Analysis of sequential treatments for hematological diseases by advanced statistical methods

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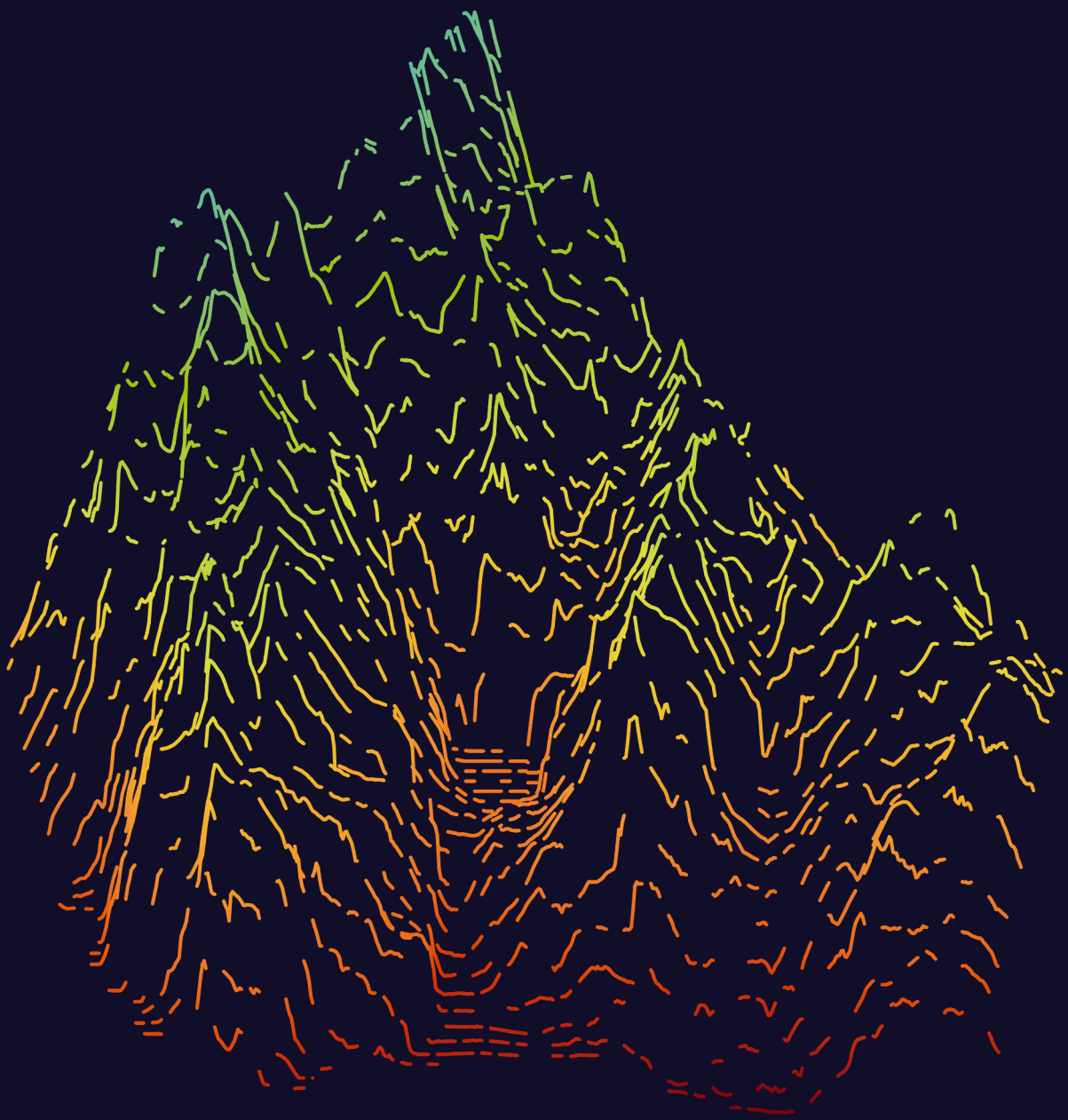

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Risk factors for graft-versus-host-disease after donor lymphocyte infusion following T-cell depleted allogeneic stem cell transplantation

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ABSTRACT

Unmodified donor lymphocyte infusions (DLI) after allogeneic stem cell transplantation (alloSCT) can boost the beneficial Graft-versus-Leukemia (GvL) effect but may also induce severe Graft-versus-Host-Disease (GvHD). To improve the balance between GvL and GvHD, it is crucial to identify factors that influence the alloreactivity of DLI. We investigated the effects of the presence of patient-derived antigen-presenting cells at time of DLI as estimated by the bone marrow (BM) chimerism status, lymphopenia as measured by the absolute lymphocyte count (ALC) at time of DLI, and the presence of a viral infection (*de novo* or reactivation) close to DLI on the risk of GvHD after DLI. The cohort consisted of patients with acute leukemia or myelodysplastic syndrome who prophylactically or pre-emptively received DLI as standard care after alemtuzumab-based alloSCT. In patients at high risk for relapse, DLI was administered at 3 months after alloSCT (n=88) with a dose of $0.3x10^6$ or $0.15x10^6$ T cells/kg in case of a related or unrelated donor, respectively. All other patients (n=76) received $3x10^6$ or $1.5x10^6$ T cells/kg, respectively, at 6 months after alloSCT. For both DLIs, patients with reduced-intensity conditioning and an unrelated donor had the highest risk of GvHD. For DLI given at three months, viral infection within 1 week before and 2 weeks after DLI was an additional significant risk factor (hazard ratio (HR) 3.66 compared to no viral infection) for GvHD. At six months after alloSCT, viral infections were rare and not associated with GvHD. In contrast, mixed BM chimerism (HR 3.63 for ≥5% mixed chimerism compared to full donor) was an important risk factor for GvHD after DLI given at six months after alloSCT. ALC of $<1000x10^6$/l showed a trend for association with GvHD after this DLI (HR 2.05 compared to $\geq 1000x10^6$/l, 95% confidence interval 0.94-4.45). Furthermore, the data suggested that the presence of a viral infection close to the DLI at three months or ≥5% mixed chimerism at time of the DLI at six months correlated with the severity of GvHD, thereby increasing their negative impact on the current GvHD-relapse-free survival. These data demonstrate that the risk factors for GvHD after DLI depend on the setting of the DLI.

INTRODUCTION

The Graft-versus-Leukemia (GvL) effect of allogeneic hematopoietic stem cell transplantation (alloSCT) results from elimination of persisting malignant hematopoietic cells by donor-derived alloreactive T cells.[1] The GvL effect can provide enduring relapse-free survival but can be accompanied by Graft-versus-Host-Disease (GvHD) when non-hematopoietic cells are targeted.[2] T-cell depletion (TCD) reduces the risk of severe GvHD, but increases the relapse risk by reduction of the GvL effect.[3,4] To boost the GvL effect, unmodified donor lymphocyte infusions (DLI) can be administered after alloSCT.[5] A third of the patients develops clinically relevant GvHD after DLI.[6] Although GvHD is a complication, it does not necessarily mean treatment failure: if GvHD resolves, the patient is unlikely to experience an eventual relapse due to the established concomitant GvL effect.[7,8] The long-term health status of patients with resolved GvHD is comparable to those who did not develop GvHD.[9] Thus, GvHD is a temporary undesired state in contrast to relapse or death as definitive failures. This is captured by the endpoint current GvHD-relapse-free survival (cGRFS) which incorporates recovery from GvHD.[10] Estimation of cGRFS requires advanced statistical methods that can take the end date of GvHD into account, such as multi-state models.[10-12]

Different DLI strategies can be applied to achieve an optimal balance between GvL and GvHD.[13] A reactive strategy is to give only therapeutic DLI to relapsed patients who need a strong alloimmune response to survive. A preemptive strategy administers DLI to patients based on biomarkers that may herald relapse such as mixed chimerism (MC) or minimal residual disease (MRD). In a prophylactic strategy, DLIs are given to all patients without any GvHD independent on additional biomarkers. Several factors known to influence the alloreactivity of DLI are usually taken into account to determine the DLI dose.[14] First, DLIs with higher T-cell doses induce more GvHD and GvL.[15] Second, patients with an unrelated donor (UD) or HLA-mismatched donor have more allo-antigens that can provoke an alloimmune response and often receive a lower dose than patients with an HLA-matched related donor (RD). Third, the DLI dose is also dependent on the timing after alloSCT, since the alloreactive potential of DLI decreases over time due to changes in the host environment.[16,17] Early after transplantation, professional antigen-presenting cells (APCs) required to activate naïve T cells are still patient-derived and therefore highly capable of activating donor-derived alloreactive T cells. Tissue damage by the conditioning regimen and infections, which occur relatively frequently during the first months after alloSCT, leads to a pro-inflammatory environment that promotes activation of alloreactive T cells.[18,19] Moreover, the conditioning-induced lymphopenia stimulates the outgrowth of (alloreactive) T cells by homeostatic proliferation and promotes activation of these T cells.[20,21] Over time after alloSCT, tissue damage is repaired, patient-derived professional APCs are replaced by donor-derived APCs, lymphopenia disappears, infections become rare, and higher T-cell doses are needed to induce a sufficient GvL effect after DLI.

Despite dose adjustments based on timing and donor type, the effect of a single DLI is highly variable between patients, ranging from patients not responding at all to patients succumbing to severe GvHD. To avoid excessive toxicity in the prophylactic or preemptive setting, it is crucial to better understand which factors influence the efficacy and toxicity of DLI. Since development of clinically relevant GvHD represents the

clearest indicator for induction of alloreactivity after DLI, we aimed to identify risk factors for GvHD after prophylactic or preemptive DLI following alemtuzumab-based TCD alloSCT. Focusing on conditions that promote T-cell activation, we investigated the effects of the presence of patient-derived APCs in the bone marrow (BM) as measured by the BM chimerism level at time of DLI, the presence of lymphopenia as measured by the absolute lymphocyte count (ALC) at time of DLI, and the occurrence of viral infections (i.e., *de novo* infections or reactivations) close to DLI. We also investigated the impact of potential risk factors on the course of GvHD: GvHD only requiring short-term therapeutic systemic immunosuppression (tIS), GvHD requiring long-term tIS, or lethal GvHD. To assess their clinical relevance, we transformed these effects into cGRFS probabilities.

METHODS

Study population

This retrospective study included all adult patients with acute myeloid leukemia, acute lymphoblastic leukemia or myelodysplastic syndrome in complete morphologic remission who received an alloSCT from a 10/10 HLA-matched donor using a standard conditioning and TCD protocol[22-24] at Leiden University Medical Center (LUMC, Leiden, The Netherlands) between 2005 and 2019. Patients scheduled to receive azacitidine or daratumumab (in 1 patient with CD38 positive acute lymphoblastic leukemia) as pharmacological maintenance therapy after alloSCT were excluded. All patients signed informed consent for data collection and analysis. Data were analyzed as of July 2021.

Transplantation and DLI protocol

The protocols for the myeloablative and reduced-intensity conditioning regimens (MAC and RIC, respectively), TCD and GvHD prophylaxis are described in the Supplemental Methods. The dose of unmodified preemptive and prophylactic DLI was based on donor type and timing after alloSCT. DLI at 3 months after alloSCT contained low doses of $0.3x10^6$ and $0.15x10^6$ T cells/kg in case of RD and UD, respectively. DLI at 6 months after alloSCT contained $3x10^6$ and $1.5x10^6$ T cells/kg, respectively. All patients could receive preemptive DLI in case of MC or MRD positivity, starting from 3 months after alloSCT. Subsequent preemptive DLI could be given in escalating doses with at least 3 months between DLI. Since May 2010, patients who were considered to have a high risk of relapse or who received the FLAMSA regimen received prophylactic low-dose DLI at 3 months. In addition, all eligible patients without any relapse or GvHD requiring systemic treatment received prophylactic DLI at 6 months after alloSCT regardless of chimerism or MRD status. Furthermore, selected patients could receive modified T-cell products within several clinical trials.

Definitions of clinical events and DLI cohorts

Relapse was defined as recurrence of at least 5% blasts on cytomorphologic BM examination, at least 1% blasts in the peripheral blood or the presence of extramedullary disease. Graft failure was defined as the occurrence of >95% mixed BM chimerism in all

lineages tested or refractory granulopenia (granulocyte count $<0.5x10^9$/l) in the absence of relapse and ongoing myelotoxic medication. To have a clear definition of clinically relevant GvHD with exact starting and stopping dates, essential for statistical modeling, we considered administration of tIS for acute or chronic GvHD instead of the grading of GvHD. For the analyses, we only considered tIS which was given for at least 14 days or until death, or which was stopped within 1 week before death from GvHD. In the latter case, the last week before death was added to the tIS episode. If a patient stopped tIS but had to restart tIS again within 2 months due to the recurrence of GvHD, both tIS episodes were combined into one episode. cGRFS was defined as the probability of being alive without relapse and currently not using any tIS for GvHD.

To investigate the clinical outcomes after DLI, two subcohorts were defined. The low-dose 3-month DLI cohort included all patients who were scheduled to receive a prophylactic or preemptive low-dose DLI at 3 months after alloSCT and received it within 6 months after alloSCT without any prior relapse, tIS for GvHD or cellular intervention besides infusion of virus-specific T cells. The 6-month DLI cohort consisted of all patients who were scheduled to receive a prophylactic or preemptive 6-month DLI as first DLI and received it within 9 months after alloSCT without any prior relapse, tIS for GvHD or cellular intervention besides infusion of virus-specific T cells. Both subcohorts were thus independent.

BM chimerism, ALC and viral infections

The methods for measuring BM chimerism, ALC and viral infections are described in the Supplemental Methods. The BM chimerism level was used as a measurement of the presence of patient-derived APCs in the BM at time of DLI. Three chimerism categories were defined: full donor chimerism (FDC; no detectable patient material), low MC (detectable patient material but <5%), and high MC (≥5% patient material).

Lymphopenia was defined as ALC $<1000x10^6$/l, the lower limit of normal in our laboratory. For patients receiving the 3-month DLI, three ALC categories were defined: ALC $<500x10^6$/l, ALC between 500 and $999x10^6$/l and ALC $\geq1000x10^6$/l. For patients who received the 6-month DLI as first DLI, only two categories were used, <1000 and $\geq1000x10^6$/l, since most patients had ALC $\geq500x10^6$/l at that time.

All viral infections (*de novo* or reactivation) confirmed by PCR that occurred within 1 week before and 8 weeks after DLI without any prior relapse, second DLI or tIS were considered.

Statistical analyses

Follow-up after alloSCT was quantified using the reversed Kaplan-Meier method.[25] The cumulative incidence of tIS after the first DLI (DLI1) was estimated in a competing risks model starting at time of DLI1 with start of tIS as the event of interest and relapse, death and second DLI (DLI2) as competing events. The cumulative incidence of death during treatment for GvHD from start of tIS was estimated in a competing risks model starting at time of start tIS after DLI1 with death as the event of interest and relapse, stop tIS and DLI2 as competing events.

To investigate risk factors for requiring tIS for GvHD and death during tIS and to

estimate cGRFS after DLI, several Markov time-inhomogeneous multi-state models were constructed. See the Supplemental Methods for a brief explanation of the methodology of multi-state modelling. The structure of the main multi-state model is shown in Figure 1. The model used DLI1 as the starting state and time and considered the following events: death, relapse, start and stop of tIS for GvHD, and DLI2. Separate states were used for events after DLI1 and for events after second DLI (e.g., 'relapse after DLI1' and 'relapse after DLI2'). The probability of cGRFS over time was calculated as the sum of the probabilities of being in one of the relevant states in the multi-state model (i.e., 'DLI1', 'stop tIS after DLI1', 'DLI2' and 'stop tIS after DLI2'). The probabilities of death after start of tIS, being alive with clinically GvHD, relapse-free survival (RFS) and overall survival (OS) were calculated analogously. The outcomes after the low-dose 3-month DLI and the 6-month DLI were analyzed using two separate versions of this

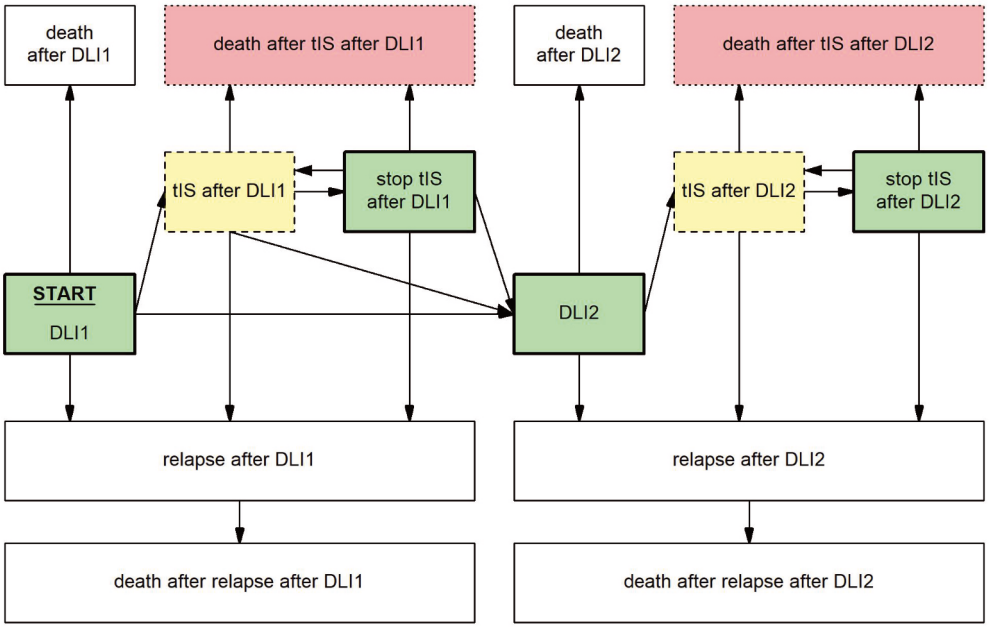

Figure 1. Multi-state model to evaluate the development and outcome of GvHD and other clinical events after DLI. Boxes represent states, arrows represent transitions. Starting state and time was DLI1. From here, patients could move to the state 'relapse after DLI1' at time of relapse, 'death after DLI1' at time of death, 'tIS after DLI1' at time of the start of tIS for GvHD and 'DLI2' at time of the administration of a second DLI, whichever occurred first. From the state 'relapse after DLI1' patients could only enter the state 'death after relapse after DLI1'. From the state 'tIS after DLI1' patients could move to 'stop tIS after DLI1' at time of stop of all tIS, 'relapse after DLI1' at time of relapse, 'death after tIS after DLI1' at time of death or 'DLI2' at time of the administration of a second DLI, whichever occurred first. From the state 'stop tIS after DLI1' patients could return to 'tIS after DLI1' when patients had to restart tIS for recurrent GvHD, 'relapse after DLI1' at time of relapse, 'death after tIS after DLI1' at time of death or 'DLI2' at time of the administration of a second DLI, whichever occurred first. After DLI2, similar states were constructed, except that any further DLIs were ignored. The cGRFS is the sum of the probabilities of all green (thick border) states, the probability of being alive with GvHD the sum of all yellow (dashed border) states, the probability of death after start of tIS for GvHD the sum of all red (dotted border) states, the RFS the sum of all green (thick border) and yellow (dashed border) states, and the OS the sum of all non-death states. For these summarizing measures, no distinction was made between states after the first DLI or after multiple DLIs.

model, omitting all transitions and states that were not used by the included patients (Supplemental Figures 1 and 2).

The effects of BM chimerism, ALC and viral infections on the risk of clinically relevant GvHD after DLI were estimated using separate multivariable Cox proportional hazards regression models for the transition from 'DLI1' to 'tIS after DLI1': 3 models were fitted for the low-dose 3-month DLI and two for the 6-month DLI (only chimerism and ALC). Since donor type and conditioning/TCD regimen have been recognized as important factors for GvHD after DLI[16], conditioning/donor type (MAC UD, RIC RD and RIC UD vs MAC RD) was included in all models, while BM chimerism (low MC and high MC vs FDC), ALC ($<500x10^6$/l and 500-$999x10^6$/l vs $\geq 1000x10^6$/l for the 3-month DLI or $<1000x10^6$/l vs $\geq 1000x10^6$/l for the 6-month DLI), or viral infection were added as the only other covariate per model. Viral infection was time-varying: patients could start as having no viral infection or as having an early viral infection if they had a viral infection during the last week before DLI. After DLI, the variable could change to 'early viral infection' at time of the first viral infection if this occurred within 2 weeks after DLI or to 'late-onset viral infection' at time of the first viral infection occurring beyond 2 weeks after DLI.

To identify risk factors for death during treatment for GvHD, univariable Cox proportional hazards regression models were fitted for the transition from 'tIS after DLI1' to 'death after tIS after DLI1' with either patient age at time of alloSCT or with the presence of early viral infection (3-month DLI) or high MC (6-month DLI). Two-sided p-values <0.05 were considered statistically significant for all Cox models. All models were based on complete cases only: patients with missing values for the included covariates were excluded.

To illustrate the impact of early viral infections on the outcome after the low-dose 3-month DLI, an extended version of the multi-state model was constructed with two starting states: 'DLI1 without early viral infection' for patients without any viral infection during the last week before DLI and 'DLI1 with early viral infection' for patients with a viral infection during this period (Supplemental Figure 3). To evaluate the impact of the identified transition-specific risk factors on the probability of cGRFS, the probability of being alive with GvHD, and the probability of death after start of tIS after the 6-month DLI, the Cox models for the two transitions were integrated as components in a multi-state model. This model was used to predict the outcomes after the 6-month DLI for reference patients with different baseline characteristics.

Software

All analyses were performed in R version 4.3.1 using the packages survival[26], prodlim[27], cmprsk[28], mstate[29], ggplot2[30], and ComplexUpset[31].

RESULTS

Cohort

388 patients were included in this study (Supplemental Table 1). Median follow-up after alloSCT was 76 months (interquartile range 32-110). 88 patients received the low-dose

3-month DLI prophylactically or pre-emptively at a median of 3.2 months after alloSCT (range 2.7-5.2) and 76 the 6-month DLI as first DLI at a median of 6.3 months after alloSCT (range 4.8-8.9; Table 1). 79 (20%) patients could not receive any DLI because of early relapse (n=44), death (n=23), or graft failure (n=12; Supplemental Figure 4). 66 (17%) other patients developed clinically relevant GvHD after alloSCT and therefore were not eligible for DLI. 42 patients received a modified T-cell product as part of a clinical study, and 9 received a DLI not according to our standard prophylactic/preemptive DLI protocol (different cell dose (n=6), DLI for a viral infection (n=2) or DLI in combination with interferon (n=1)). The remaining 28 patients did not receive any DLI within the first 9 months after alloSCT because of alloSCT before May 2010 (n=12), (temporary) donor unavailability (n=3) or physician's decision (n=13).

Similar incidences of GvHD after low-dose 3-month DLI and 6-month DLI

The 3-month cumulative incidence of clinically relevant GvHD was 28% (95%-CI 20-40) after the low-dose 3-month DLI and 30% (95%-CI 22-43) after the 6-month DLI.

	Low-dose 3-month DLI (N = 88)	**6-month DLI (N = 76)**
Age at alloSCT (years)		
median (range)	58 (18-74)	57 (19-76)
Disease		
AML	59 (67%)	56 (74%)
ALL	23 (26%)	9 (12%)
MDS	6 (7%)	11 (14%)
Conditioning		
MAC: Cyclo/TBI	35 (40%)	33 (43%)
MAC: Cyclo/Bu	1 (1%)	1 (1%)
RIC: Flu/Bu*	38 (43%)	42 (55%)
RIC: Flu/Bu/Ara-C/Amsa	14 (16%)	0
Donor		
RD	39 (44%)	30 (39%)
UD	49 (56%)	46 (61%)
Graft source		
G-CSF mobilized PBSC	84 (95%)	70 (92%)
BM	4 (5%)	6 (8%)
CMV serostatus patient/donor		
+/+	43 (49%)	33 (43%)
+/-	13 (15%)	12 (16%)
-/+	6 (7%)	4 (5%)
-/-	26 (30%)	27 (36%)
EBV serostatus patient/donor		
+/+	78 (89%)	59 (78%)
+/-	6 (7%)	7 (9%)
+/unknown	0	4 (5%)
-/+	3 (3%)	6 (8%)
-/-	1 (1%)	0

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The probability of death during tIS after one DLI was 15% (95%-CI 9-24) and 16% (95%-CI 9-27) at 12 months after the 3- and 6-month DLI, respectively (Supplemental Figures 5 and 6). Figures 2 and 3 show how the state probabilities add up to the overall survival, relapse-free survival, and cGRFS probabilities. For example, the cGRFS decreased during the first months after DLI but later increased as patients with GvHD could stop their tIS after the GvHD was resolved. Notably, none of the patients with GvHD after DLI relapsed, demonstrating the concomitant GvL effect. 1- and 5-year cGRFS probabilities were 55% (95%-CI 45-66) and 48% (95%-CI 38-61) after 3-month DLI and 57% (95%-CI 46-69) and 67% (95%-CI 57-79) after 6-month DLI, respectively. Together, these data show that the tenfold dose difference effectively equalized the

Main indication of first DLI		
ALL: t(9;22)	11 (12%)	-
ALL: hypodiploidy, complex karyotype, or t(4;11)	3 (3%)	-
ALL: high white blood cell count at diagnosis	4 (5%)	-
ALL: no CR1	2 (2%)	-
AML: monosomal karyotype	10 (11%)	-
AML: complex karyotype	1 (1%)	
AML/MDS: EV1 overexpression	15 (17%)	-
AML: ASXL mutation	2 (2%)	
AML: FLT3 mutation	1 (1%)	-
AML/MDS: FLAMSA regimen	14 (16%)	
AML: progression during remission-induction	1 (1%)	-
AML/MDS: no intensive treatment or no consolidation	4 (5%)	-
AML/MDS: persisting CMML	1 (1%)	-
MRD+ at time of alloSCT	11 (12%)	-
Preemptive for MC	8 (9%)	34 (45%)
Standard prophylactic DLI	-	42 (55%)
BM chimerism at time of first DLI		
FDC	28 (33%)	25 (34%)
Low MC (1-4% mixed chimerism)	32 (38%)	30 (41%)
High MC (≥5% mixed chimerism)	24 (29%)	19 (26%)
Unknown	4	2
ALC at time of first DLI (x10^6/l)		
≥1000	41 (47%)	45 (61%)
500-999	29 (33%)	20 (27%)
<500	17 (20%)	9 (12%)
Unknown	1	2

Table 1. Baseline characteristics of the patients who received either a low-dose 3-month DLI or 6-month DLI as first DLI. Characteristics are given at time of alloSCT unless otherwise indicated. DLI, donor lymphocyte infusion; alloSCT, allogeneic stem cell transplantation; AML, acute myeloid leukemia; ALL, acute lymphoblastic leukemia; MDS, myelodysplastic syndrome; MAC, myeloablative conditioning; RIC, reduced-intensity conditioning; Cyclo, cyclophosphamide; TBI, total body irradiation; Bu, busulfan; Flu, fludarabine; Ara-C, cytarabine; Amsa, amsacrine; RD, related donor; UD, unrelated donor; G-CSF, granulocyte-colony stimulation factor; PBSC, peripheral blood stem cells; BM, bone marrow; CMV, cytomegalovirus; EBV, Epstein-Barr virus; CR, complete morphological remission; CMML, chronic myelomonocytic leukemia; MRD, minimal residual disease; MC, mixed chimerism; FDC, full donor chimerism; ALC, absolute lymphocyte count. *One patient had not received a second consolidation course before transplant and received 2 days cyclophosphamide 750 mg/m^2 intravenously additionally to the conditioning regimen.

GvHD risk between low-dose 3-month DLI and 6-month DLI. Because 16% of patients died within 1 year after DLI during treatment for GvHD (Figures 2 and 3), we investigated risk factors for the development of clinically relevant GvHD and the occurrence of death during tIS.

Viral infections close to low-dose 3-month DLI increase the risk of GvHD after this DLI

First, we analyzed the low-dose 3-month DLI. To investigate whether the presence of patient-derived APCs in the BM increased the risk of GvHD after this DLI, we examined the chimerism model (Figure 4A). RIC patients with an UD had a hazard ratio (HR) of 3.2 (95%-CI 1.1-9.1) for developing GvHD compared to MAC RD patients. However, there was no significant effect of chimerism (p-values 0.9 and 0.8 for low and high MC compared to FDC, respectively) on the risk of clinically relevant GvHD after this DLI. To investigate whether lymphopenia increased the risk of GvHD after the 3-month DLI, we examined the ALC model (Figure 4B). Again, RIC UD was a significant

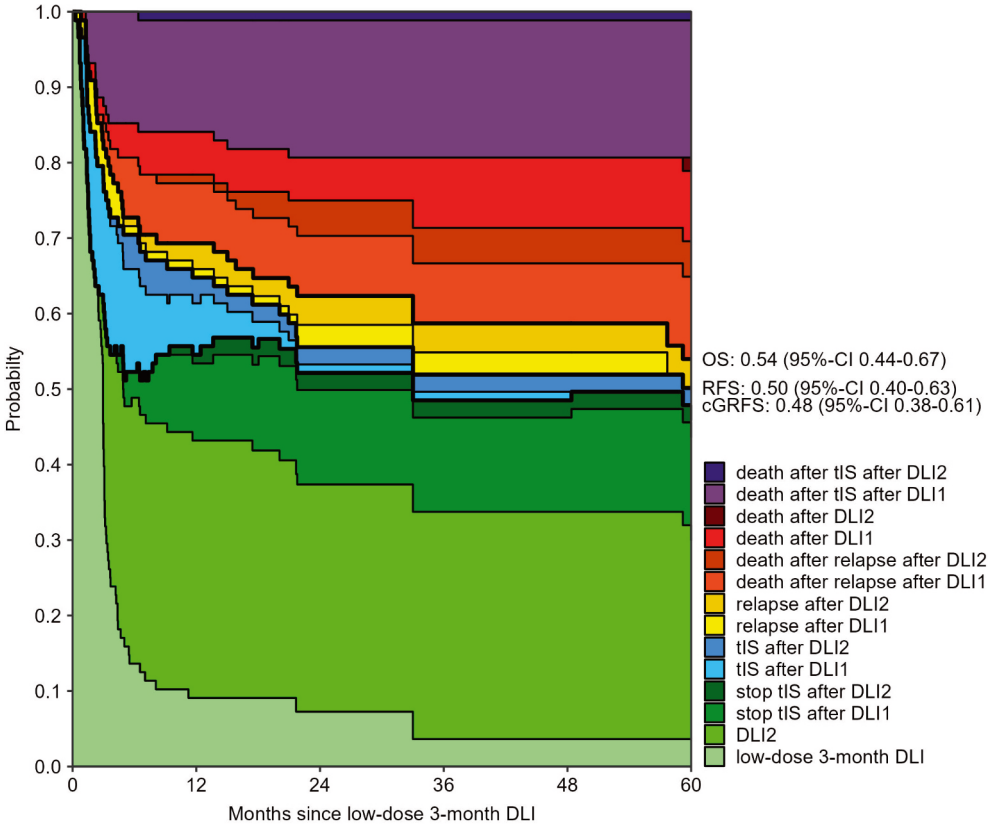

Figure 2. Outcomes after low-dose 3-month DLI. Stacked transition probabilities from state DLI1 (low-dose 3-month DLI) estimated in the non-parametric model in Supplemental Figure 1. The difference between two adjacent curves represents the probability of being in the corresponding state. 39 patients reached the second DLI as planned. Bold lines show the overall survival (OS), relapse-free survival (RFS) and current GvHD-relapse-free survival (cGRFS), of which the 5-year probabilities with 95%-CI are stated next to the figure.

risk factor while ALC showed no significant effect on GvHD after DLI (p-values 0.9 and 0.6 for ALC 500-999x10^6/l and <500x10^6/l compared to ≥1000x10^6/l, respectively). We then investigated the correlation between viral infections close to the 3-month DLI and the development of GvHD after DLI. 34 of the 88 patients with a 3-month DLI had a viral infection within the last week before and first 8 weeks after DLI: 28 had an early viral infection (25 before or at time of DLI and 3 within 2 weeks after DLI) and 6 a late-onset viral infection (>2 weeks after DLI). Most common pathogens were cytomegalovirus (CMV; n=15), adenovirus (n=7) and Epstein-Barr virus (EBV; n=5; Supplemental Figure 7A). The model with viral infection revealed that patients with an early viral infection had a HR of 3.7 (95%-CI 1.7-7.9) for developing clinically relevant GvHD compared to those without any viral infection (Figure 4C). Patients with a late-onset viral infection did not have a higher risk of GvHD (p-value 0.7).

Since the ALC at time of the low-dose 3-month DLI was higher in patients with a viral infection (Supplemental Figure 8), viral infections may have confounded the correlation between ALC and GvHD. Therefore, to explore whether ALC is a risk factor for GvHD

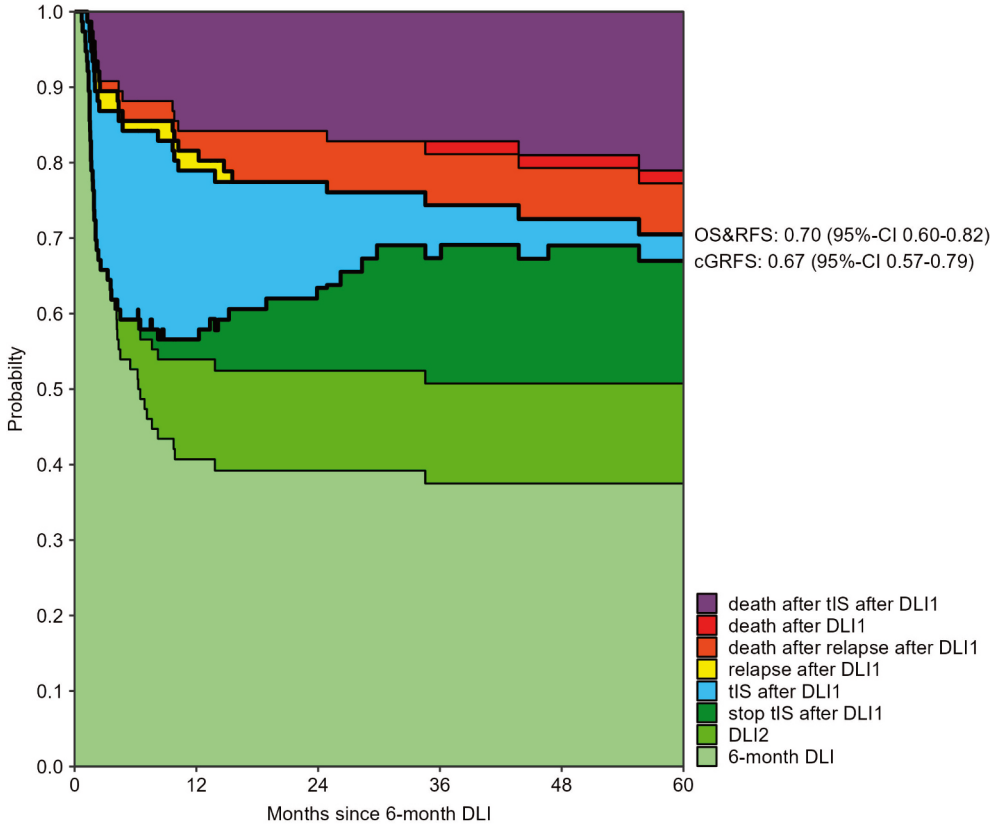

Figure 3. Outcomes after 6-month DLI. Stacked transition probabilities from state DLI1 (6-month DLI) estimated in the non-parametric model in Supplemental Figure 2. The difference between two adjacent curves represents the probability of being in the corresponding state. Nine patients required a second DLI because of MC. The legend only shows the states which were occupied within 5 years after the 6-month DLI. Bold lines show the overall survival (OS), relapse-free survival (RFS) and current GvHD-relapse-free survival (cGRFS), of which the 5-year probabilities with 95%-CI are stated next to the figure.

in the absence of viral infections, we compared the cumulative incidences of tIS for GvHD between ALC <1000x10^6/l and ≥1000x10^6/l in the 63 patients without any viral infection during the last week before the 3-month DLI. As we did not observe a significant difference (Supplemental Figure 9), there was no clear indication that viral infection acted as confounding factor. Together, these data show that viral infections close to the low-dose 3-month DLI increased the alloreactivity of this DLI leading to significantly more clinically relevant GvHD.

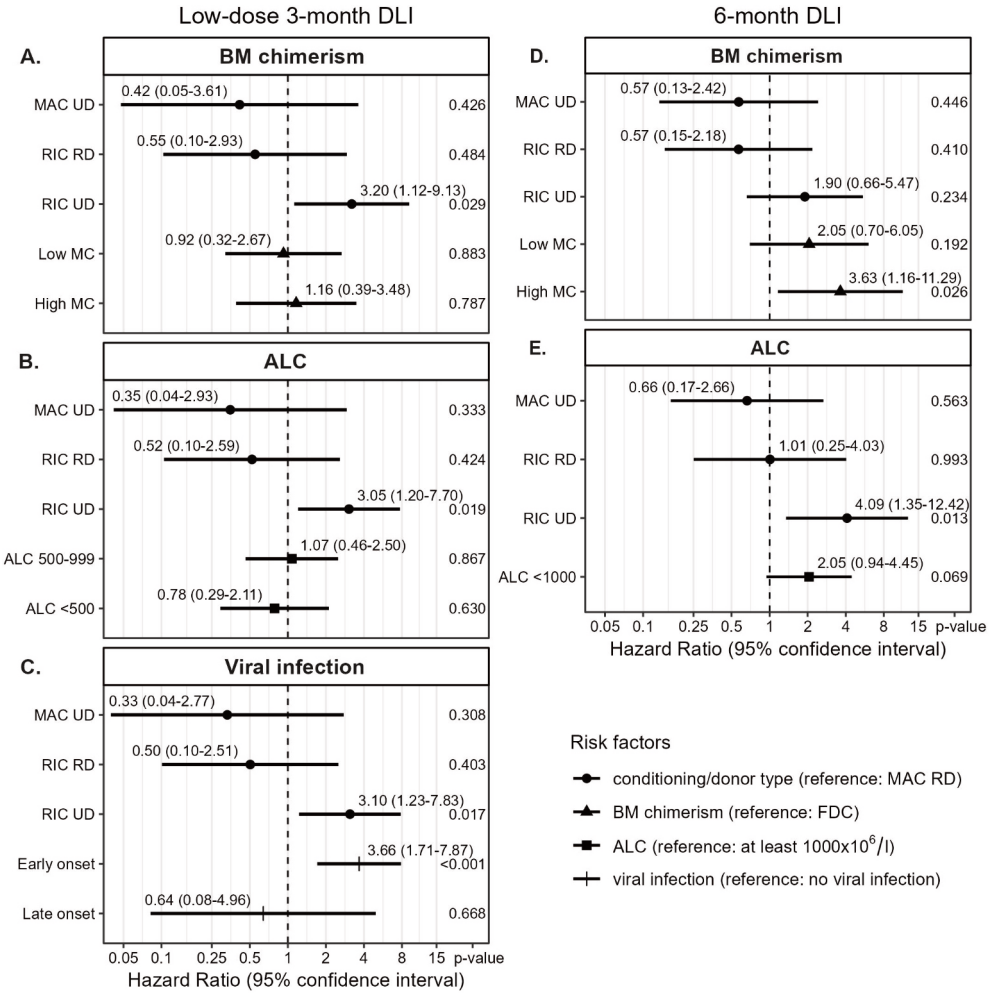

Figure 4. Cox proportional hazards models for the transition from first DLI to requiring tIS for GvHD (see Figure 1). Based on complete case analysis (A: n=84, B: n=87, C: n=88, D and E: n=74). Viral infection was treated as a time-varying covariate. DLI, donor lymphocyte infusion; BM, bone marrow; MAC, myeloablative conditioning; RIC, reduced-intensity conditioning; UD, unrelated donor; RD, related donor; low MC, 1-4% mixed chimerism; high MC, ≥5% mixed chimerism; FDC, full donor chimerism (no patient material detectable); ALC, absolute lymphocyte count (x10^6/l)

Mixed BM chimerism and lymphopenia increase the risk of GvHD after the 6-month DLI

We then investigated which risk factors were associated with the alloreactivity of the 6-month DLI. Viral infections were uncommon at the time of this DLI: of the 76 patients receiving this DLI, only 11 had a viral infection (3 early and 8 late-onset), most often EBV (n=3; Supplemental Figure 7B). The presence of high MC in the BM at time of DLI was a strong predictor for GvHD with a HR of 3.6 (95%-CI 1.2-11.3) compared to FDC, while patients with low MC had a nonsignificant higher risk of GvHD (HR 2.1, 95%-CI 0.7-6.1, p-value 0.19, Figure 4D). In the ALC model (Figure 4E), RIC UD was a significant risk factor for GvHD (HR 4.1, 95%-CI 1.3-12.4 compared to MAC RD). Additionally, a trend was observed for higher GvHD risk in lymphopenic patients compared to ALC $\geq 1000 \times 10^6$/l (HR 2.1, 95%-CI 0.9-4.5, p-value 0.07). Together, these data show for both the low-dose 3-month DLI and the 6-month DLI, with 50% dose reduction in case of an UD, comparable risks of GvHD between patients with RD and UD after MAC but not RIC. The data indicate that mixed BM chimerism increased the risk of clinically relevant GvHD after the 6-month DLI, and suggest a similar effect of lymphopenia.

Risk factors for death during treatment for GvHD after DLI

To identify risk factors for death during tIS for GvHD (Supplemental Figure 10), we first investigated the effect of patient age. As expected, older patients seemed to have a higher risk of dying from severe GvHD after the 6-month DLI (HR 2.1 per decade, 95%-CI 0.9-5.1, p-value 0.10). Remarkably, we did not observe this association after the low-dose 3-month DLI (p-value 0.7).

Next we investigated whether the main risk factors for clinically relevant GvHD also correlated with the risk of death among those who required treatment for GvHD. For the low-dose 3-month DLI, we considered the presence of an early viral infection. We observed a nonsignificant increase in the risk of dying during tIS for GvHD for patients with an early viral infection compared to those without an early viral infection close to DLI (HR 1.8, 95%-CI 0.6-5.6, p-value 0.28, Supplemental Figure 11). For the 6-month DLI we considered the presence of high mixed BM chimerism at time of DLI. Patients with high MC had a nonsignificant higher risk of death during tIS for GvHD compared to those with GvHD who had FDC or low MC at time of DLI (HR 2.0, 95%-CI 0.6-6.4, p-value 0.23, Supplemental Figure 12). In conclusion, among those who required tIS for GvHD, older patients had a higher risk of dying during treatment after the 6-month but not the low-dose 3-month DLI. We did not observe significant associations between the risk of death during tIS and BM chimerism or viral infections. However, only one of the 53 patients with FDC at time of the low-dose 3-month DLI or 6-month DLI developed lethal GvHD.

Impact of early viral infection and mixed BM chimerism on the cGRFS after the low-dose 3-month DLI and 6-month DLI

The probability of having clinically relevant GvHD at 6 months after the 3-month DLI was 15% (95%-CI 9-26) for the patients without any viral infection during the last week before DLI compared to 25% (95%-CI 14-46) for the patients with a viral infection

(Figure 5). The probability of death after start of tIS was 8% (95%-CI 4-17) compared to 32% (95%-CI 19-55), respectively. The cGRFS was 61% (95%-CI 50-73) and 31% (95%-CI 19-52), respectively.

For a MAC patient receiving a 6-month DLI from a RD, the predicted probability of having clinically relevant GvHD at 6 months after DLI was 14% (95%-CI 5-44) if the patient had FDC compared to 30% (95%-CI 11-80) if the patient had high MC, respectively (Figure 6). The probability of death after start of tIS was 4% (95%-CI 1-16) and 23% (95%-CI 9-58), respectively. The cGRFS for these reference patients was 77% (95%-CI 60-98) and 44% (95%-CI 19-100) at 6 months after DLI, respectively.

DISCUSSION

In this retrospective study we investigated the outcomes after prophylactic and preemptive DLI following alemtuzumab-based TCD alloSCT. The tenfold dose difference between the 3- and 6-month DLI resulted in comparable risks of GvHD. For both DLIs, the 50% dose reduction in case of an UD sufficed for patients with MAC but not RIC. We demonstrate that the risk factors for GvHD after DLI depend on the setting of the DLI: at time of the 3-month DLI, the occurrence of viral infections played a major role, while for the 6-month DLI the presence of high MC in the BM was an important risk factor. The strong impact of both factors on cGRFS underlines the clinical relevance of these findings. Additionally, we observed trends for higher GvHD risk in patients with low MC or lymphopenia at time of the 6-month DLI. The very low

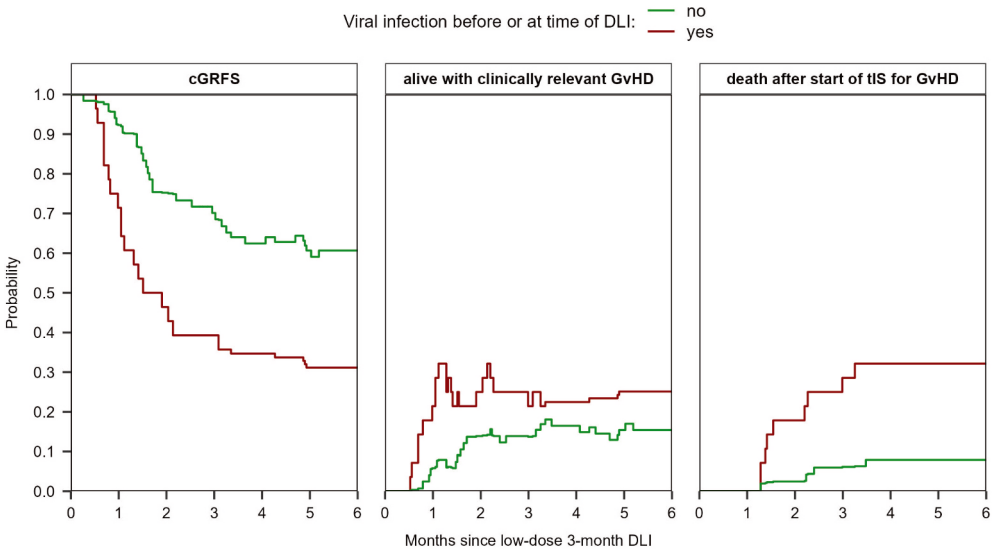

Figure 5. Estimated probabilities of cGRFS, being alive with clinically relevant GvHD, and of death after start of tIS for GvHD after the low-dose 3-month DLI based on the viral status at time of DLI (viral infection during the last week before DLI (n=25) or no viral infection until DLI (n=63)). The estimates are based on the non-parametric multi-state model in Supplemental Figure 3 which has two starting states ('DLI1 without early viral infection' and 'DLI1 with early viral infection'). See Supplemental Figure 13 for the probabilities of all states separately.

risk of lethal GvHD for patients with FDC at time of either DLI provides further evidence for the important role of patient-derived APCs and demonstrates the safety of DLI in these patients, consistent with the matched-pair analysis by Schmid et al.[32]

Viral infection and the concomitant antiviral immune response lead to tissue damage and upregulation of HLA class II expression by non-hematopoietic cells, and induce a pro-inflammatory environment promoting activation of professional APCs and immune cells. Miller et al. showed that the occurrence of any infection (bacterial, viral or fungal) increased the risk of acute GvHD after alloSCT.[33] We only considered viral infections, since these were most common in the relevant time period and most of the patients with a bacterial or fungal infection had a viral infection at the same time (data not shown). Other studies have reported associations specifically between CMV and GvHD.[19,34,35] Previously, we demonstrated activation of alloreactive HLA-DP1-specific CD4+ T cells leading to GvHD in two patients with a CMV reactivation after a CD4+ T-cell infusion from an HLA-DP1 mismatched donor.[19] Since about 80% of the patients with a 10/10 HLA-matched unrelated donor are HLA-DP mismatched[36,37] and CMV was the most common pathogen, this mechanism could play a role in our cohort. Due to the limited number of events, we could not differentiate between the different viral pathogens.

While the role of patient-derived professional APCs in the induction of alloreactivity has been clearly demonstrated in mice[38-41], results of human studies are conflicting.[42-48] This may be due to the cell subsets used for the chimerism measurement, possible bias by overrepresentation of patients with multiple DLIs, and the clinical setting. For example, Bar et al.[48] did not observe a significant correlation between BM chimerism and GvHD

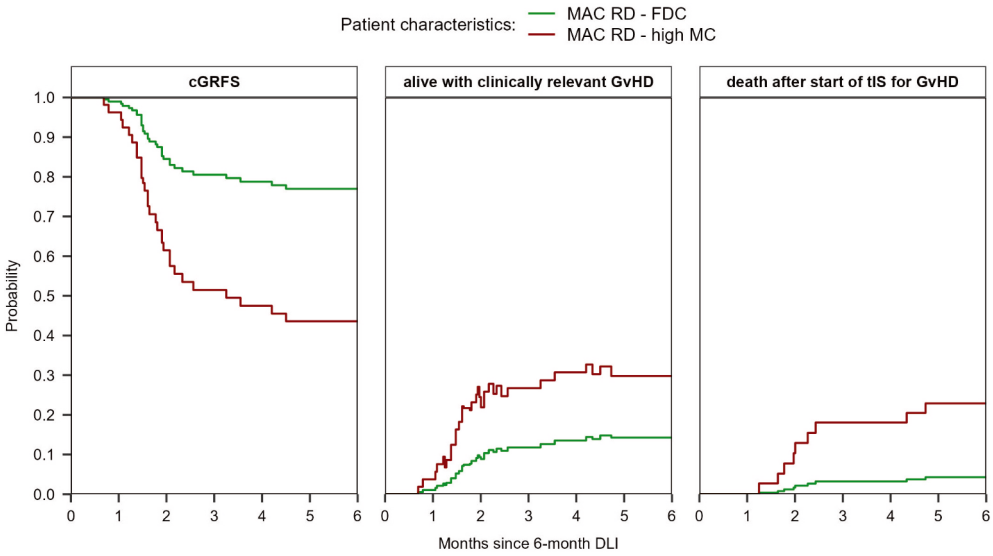

Figure 6. Prediction of cGRFS, being alive with clinically relevant GvHD, and of death after start of tIS for GvHD after the 6-month DLI for reference patients with different characteristics. The prediction is based on the multi-state model in Supplemental Figure 2 with semi-parametric transition-specific proportional hazards models with BM chimerism and conditioning/donor combination as covariates for the transition from 'DLI1' to 'tIS for GvHD after DLI1' and BM chimerism (high MC vs other) for the transition from 'tIS for GvHD after DLI1' to 'death after tIS after DLI1'. No covariates were assessed for the other transitions of the model.

(HR 1.26, p-value 0.46). They however analyzed therapeutic DLI in patients who often received disease-specific treatment or cytoreduction before DLI, which most likely resulted in a more pro-inflammatory environment at time of DLI. Under these circumstances, non-hematopoietic tissues from the patient express HLA class II molecules and can act as APCs to activate donor-derived alloreactive T cells.[49,50] The presence of a pro-inflammatory environment may also be an explanation for the absent association between BM chimerism and GvHD after the 3-month DLI, as tissue damage from the conditioning and recent viral infections may still be present. Another explanation may lie in the persistence of professional patient-derived APCs in the peripheral tissues at that time. The replacement of these APCs lags behind the donor-derived BM repopulation, as long as GvHD and severe inflammation as caused by myeloablative conditioning are absent.[51-53]

The relation between lymphopenia and alloreactivity of DLI has mostly been investigated in relapsed patients who often received (lymphodepleting) chemotherapy before DLI.[44,48,54] In this context, the effects of tissue damage and APC activation interfere with estimating the effect of the lymphopenia itself on the risk of GvHD. In our setting, patients received their DLI in the absence of relapse, tissue damage and chemotherapy. Here, we observed a trend for higher GvHD risk in lymphopenic patients at time of the 6-month DLI, but not at time of the 3-month DLI.

Multi-state modeling allowed us to not only estimate the effects of risk factors on the development of GvHD and death during treatment, but also assess the impact of these factors on the probabilities of different outcomes after DLI while taking into account the hazards of all clinical events. This is a major advantage compared to less advanced statistical methods since these probabilities are more relevant for patients than HRs. Multi-state models can capture recovery after GvHD and thereby model the current GvHD burden over time, which makes cGRFS a better estimate of treatment success than GvHD-relapse-free survival.[11,12] In 2016, we introduced the endpoint treatment success, which equals cGRFS.[10] During the last years, cGRFS and current immunosuppression-relapse-free survival have become more popular as outcome measures.[11,12,55-58] However, to our knowledge, we are the first who have applied semi-parametric multi-state modeling in this context. For this, detailed data collection regarding posttransplant events and interventions as performed in this study is essential.

Our observations may eventually lead to refinement of the DLI strategy. In the prophylactic or preemptive setting, there is room to lower the initial DLI dose, delay the DLI or start immunosuppressive treatment on early signs of GvHD based on the anticipated risk of severe GvHD. Before implementation, our results should be validated in other clinical settings, since BM chimerism, ALC and viral infections all depend on the conditioning, donor and use or method of TCD.[59-61] Larger cohorts with more events will allow for more precise prediction of alloimmune responses after DLI, not only GvHD but also the prevention of relapse. Especially the effect of BM chimerism on the risk of relapse should be investigated to confirm the correlation between MC and alloreactivity after DLI. If this is the case, the presence of MC at time of DLI can be considered for the determination of the dose of prophylactic or preemptive DLI.

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SUPPLEMENTAL METHODS

Transplantation protocol

Myeloablative conditioning (MAC) consisted of cyclophosphamide (2 days 60 mg/kg intravenously) combined with either 9 Gy total body irradiation or busulfan (4 days 4x0.8 mg/kg intravenously). Reduced-intensity conditioning (RIC) consisted either of fludarabine (6 days 50 mg/m^2 orally or 30 mg/m^2 intravenously) and busulfan (2 days 4x0.8 mg/kg intravenously), or the FLAMSA regimen: fludarabine (5 days 30 mg/m^2 intravenously), cytarabine (4 days 2000 mg/m^2 intravenously), amsacrine (4 days 100 mg/m^2 intravenously) and busulfan (4 days 4x0.8 mg/kg intravenously).

Standard *in vitro* TCD was performed by adding 20 mg alemtuzumab (Sanofi Genzyme) to the graft. (1) Additional *in vivo* TCD depended on the donor type and conditioning regimen: MAC patients with a RD did not receive any *in vivo* TCD. All other patients received 15 mg alemtuzumab intravenously on days -6 and -5 (MAC) or on days -4 and -3 (RIC). Before June 2007, RIC patients with an UD received 10 mg/kg horse-derived anti-thymocyte globulin (Lymphoglobulin, Genzyme) additionally on day -4 until day -1. After Lymphoglobulin was withdrawn from the market, RIC patients with an UD first received no anti-thymocyte globulin (alloSCT between June 2007 and September 2009) and later received rabbit-derived anti-thymocyte globulin (Thymoglobulin, Sanofi Genzyme) additionally on day -2 (until April 2010 2mg/kg and thereafter 1mg/kg). Only MAC patients with an UD received posttransplant ciclosporin as GvHD prophylaxis, which was tapered from 1 month with the aim to stop within 3 months after alloSCT.

BM chimerism, ALC and viral infections

For the BM chimerism at time of DLI, we used the BM sample that was closest to DLI and taken within 5 weeks before and 1 week after DLI. BM chimerism was measured in total BM leukocytes by short-tandem-repeat PCR or, for patients transplanted before 2007 with a sex-mismatched donor, by FISH analysis using Vysis CEP X/Y probes. The lower detection limit of the chimerism analyses was 1-2%, depending on the method and the selected markers. For patients without any evaluable BM chimerism measurement during this period but whose last measurement before and first measurement after DLI belonged to the same chimerism category (FDC, low MC or high MC), this category was taken as the BM chimerism status at time of DLI.

ALC was calculated by the sum of the absolute numbers of circulating T cells, B cells and NK cells as measured on anticoagulated fresh venous blood by flow cytometry with bead calibration (Trucount tubes, Becton Dickinson) with a lower detection limit of $0.5x10^6$ cells/l. If these counts were unavailable, the lymphocyte count by manual blood smear was used. For the ALC at time of DLI, the closest measurement within 2 weeks before and 1 week after DLI was taken. For patients without any ALC measurement during this period but whose last ALC before and first ALC after DLI belonged to the same category (<500, 500-999 or $\geq 1000x10^6$/l for the low-dose 3-month DLI and <1000 or $\geq 1000x10^6$/l for the 6-month DLI), this category was taken as the ALC at time of DLI.

Cytomegalovirus (CMV) and Epstein-Barr virus (EBV) were monitored weekly by PCR on peripheral blood samples in all patients. Single positive values of CMV or EBV below log 2.4 were not considered. PCRs on other viruses were only performed in symptomatic patients. For the analyses, only the first viral infection was used.

Multi-state modelling

In a multi-state model patients move between states at the occurrence of clinical events or treatments. Transitions define which routes between states are allowed (for instance the transition from the state DLI to the state GvHD). (2) In a Markov model, the hazard of making a certain transition only depends on the current state and the time since start, which is in this case the first DLI. Each transition hazard can either be estimated without taking covariates into account (non-parametrically) or can be analyzed by means of a transition-specific Cox proportional hazards model (semi-parametric approach). The baseline hazards and the hazard ratios are the building blocks for the calculation of the transition probabilities, which represent the probabilities of being in each of the states over time. For example, in a semi-parametric model the probability of being alive with GvHD depends on the baseline hazard of GvHD, the effects of risk factors for GvHD, and the risks of death and disappearance of GvHD symptoms. Confidence intervals for the probabilities of cGRFS, death after start of tIS, being alive with clinically relevant GvHD, RFS and OS were calculated based on the estimated variance-covariance matrix of all transition probabilities.

References

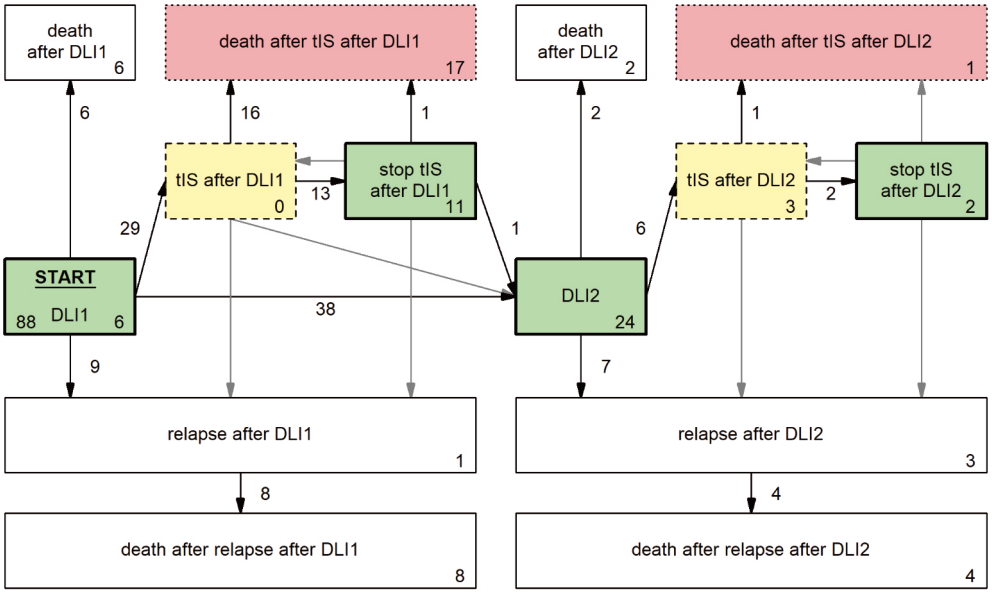
1. von dem Borne, P. A., Beaumont, F., Starrenburg, C. W. et al. Outcomes after myeloablative unrelated donor stem cell transplantation using both in vitro and in vivo T-cell depletion with alemtuzumab. Haematologica 91, 1559-1562 (2006).
2. Putter, H., Fiocco, M. & Geskus, R. B. Tutorial in biostatistics: competing risks and multi-state models. Stat Med 26, 2389-2430 (2007). doi: 10.1002/sim.2712

SUPPLEMENTAL TABLES

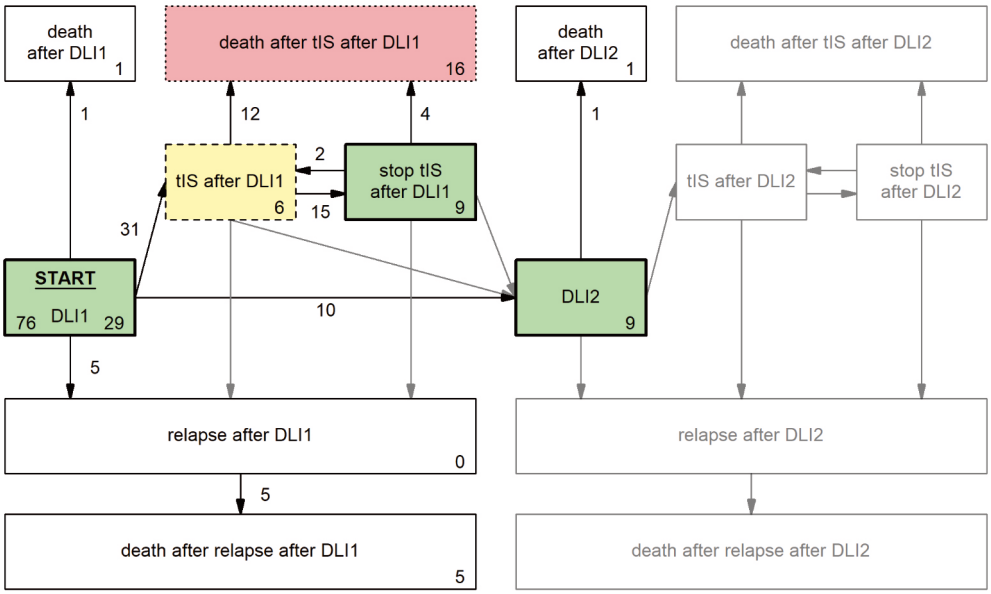
	Total cohort (all included patients with alloSCT; N = 388)
Age at alloSCT (years)	
median (range)	54 (18-78)
Disease	
acute myeloid leukemia	260 (67%)
acute lymphoblastic leukemia	85 (22%)
myelodysplastic syndrome	43 (11%)
Conditioning	
MAC: Cyclo/TBI	196 (51%)
MAC: Cyclo/Bu	9 (2%)
RIC: Flu/Bu*	167 (43%)
RIC: Flu/Bu/Ara-C/Amsa	16 (4%)
Donor	
RD	165 (43%)
UD	223 (57%)
Graft source	
G-CSF mobilized PBSC	368 (95%)
BM	20 (5%)
CMV serostatus patient/donor	
+/+	169 (44%)
+/-	70 (18%)
-/+	29 (7%)
-/-	120 (31%)
EBV serostatus patient/donor	
+/+	323 (83%)
+/-	30 (8%)
+/unknown	15 (4%)
-/+	18 (5%)
-/-	2 (1%)

Supplemental Table 1. Baseline characteristics of all included patients who received an alloSCT. alloSCT, allogeneic stem cell transplantation; MAC, myeloablative conditioning; RIC, reduced-intensity conditioning; Cyclo, cyclophosphamide; TBI, total body irradiation; Bu, busulfan; Flu, fludarabine; Ara-C, cytarabine; Amsa, amsacrine; RD, related donor; UD, unrelated donor; G-CSF, granulocyte-colony stimulation factor; PBSC, peripheral blood stem cells; BM, bone marrow; CMV, cytomegalovirus; EBV, Epstein-Barr virus. *One patient had not received a second consolidation course before transplant and received 2 days cyclophosphamide 750 mg/m^2 intravenously additionally to the conditioning regimen.

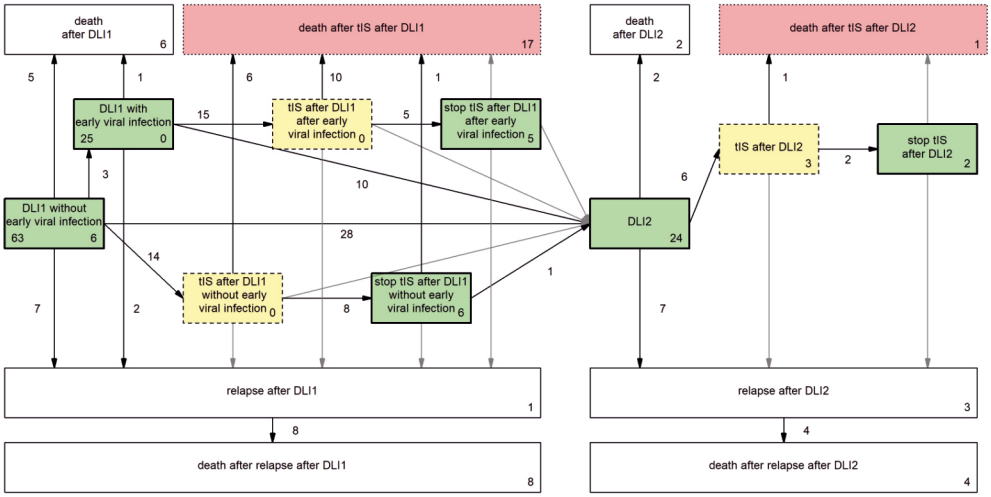
SUPPLEMENTAL FIGURES



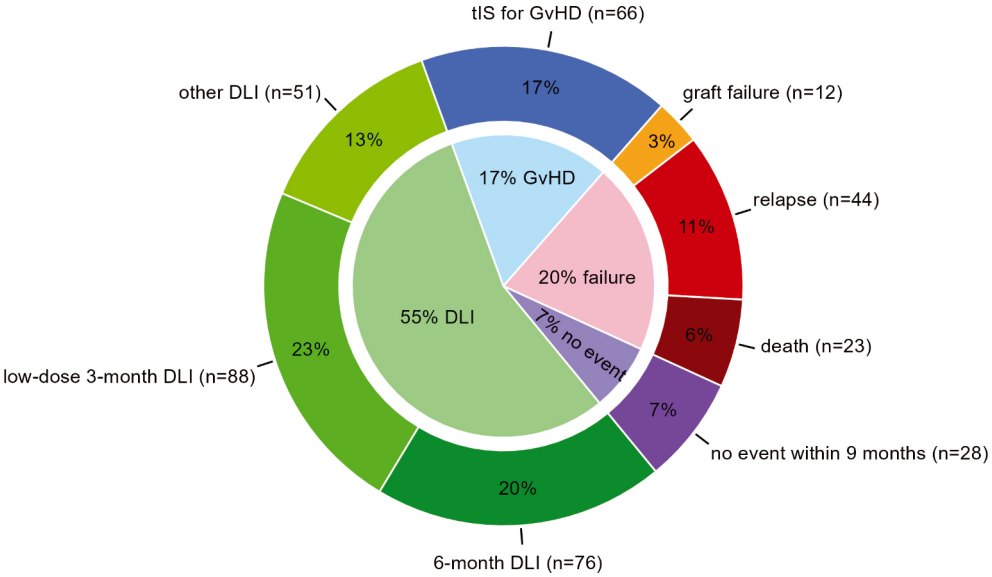
Supplemental Figure 1. Multi-state model for low-dose 3-month DLI. Boxes represent states and arrows represent the transitions between the states. Grey transitions were not used by any of the included patients and omitted from the final model. All patients started in the state 'DLI1'. The number at the bottom left corner of the starting state shows the number of patients included in the model. The numbers at the bottom right corner of the boxes show the numbers of the patients who were in that state at the end of their follow-up. The numbers next to the arrows show the numbers of the patients who made that transition during their follow-up. The cGRFS is the sum of the probabilities of all green (thick border) states, the probability of being alive with GvHD the sum of all yellow (dashed border) states, the probability of death after start of tIS for GvHD the sum of all red (dotted border) states, the RFS is the sum of all green (thick border) and yellow (dashed border) states, and the OS the sum of all non-death states. For these summarizing measures, no distinction was made between states after the first DLI or after multiple DLIs.



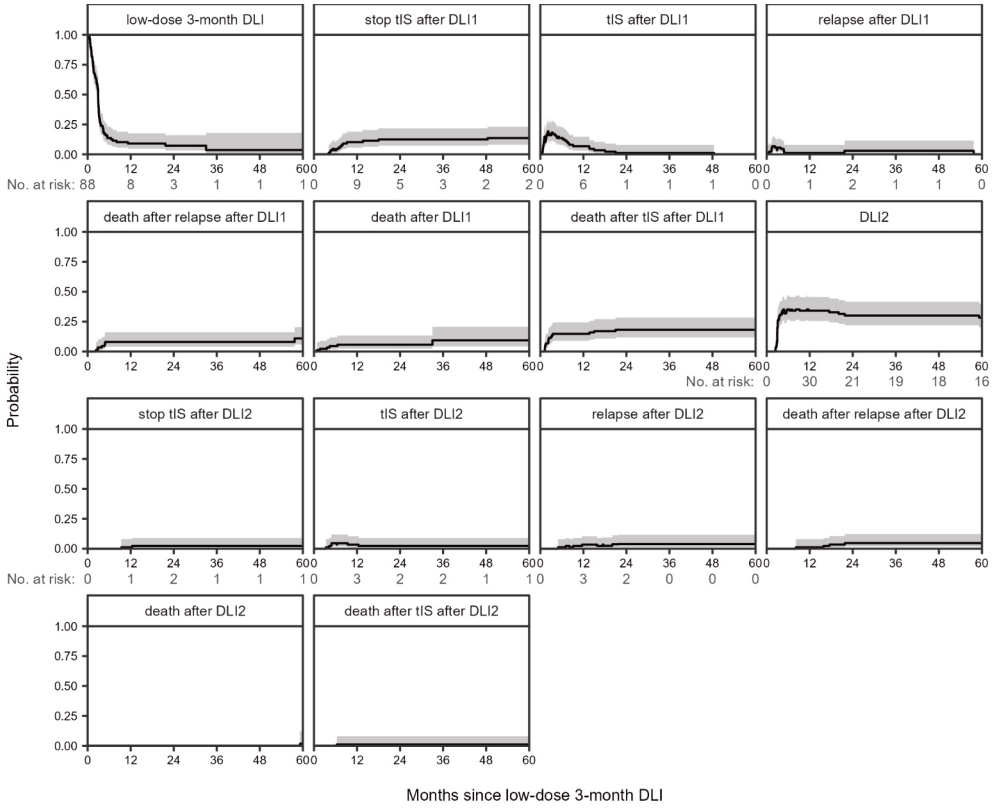
Supplemental Figure 2. Multi-state model for 6-month DLI. Boxes represent states and arrows represent the transitions between the states. Grey states and transitions were not used by any of the included patients and omitted from the final model. All patients started in the state 'DLI1'. The number at the bottom left corner of the starting state shows the number of patients included in the model. The numbers at the bottom right corner of the boxes show the numbers of the patients who were in that state at the end of their follow-up. The numbers next to the arrows show the numbers of the patients who made that transition during their follow-up. The cGRFS is the sum of the probabilities of all green (thick border) states, the probability of being alive with GvHD the sum of all yellow (dashed border) states, the probability of death after start of tIS for GvHD the sum of all red (dotted border) states, the RFS the sum of all green (thick border) and yellow (dashed border) states, and the OS the sum of all non-death states. For these summarizing measures, no distinction was made between states after the first DLI or after multiple DLIs.



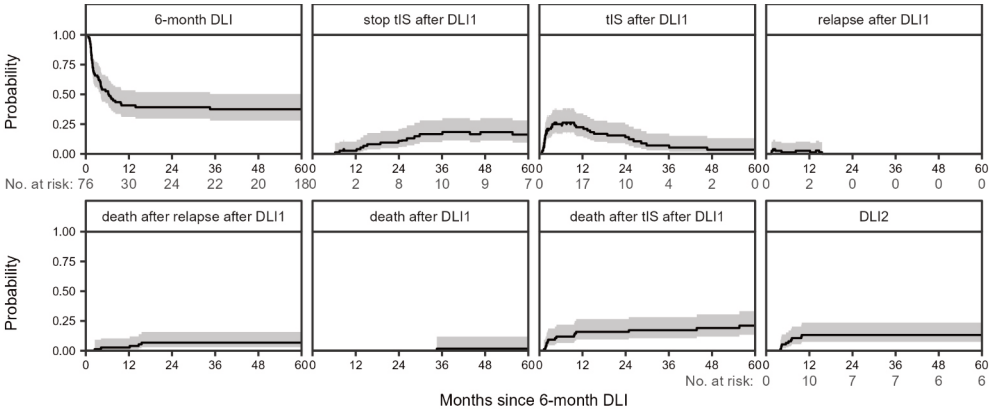
Supplemental Figure 3. Multi-state model for low-dose 3-month DLI considering early viral infections. Boxes represent states and arrows represent the transitions between the states. Grey transitions were not used by any of the included patients and omitted from the final model. 63 patients had no viral infection during the last week before DLI and started in the state 'DLI1 without early viral infection', while the 25 patients with a viral infection during the last week before DLI started in the state 'DLI1 with early viral infection' (see the numbers at the bottom left corner of the two starting states). Patients who had an early viral infection during the first 2 weeks after DLI without any prior event moved from 'DLI1 without early viral infection' to 'DLI1 with early viral infection' at time of the viral infection. The numbers at the bottom right corner of the boxes show the numbers of the patients who were in that state at the end of their follow-up. The numbers next to the arrows show the numbers of the patients who made that transition during their follow-up. The cGRFS is the sum of the probabilities of all green (thick border) states, the probability of being alive with GvHD the sum of all yellow (dashed border) states, the probability of death after start of tIS for GvHD the sum of all red (dotted border) states, the RFS the sum of all green (thick border) and yellow (dashed border) states, and the OS the sum of all non-death states. For these summarizing measures, no distinction was made between states after the first DLI or after multiple DLIs.



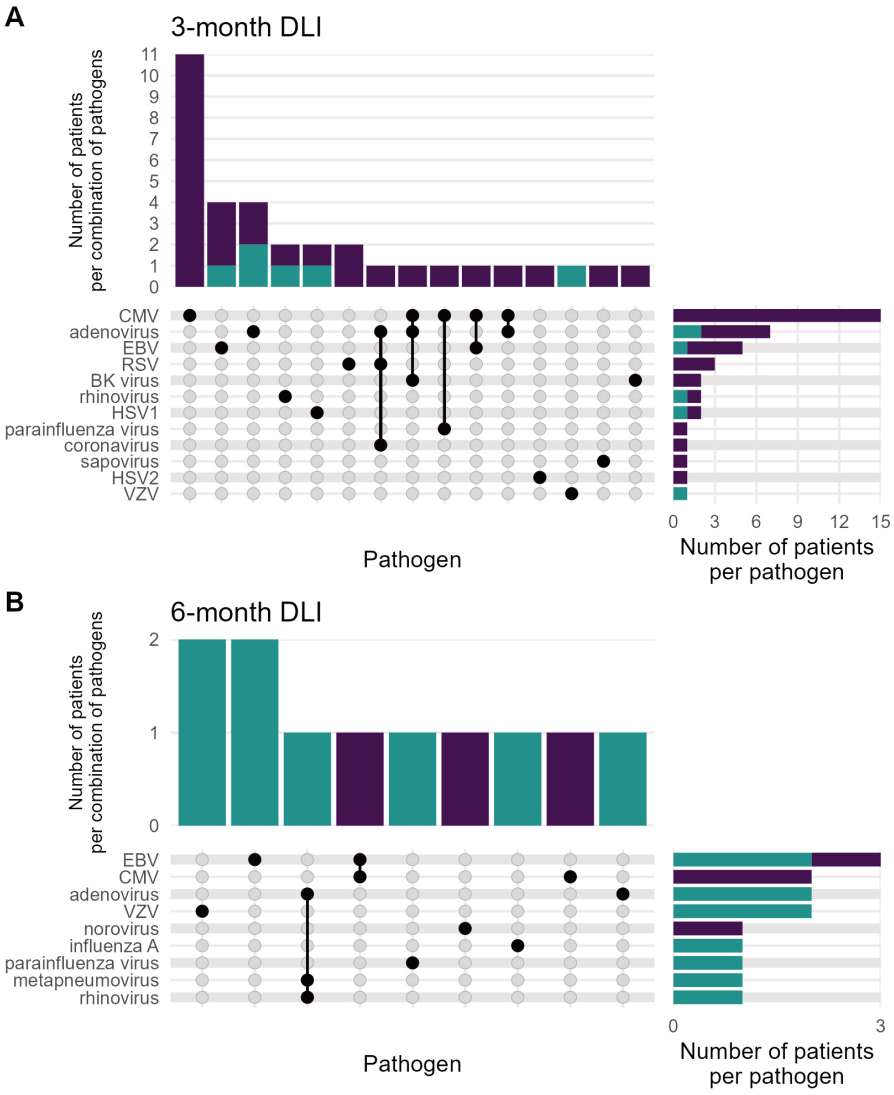
Supplemental Figure 4. Selection of the DLI cohorts. Events during the first 9 months after alloSCT for the total cohort. Per patient only the first occurring event was taken into account. The inner circle describes the main event categories (DLI, GvHD, treatment failure (i.e., death, relapse or graft failure), no event), while the outer circle further specifies the kind of DLI or treatment failure. The 88 patients who received the low-dose 3-month DLI and the 76 patients who received the 6-month DLI as first DLI were included in the DLI analyses.



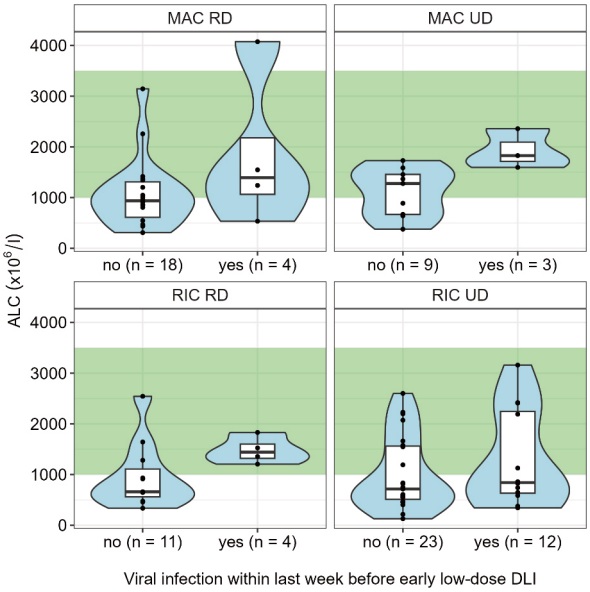
Supplemental Figure 5. Outcomes after low-dose 3-month DLI: probabilities with associated 95% confidence intervals per state. Probabilities with associated 95% confidence intervals for each state. The at risk numbers are shown for all non-death states and indicate the numbers of uncensored patients present in each state at different timepoints.



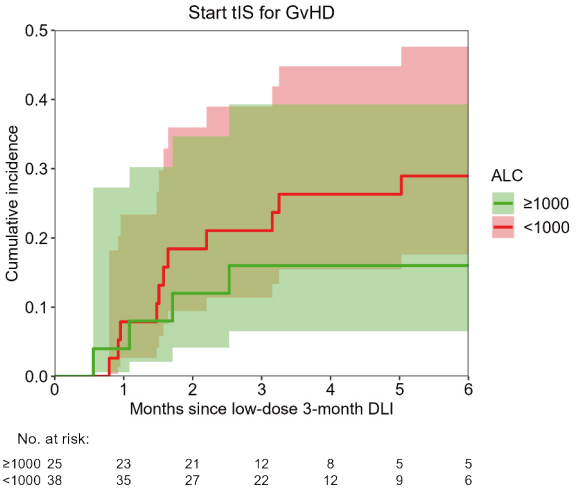
Supplemental Figure 6. Outcomes after 6-month DLI: probabilities with associated 95% confidence intervals per state. Probabilities with associated 95% confidence intervals for each state. The at risk numbers are shown for all non-death states and indicate the numbers of uncensored patients present in each state at different timepoints. Only states that were occupied within 5 years after 6-month DLI are shown.



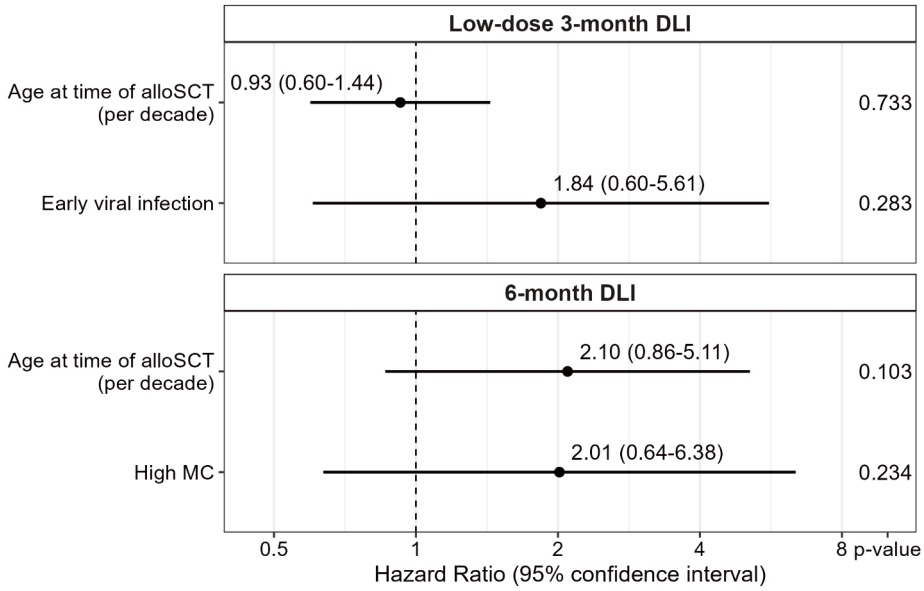
Supplemental Figure 7. Pathogens of viral infections close to the first DLI. UpSet plots of all viral pathogens present within 1 week before and 8 weeks after the low-dose 3-month DLI (panel A) or the 6-month DLI (panel B). The horizontal bar charts show for each of the pathogens the number of patients with this pathogen. As can be seen by the dot-connecting lines, some patients had multiple pathogens during this period. The vertical bar charts show the numbers of patients for each of the combinations. Purple indicates early onset (<2 weeks after DLI) viral infections, turquoise late onset (>2 weeks after DLI) infections. For instance, 3 patients had an EBV viremia close to the 6-month DLI, of whom two beyond 2 weeks after DLI without any other pathogen. The other patient had an early EBV viremia and a CMV viremia.



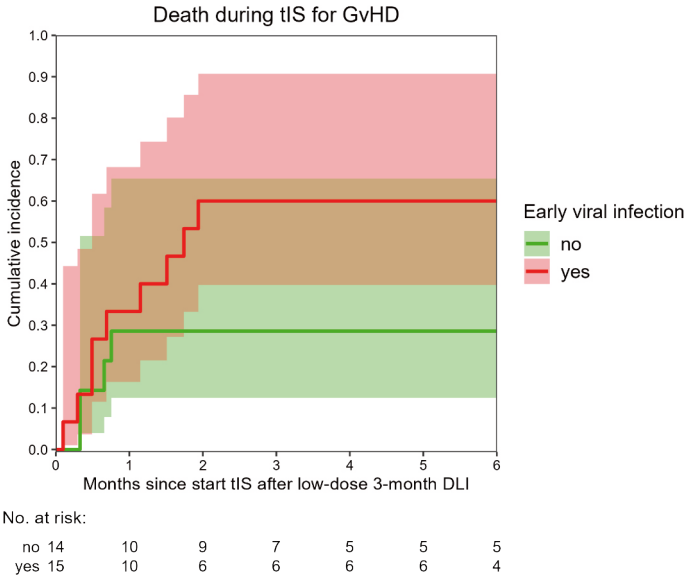
Supplemental Figure 8. ALC per conditioning/donor type and viral status at time of low-dose 3-month DLI. ALC at time of low-dose 3-month DLI per conditioning/donor type and the presence of a viral infection within the last week before this DLI. The boxplots are combined with violin plots showing the kernel probability density to visualize the distribution of the data. The lower and upper hinges of the boxplots correspond to the 25th and 75th percentiles. The green area shows the normal range used in our laboratory. Four patients for whom the exact ALC at time of DLI was unknown, were excluded (1 MAC RD without viral infection, 1 MAC RD with viral infection, 1 RIC UD without viral infection, 1 RIC UD with viral infection).



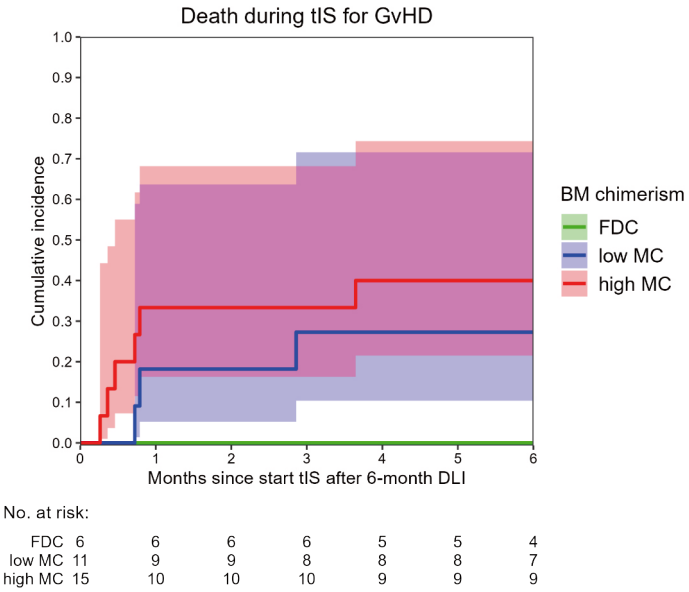
Supplemental Figure 9. Cumulative incidence of tIS for GvHD after low-dose 3-month DLI in the absence of viral infections within the last week before DLI. Cumulative incidences with associated 95% confidence intervals of tIS for GvHD after the low-dose 3-month DLI for patients with ALC ≥1000x10^6/l (n=25) or lower (n=38). This was calculated in a competing risks model starting at time of low-dose 3-month DLI with start tIS, relapse, death and DLI2 as competing events. The 25 patients with a viral infection during the last week before the low-dose 3-month DLI were excluded.



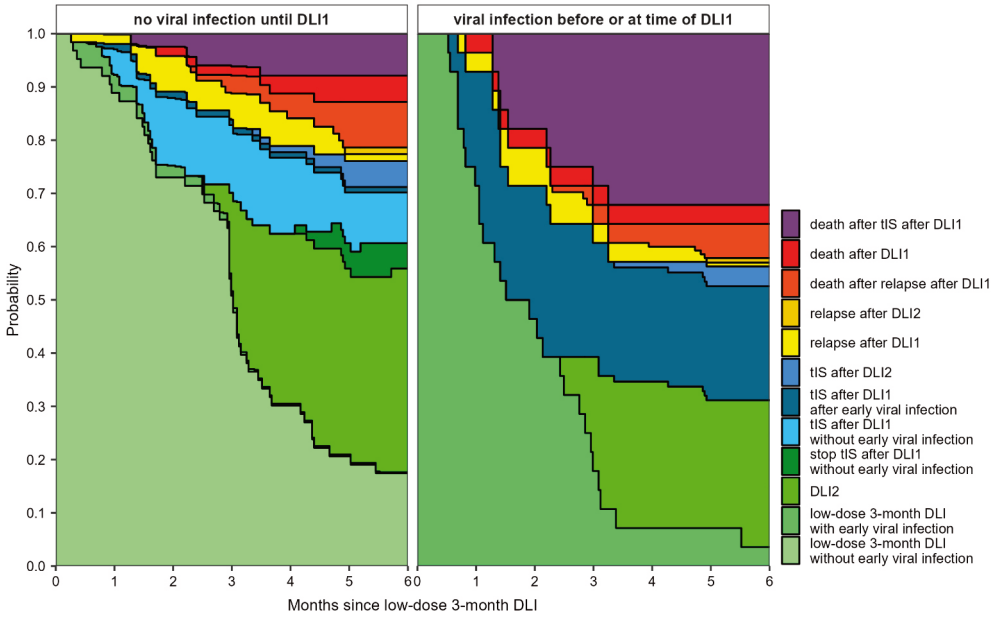
Supplemental Figure 10. Risk factors for death during tIS for GvHD. Cox proportional hazards models for the transition from tIS for GvHD after DLI1 to death (see Figure 1). Based on complete case analysis (n=29 for low-dose 3-month DLI and n=31 (age) or n=30 (chimerism) for 6-month DLI). DLI, donor lymphocyte infusion; alloSCT, allogeneic stem cell transplantation; high MC, ≥5% mixed chimerism in the bone marrow



Supplemental Figure 11. Cumulative incidence of death during tIS for GvHD after low-dose 3-month DLI. Cumulative incidences with associated 95% confidence intervals of death during tIS for GvHD for patients who developed GvHD after an early viral infection and those without any early viral infection. This was calculated in a competing risks model starting at time of start tIS for GvHD after DLI with death, DLI2, relapse and stop tIS as competing events.



Supplemental Figure 12. Cumulative incidence of death during tIS for GvHD after 6-month DLI. Cumulative incidences with associated 95% confidence intervals of death during tIS for GvHD per BM chimerism status at time of DLI for patients who developed GvHD after the 6-month DLI. This was calculated in a competing risks model starting at time of start tIS for GvHD after DLI with death, DLI2, relapse and stop tIS as competing events. One patient with FDC and one with high MC had two tIS episodes and entered the risk set twice.



Supplemental Figure 13. Outcomes after low-dose 3-month DLI based on the viral status at time of DLI. Stacked state occupation probabilities after low-dose 3-month DLI based on the viral status at time of DLI (viral infection during the last week before DLI (n=25) or no viral infection until DLI (n=63). The estimates are based on the non-parametric multi-state model in Supplemental Figure 3 which has two starting states ('DLI1 without early viral infection' and 'DLI1 with early viral infection'). The difference between two adjacent curves represents the probability of being in the corresponding state. States that were not used within 6 months after DLI were omitted from the legend.