van DLI is niet gemakkelijk te bepalen als T-cellen en chemotherapie tegelijkertijd worden toegediend. Na effectieve TCD is de kans op GvHD laag en kan DLI gepland toegediend worden. Het meten van een daadwerkelijk effect van DLI is niet eenvoudig. Omdat DLI later na transplantatie toegediend wordt, is dit per definitie een tijdsafhankelijke gebeurtenis (gemeten vanaf transplantatie). De meest toegepaste (traditionele) statistische methoden bij alloSCT richten zich op een enkel start- en eindpunt, zoals de tijd vanaf transplantatie tot recidief, het ontwikkelen van GvHD of overlijden. Hiermee worden o.a. de progressievrije en algemene overleving bepaald. Hiermee is het echter niet mogelijk om het effect van een tussenliggende tijdsafhankelijke gebeurtenis (zoals toediening van DLI) op deze uitkomsten te bepalen. Daarvoor zijn meer geavanceerde statistische methodes nodig. Verder zal DLI niet tijdens het optreden van GvHD toegediend worden. Daardoor zijn deze gebeurtenissen afhankelijk van elkaar. Het ene event kan niet tegelijkertijd plaatsvinden met het andere event. Dit komt overeen met 'competing risk'. Al deze gebeurtenissen kunnen wel in een multi-state model ingebouwd worden. Hiermee kunnen zowel de tijdsafhankelijke effecten van de toediening van DLI, als het ontwikkelen van (wel of geen) GvHD op verschillende uitkomsten zoals recidief ziekte of overlijden bepaald worden.

Een multi-state model bestaat uit een (of meerdere) startpunt(en), bijvoorbeeld infusie van stamcellen en minimaal 1 eindpunt, bijvoorbeeld overlijden. Een patiënt blijft in een bepaalde 'state' totdat een volgende gebeurtenis, een 'event', optreedt. Tevens kunnen intermediaire 'states' ingebouwd worden, bijvoorbeeld 'toediening van DLI' of 'begin van GvHD'. Een patiënt kan vervolgens afhankelijk van de grootte van het model van 'state' naar 'state' gaan, bijvoorbeeld van 'begin van GvHD' naar 'stoppen van GvHD'. Tenslotte worden eindpunten oftewel 'absorbing states' ingebouwd, bijvoorbeeld sterfte of recidief ziekte. Met behulp van een multi-state model kan bepaald worden wat de kans op een bepaalde uitkomst (bijvoorbeeld sterfte of recidief ziekte) is als men op een bepaald moment (bijvoorbeeld een half jaar na transplantatie) een bepaald 'event' (bijvoorbeeld toediening van DLI) heeft gehad. Met behulp van het multi-state model kunnen de verschillende gebeurtenissen ook in grafische vorm inzichtelijk worden gemaakt en kunnen verschillende uitkomsten naar wens worden gecombineerd. Een

belangrijk verschil met meer traditionele statistische methoden is dat patiënten van 'state' naar 'state' kunnen gaan en dat de krommes die de kans weergeven om in een bepaalde 'state' te verblijven omhoog of omlaag gaan al naar gelang er zich meer of minder patiënten in een bepaalde 'state' bevinden.

2. Dit proefschrift

Effectieve TCD voorkomt het ontwikkelen van GvHD en bevordert zo het herstel van beschadigde weefsels na de conditionering. In het Leids Universitair Medisch Centrum (LUMC) worden patiënten getransplanteerd m.b.v. *ex vivo* TCD door het transplantaat gedurende 30 minuten met alemtuzumab te incuberen. Hierdoor is de kans op GvHD erg laag en heeft de meerderheid van patiënten geen aanvullende immunosuppressie nodig. Na de transplantatie is het belangrijk om infecties vroeg te behandelen. Op deze manier voorkomt men morbiditeit door infecties, maar het verbetert ook de omstandigheden voor veilige toediening van DLI. Een langere tijd tussen transplantatie en toediening van DLI onder niet-inflammatoire omstandigheden vermindert de incidentie van ernstige GvHD na DLI. Dit maakt het mogelijk om grotere hoeveelheden T-cellen te infunderen. Deze hogere aantallen T-cellen verhogen de kans dat de maligne kloon met een hoge aviditeit wordt herkend en opgeruimd. Een te lange tijd tussen transplantatie en DLI verhoogt het risico op recidief ziekte. De klinische respons van DLI op recidieven van verschillende ziektebeelden varieert sterk. Recidieven van een acute leukemie reageren bijvoorbeeld zelden op DLI als monotherapie. Het op tijd toedienen van profylactische DLI kan de uitkomst van hoog-risico patienten verbeteren. In dit proefschrift worden de klinische effecten van DLI in verschillende klinische situaties na TCD-alloSCT beschreven.

In **hoofdstuk 2** worden de veiligheid en effectiviteit van de stategie van vroege DLI na TCD-alloSCT in een cohort van patiënten met ALL onderzocht. Recent onderzoek toont aan dat de overleving van volwassen patiënten met ALL blijft achter op die van kinderen en jongvolwassen patiënten. De 25 volwassen patiënten met ALL in onze studie hadden met name hoog-risico ALL (hoog risico op recidief). Ze ondergingen myeloablatieve TCD-alloSCT met vroeg-geplande DLI. Ze kwamen in aanmerking voor profylactische DLI als ze tussen 3 tot 6 maanden na transplantatie nog in leven waren, geen recidief hadden en geen immuunsuppressie voor GvHD nodig hadden. Patiënten met een familiedonor kregen 3,0 x 10^6 T-cellen/kg en patiënten met een onverwante donor kregen 1,5 x 10^6 T-cellen/kg. In totaal kon profylactische DLI na gemiddeld 185 dagen na transplantatie aan 14 patiënten toegediend worden. De strategie was succesvol in het voorkomen van ernstige GvHD: slechts 17% van alle patiënten hadden meer dan een jaar immuunsuppressie nodig. Drie patiënten kregen een recidief van de leukemie. Bij 1 van deze 3 kon een recidief van een Philadelphia-chromosoom positieve leukemie (Ph+ ALL) succesvol behandeld worden met een combinatie van DLI, interferon-alpha

en een tyrosine-kinase remmer. De mediane follow-up van het hele cohort bedroeg 50 maanden en de kans op 2-jaar overleving was 68%. Er was geen duidelijk verschil tussen patiënten met een verwante of onverwante donor en ook de kans op 2-jaar overleving van patiënten met een Ph+ ALL was minstens zo goed als die van patiënten met een Ph- ALL (respectievelijk 80% en 60%, p=0,27). Samenvattend toont deze single-center studie van een cohort van hoofdzakelijk hoog-risico ALL patiënten aan dat het op tijd toedienen van DLI na TCD-alloSCT leidt tot een laag risico op recidief ziekte zonder een hoge incidentie van ernstige GvHD.

In **hoofdstuk 3** wordt de rol van DLI bij recidief AML onderzocht. De prognose van recidief AML na alloSCT is somber en toediening van DLI werkt onvoldoende om een lange remissie te verkrijgen. Om de overleving van patiënten te verbeteren hebben we een strategie ontwikkeld waarbij gestreefd werd naar de inductie van een optimale immuunrespons. Na effectieve cytoreductie voor recidief van de leukemie werd DLI toegediend tijdens de lymfopene fase na chemotherapie. Indien er geen GvHD optrad gedurende 3 weken na DLI, werd gepoogd om m.b.v. interferon-alfa de immuunrespons te versterken. Het tijdsafhankelijke effect van uitblijven van GvHD, behandelbare graad I tot III GvHD en levensbedreigende graad IV GvHD op tweede recidief en overleving werd onderzocht in een cohort van 44 patienten met recidief AML na transplantatie. Bij 7 patiënten was er geen behandeling mogelijk door een slechte algehele conditie (n=3) of ernstige GvHD (n=4) ten tijde van het recidief. Nog eens 10 patiënten konden geen DLI krijgen door overlijden na chemo (n=1) of chemotherapie-refractaire ziekte (n=9). De kans op 2-jaar overleving na DLI bedroeg 36%. Ten opzichte van patiënten die geen GvHD ontwikkelden was de kans op overleven hoger bij patiënten die graad I tot III GvHD ontwikkelden (hazard ratio 0,22, p=0,03), maar graad IV GvHD resulteerde niet in een betere overleving. Samenvattend toont deze studie aan dat DLI een belangrijke rol kan spelen bij de behandeling van patiënten met recidief AML na alloSCT. De grootste kans op genezing hebben patiënten bij wie de ziekte in remissie werd gebracht en bij wie in korte tijd een behandelbare GvHD-respons opgewekt kon worden.

In **hoofdstuk 4** worden de veiligheid en effectiviteit van profylactische DLI na myeloablatieve TCD-alloSCT bij patiënten met AML of hoog-risico MDS onderzocht. Over het algemeen focust onderzoek na tranplantaties zich op het ontwikkelen van GvHD, recidief ziekte en sterfte. Echter als men het effect van interventies na de transplantatie wil onderzoeken, schieten traditionele statistische methodes, zoals algemene of progressie-vrije overleving, tekort. Deze richten zich immers op een enkel start- en eindpunt. Wij wilden onderzoeken of een multi-state model het inzicht in de gebeurtenissen na de transplantatie zou kunnen verbeteren. Op deze manier wilden we analyseren wat de impact van DLI en GvHD op de kans van recidief ziekte en overlijden aan andere oorzaken dan een recidief was. Het onderzoek werd verricht bij patiënten met AML/MDS die profylactische DLI konden krijgen, mits ze tussen 3 tot 6 maanden na TCD-alloSCT

in remissie waren en geen immuunsuppressie voor GvHD nodig hadden. We bouwden een multi-state model met de dag van stamcelinfusie als startpunt: 'SCT'. Patiënten die immunosuppressie voor GvHD nodig hadden gingen over naar de staat 'Start IS' op de dag dat de immuunsuppressie gestart werd en vervolgens naar de staat 'Stop IS' als de immuunsuppressie weer gestaakt werd. Op de dag van DLI gingen ze over naar de staat 'DLI'. Overgang naar de staat 'DLI' was (conform de praktijk) mogelijk vanuit de staten 'SCT' en 'Stop IS'. Na de DLI was overgang naar de staten 'start IS after DLI' en 'stop IS after DLI' mogelijk als er respectievelijk immuunsuppressie nodig was voor de behandeling van GvHD en die behandeling vervolgens gestaakt werd. Door het meten van het aantal transities naar de verschillende staten in combinatie met de uiteindelijke uitkomst aan het einde van de follow-up (nog in leven, optreden van recidief of overleden zonder recidief) kon de impact van het verblijf in de verschillende staten op verschillende momenten van follow-up na transplantatie op de uiteindelijke uitkomst berekend worden. Aan de hand van een multistate model werd een nieuwe uitkomstmaat gedefinieerd: 'treatment success'. Dit werd gedefinieerd als het in leven zijn zonder een recidief en zonder immuunsuppressie voor GvHD. Uit het onderzoek bleek dat de kans op recidief en sterfte zonder recidief ongeveer even groot waren. Echter de kans op recidief was vooral aanwezig na transplantatie (zonder ontwikkelen van GvHD) en werd lager na het ontwikkelen van GvHD, maar vooral na DLI. De kans op ernstige GvHD na DLI was laag: na DLI had slechts 11% van de patiënten behandeling met immuunsuppressie nodig. In de hele populatie bedroeg de lange-termijn kans op 'treatment success' ongeveer 40%, maar na DLI ging deze kans duidelijk omhoog: ongeveer 70% van de patiënten die uiterlijk op 6 maanden na transplantatie DLI hadden gekregen en op 6 maanden nog in leven waren had 'treatment success' op 5 jaar na transplantatie. Op basis van deze studie lijkt toediening van profylactische DLI gunstig voor de algehele uitkomst na transplantatie.

In **hoofdstuk 5** wordt het effect van DLI op ziekteprogressie van multipel myeloom (MM) onderzocht. Op dit moment is de rol van alloSCT bij de behandeling van MM door niet-recidief mortaliteit (NRM) en late recidieven onduidelijk, maar er kan wel veel geleerd worden van het bestuderen van effect van donor T-cellen op de ziekte. De T-cellen kunnen een graft-versus-myeloom effect bewerkstellen, maar dit effect lijkt minder sterk in het extramedullaire compartiment. Wij brachten 43 patiënten met MM in kaart die RIC TCD-alloSCT (evt. gevolgd door DLI) ondergingen. Ook werd de ziekteprogressie bij een cohort in kaart gebracht van 12 patiënten waarbij wel een donor gezocht werd, maar die geen transplantatie ondergingen. De incidenties van beenmerg en focale (radiologische) progressie (respectievelijk na de allogene stamceltransplantatie of na de start van de donor search) werden separaat in alle patiënten bepaald. Ook werden deze incidenties na DLI bepaald. Met behulp van een multi-state model werden de beenmerg-progressie- en focale progressievrije overleving bepaald. Vervolgens konden de verschillen tussen beenmerg- en focale progressie onderzocht worden. DLI bleek

effectief in het opruimen van myeloom in het beenmerg: 86% van de patiënten met myeloom in het beenmerg toonden een complete remissie na toediening van DLI. De kans op 2-jaar beenmerg-progressievrij overleven na transplantatie en na DLI bedroegen respectievelijk 51% en 62%. Bij het controle-cohort was dit veel lager: 17% op 2 jaar na het starten van de donor search. Het effect van DLI op de focale progressie was beperkt. De kansen op focale progressievrije overleving 2 jaar na donor-search, alloSCT en DLI bedroegen respectievelijk 17%, 30% en 28%. Concluderend lijkt het effect van donor T-cellen op MM in het beenmerg gunstig, maar bij het voorkomen van focale lesies juist beperkt. Het multi-state model maakt deze verschillende progressiepatronen goed inzichtelijk.

Samenvattend toont het onderzoek in dit proefschrift met behulp van diverse statistische methoden dat de combinatie van myeloablatieve T-cell gedepleteerde alloSCT gevolgd door tijdige toediening van DLI een veilige en effectieve behandelstrategie kan zijn bij ALL en AML. Het toepassen van een multi-state model heeft toegevoegde waarde bij het bepalen van het effect van DLI. Wanneer effectieve cytoreductie gevolgd door DLI leidt tot inductie van behandelbare GvHD kan dit curatie geven van een recidief van AML. Ten slotte lijkt het therapeutische effect van DLI bij de behandeling van multipel myeloom het sterkst in het beenmergcompartiment.

DANKWOORD

"Tachtig procent van promotie-onderzoek doen is afzien." Met deze opbeurende woorden werd ik in 2007 door professor Willemze welkom geheten op de afdeling hematologie van het LUMC. Alhoewel dit een puntschatting is, denk ik dat hij er niet ver vanaf zat. Vervolgens stapte ik in 2010 in de achtbaan die promoveren heet…

De begeleiding onder leiding van professor Falkenburg was van topkwaliteit. Dankzij zijn geduld heb ik bijzonder veel geleerd. Met iedere versie werd een manuscript beter en vooral duidelijker. Alsof de mist opklaarde. Van Liesbeth de Wreede leerde ik dat statistiek het fundament vormt van goed onderzoek. Een looping of een kurkentrekker? Geen probleem, zij wist welk model we moesten toepassen. En met Stijn Halkes kun je niet alleen veel plezier beleven, dankzij hem lukte het om al het onderzoek in het juiste klinische perspectief te plaatsen. Ook de overige co-auteurs van de artikelen in dit proefschrift ben ik dank verschuldigd: Erik, Peter, Sabina, Johannes (danke schön!), Hein, Hendrik (danke schön!) en Roel.

Ik zag de afdeling experimentele hematologie van het LUMC als een warm bad waar kwaliteit voor alles gaat. Een mooier zingende afdeling ben ik tot nu toe niet tegengekomen. Ik hoop dat één van de stafleden (Inge, Marieke, en Mirjam, go girls!) uiteindelijk de afdeling en wellicht het ziekenhuis zal gaan leiden. Ook met Dirk, Hetty, Renate, Renate (ja 2 keer), Edith, Marian, Michel, Willy, Kees, Ellie, Nelleke, Marianke, Bart, Simone, Marleen, Margot, Caroline, Jessica, Anita, Judith, Hetty, Roos, Sanja, Pim, Floris, Marjolein, Peter, Lorenz, Marcelo, Valery, Avital, Monique en Pleun heb ik '3 fantastische dagen' op het lab gehad!

De studenten Michael, Stephanie en Marlies wil ik erg bedanken voor hun inbreng. Ik was erg onder de indruk van hun slimme opmerkingen en enthousiasme. Ook alle mensen van het secretariaat en het datamanagement wil ik van harte bedanken: in het bijzonder Karien, Gerrie en Sylwia. Dank zij hen kon ik me soepeltjes door het vele papierwerk worstelen.

Met mijn collega's van de afdelingen endocrinologie en vasculaire geneeskunde heb ik met plezier in de wachtrij voor het promotieschap gestaan. Dit zijn ware helden van de EVVMB.

Mijn vrienden wil ik danken voor hun interesse in alle vorderingen. Dankzij Douwe, Michel, Jasper, Ivan, Maarten, Jimte, Aart-Jan, Ralf, Rolf, Branko, Emiel, Jaap, Ronnie, Sergio, Tim, Vincent en Wouter kon ik me telkens opladen voor het voortzetten van mijn het promotietraject. Ook alle lieve vrienden en vriendinnen die ik via Mariëlle heb leren kennen, wil ik bedanken voor hun niet-aflatende interesse!

Op een gegeven moment werd mijn promotie-traject een inspirerende hobby waarvoor flink bijgeklust moest worden. Mijn vele collega's uit het Erasmus verdienen lof voor de tijd die ik kreeg om aan mijn onderzoek te werken en me verder te verdiepen in

de hematologie. Zo leerde ik via Jan Cornelissen en Annoek Broers dat transplanteren met T-cellen in het transplantaat een alternatief kan zijn voor in vitro T-cel depletie. Maar ook het team in het Beatrixziekenhuis heeft me erg gesteund. Met Giel, Pascal, Martijn, Maikel, Hannah, Marjan, Yasser, Daniëlle, Maarten, Lyuba, alle secretaresses en verpleegkundigen als collega's mag iedereen zich gelukkig prijzen.

Dan kom ik nu bij een paar extra bijzondere mensen. Allereerst Boris en Frans, mijn paranimfen. Een paranimf is je beschermer voor de promotiecommissie en ze hebben hun werk met verve gedaan. Ze hebben een luisterend oor geboden, maar ik wil ze ook danken voor het kritisch doorlezen van het manuscript.

Natuurlijk komt nu mijn familie: Daniël en Harmen, ik bof met zulke broers! Jullie hebben voor fijne aanwas van de familie gezorgd: Simone, Katelijn, Liselot en Hannah. Ook Maarten en Lydia wil ik heel erg bedanken voor alle interesse in mijn onderzoek, het vele oppassen op maandag en bovenal de gezelligheid in Zandvoort!

Pap, ik hoop dat je een beetje trots op me bent en ik denk dat mam ook trots geweest zou zijn. Dit proefschrift wil ik dan ook aan jullie opdragen. Het is mooi dat je weer een nieuwe liefde, Marianne, hebt gevonden!

Zo bereik je uiteindelijk het einde van de lijst en daar kan nog maar 1 iemand staan: Mariëlle, de liefde van mijn leven! Ooit stonden we samen op de Tafelberg en keken naar het moois van de wereld onder ons. Het was 1 van de vele hoogtepunten uit ons leven. En het is heerlijk om elke dag samen met jou van Wessel, Ruben en Ariadne te mogen genieten.

CURRICULUM VITAE

Matthias Eefting werd geboren op 25 augustus 1978 te IJmuiden, gemeente Velsen. Op 4-jarige leeftijd zat hij voorin de verhuiswagen naar Alphen aan den Rijn, alwaar hij aan de J.P. Sweelinckschool en vervolgens aan het Christelijk Lyceum, later Groene Hart Lyceum, respectievelijk zijn basis- en middelbare school zou afronden. Vervolgens startte hij in 1996 met de opleiding Biomedische Wetenschappen aan de Universiteit Leiden en behaalde zijn propedeuse in 1997. Datzelfde jaar startte hij met de opleiding Geneeskunde aan de Universiteit Leiden. Tijdens zijn studie Geneeskunde volgde hij enkele keuzevakken bij de studie Culturele Antropologie en Sociologie, waaronder Medische Antropologie. Op de afdeling Moleculaire Virologie deed hij onder begeleiding van prof. dr. R.C. Hoeben onderzoek naar 'Adenovirus-associated gene therapy'. Tijdens zijn co-schappen leek het alsof hij vaker in het buitenland zat dan in Nederland. Zo liep hij stages bij St. James' Mission Hospital te Mantsonyane, Lesotho, bij Princess Alexandra's Eye Pavilion, Royal Infirmary te Edinburg, Schotland, en ook bij het Gasthuisberg Ziekenhuis, Katholieke Universiteit Leuven, België. Direct na afronding van de studie Geneeskunde in 2003 startte hij met de opleiding tot internist aan het Leids Universitair Medisch Centrum; aanvankelijk onder leiding van prof. dr. A.E. Meinders en later prof. dr. H. Romijn. Van 2004 tot 2007 liep hij onder leiding van dr. R.H. Kauffmann stage in het Leyenburg Ziekenhuis, later Haga Ziekenhuis. Dr. R.H. Kauffmann maakte het ook mogelijk dat hij onder leiding van prof. dr. E. Irusen stage Longziekten/Intensive Care kon lopen in het Tygerberg Hospital, Universiteit van Stellenbosch, Zuid-Afrika. Tijdens het werken in het Haga Ziekenhuis werd o.a. door dr. P.W. Wijermans zijn interesse voor de hematologie gewekt. Vervolgens mocht hij in 2008 onder leiding van prof. dr. R. Willemze en prof. dr. J.H.F. Falkenburg starten met het aandachtsgebied Hematologie. De opleiding tot internist-hematoloog ging begin 2010 naadloos over in het hier beschreven promotieonderzoek onder begeleiding van prof. dr. J.H.F. Falkenburg.

Tijdens het werken aan het promotieonderzoek was hij als internist-hematoloog werkzaam op de afdeling Hematologie van het LUMC bij prof. dr. J.H. Veelken en vanaf 2013 op de afdeling Hematologie van het Erasmus MC - locatie Daniël den Hoed bij prof. dr. J.J. Cornelissen en prof. dr. P. Sonneveld. Sinds 2015 werkt hij als internist-hematoloog bij het Beatrixziekenhuis van de RIVAS zorggroep, te Gorinchem.

In 2012 vroeg hij op de Tafelberg in Zuid-Afrika zijn vrouw Mariëlle Aleida Koper ten huwelijk en samen hebben ze twee zoontjes en een dochter: Wessel, Ruben en Ariadne.

LIST OF PUBLICATIONS

Donor T-cell responses and disease progression patterns of multiple myeloma. **Eefting M***, de Wreede LC*, Halkes CJ, von dem Borne PA, Kersting S, Marijt EW, Putter H, Veelken H, Schetelig J, Falkenburg JH. Bone Marrow Transplant. 2017 Dec;52(12):1609-1615. Epub 2017 Oct 2.
*Contributed equally.

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Intentional donor lymphocyte-induced limited acute graft-versus-host disease is essential for long-term survival of relapsed acute myeloid leukemia after allogeneic stem cell transplantation. **Eefting M**, von dem Borne PA, de Wreede LC, Halkes CJ, Kersting S, Marijt EW, Veelken H, Falkenburg JF. Haematologica. 2014 Apr;99(4):751-8. Epub 2013 Nov 15.

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LIST OF PUBLICATIONS NOT IN THIS THESIS

Predictors of short-term and long-term mortality in critically ill patients admitted to the intensive care unit following allogeneic stem cell transplantation. van der Heiden PLJ, Arbous MS, van Beers EJ, van den Bergh WM, le Cessie S, Demandt AMP, **Eefting M**, Hess C, Kusadasi N, Marijt WAF, van Mook WNKA, Müller MCA, Tuinman PR, van Vliet M, van Westerloo DJ, Blijlevens NMA; HEMA-ICU Study Group. Bone Marrow Transplant. 2018 Aug 6. [Epub ahead of print]

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Selective graft-versus-leukemia depends on magnitude and diversity of the alloreactive T cell response. van Bergen CA, van Luxemburg-Heijs SA, de Wreede LC, **Eefting M**, von dem Borne PA, van Balen P, Heemskerk MH, Mulder A, Claas FH, Navarrete MA, Honders WM, Rutten CE, Veelken H, Jedema I, Halkes CJ, Griffioen M, Falkenburg JH. J Clin Invest. 2017 Feb 1;127(2):517-529. Epub 2017 Jan 9.

Integrated whole genome and transcriptome analysis identified a therapeutic minor histocompatibility antigen in a splice variant of ITGB2. Pont MJ, van der Lee DI, Van Der Meijden ED, Van Bergen CA, Kester MG, Honders MW, Vermaat M, **Eefting M**, Marijt EW, Kielbasa SM, 't Hoen PA, Falkenburg JH, Griffioen M. Clin Cancer Res. 2016 Aug 15;22(16):4185-96. Epub 2016 Mar 10.

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