Analysis of sequential treatments for hematological diseases by advanced statistical methods

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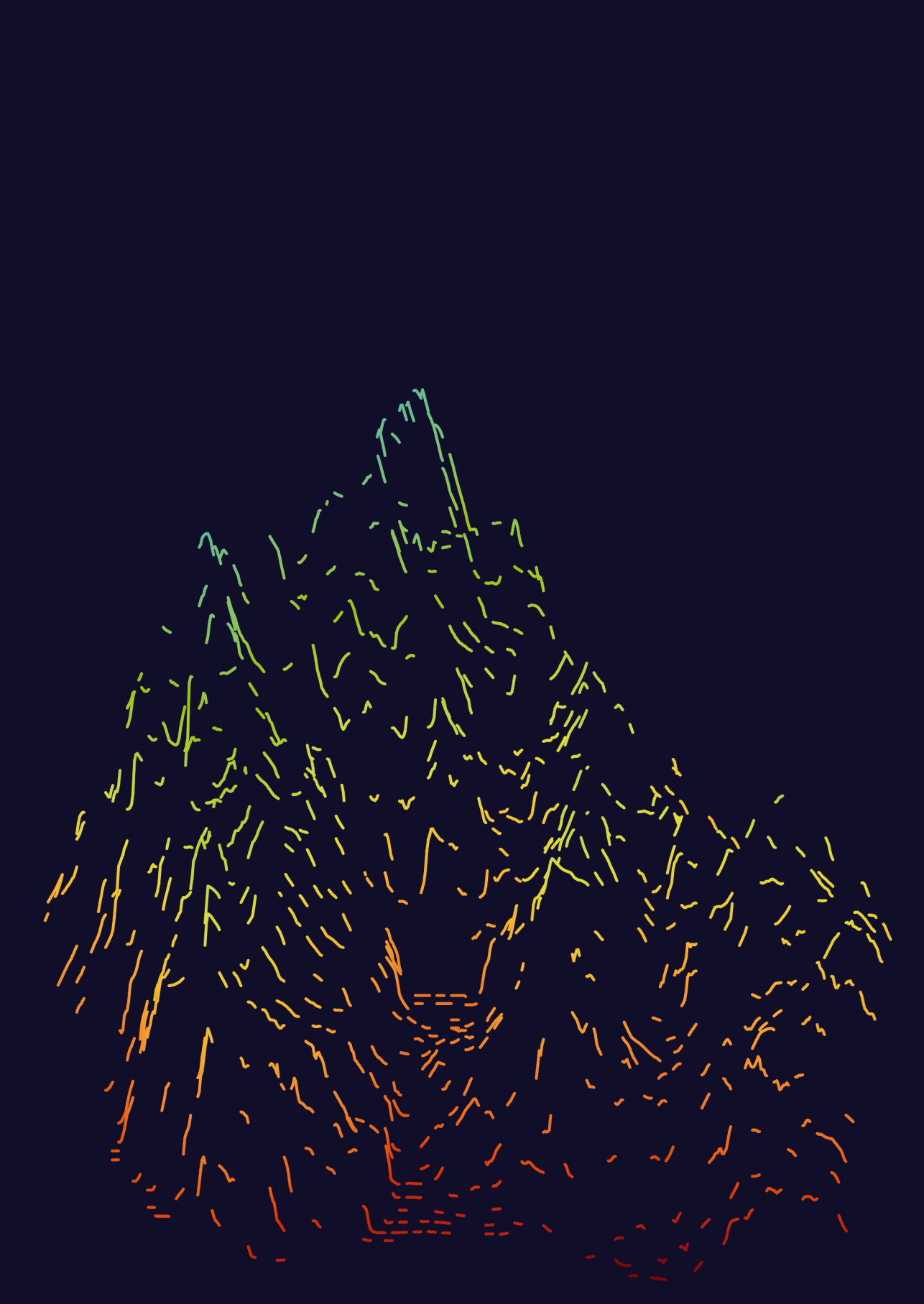

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Competitive repopulation and allo-immunological pressure determine chimerism kinetics after T-cell depleted allogeneic stem cell transplantation and donor lymphocyte infusion

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ABSTRACT

After allogeneic stem cell transplantation (alloSCT), patient-derived stem cells that survived the pretransplant conditioning compete with engrafting donor stem cells for bone marrow (BM) repopulation. In addition, donor-derived alloreactive T cells present in the stem cell product may favor establishment of complete donor-derived hematopoiesis by eliminating patient-derived lymphohematopoietic cells. T-cell depleted alloSCT with sequential transfer of potentially alloreactive T cells by donor lymphocyte infusion (DLI) provides a unique opportunity to selectively study how competitive repopulation and allo-immunological pressure influence lymphohematopoietic recovery. This study aimed to determine the relative contribution of competitive repopulation and donor-derived anti-recipient allo-immunological pressure on the establishment of lymphohematopoietic chimerism after alloSCT. In this retrospective cohort study of 281 acute leukemia patients treated according to a protocol combining alemtuzumab-based T-cell depleted alloSCT with prophylactic DLI, we investigated engraftment and quantitative donor chimerism in the BM and immune cell subsets. DLI-induced increase of chimerism and development of Graft-versus-Host-Disease (GvHD) were analyzed as complementary indicators for donor-derived anti-recipient allo-immunological pressure. Profound suppression of patient immune cells by conditioning sufficed for sustained engraftment without necessity for myeloablative conditioning or development of clinically significant GvHD. Although 61% of the patients without any DLI or GvHD showed full donor chimerism (FDC) in the BM at 6 months after alloSCT, only 24% showed FDC in the CD4+ T-cell compartment. In contrast, 75% of the patients who had received DLI and 83% of the patients with clinically significant GvHD had FDC in this compartment. In addition, 72% of the patients with mixed hematopoiesis receiving DLI converted to complete donor-derived hematopoiesis, of whom only 34% developed clinically significant GvHD. Our data show that competitive repopulation can be sufficient to reach complete donor-derived hematopoiesis, but that some allo-immunological pressure is needed for the establishment of a completely donor-derived T-cell compartment, either by the development of GvHD or by administration of DLI. We illustrate that it is possible to separate the Graft-versus-Leukemia effect from GvHD, as conversion to durable complete donor-derived hematopoiesis following DLI did not require induction of clinically significant GvHD.

INTRODUCTION

The goal of allogeneic hematopoietic stem cell transplantation (alloSCT) in patients with hematological malignancies is to eradicate the disease by replacing patient hematopoiesis with donor-derived hematopoiesis and introducing donor alloreactive T cells capable of eliminating residual malignant cells. After alloSCT, patient hematopoietic stem cells (HSCs) that survived the pretransplant conditioning compete with engrafting donor HSCs for bone marrow (BM) repopulation.[1] Patient-derived alloreactive T cells may reject the graft[2], but donor engraftment can be supported by alloreactive donor-derived T cells recognizing nonself antigens on patient immune cells.[3,4] These alloreactive donor T cells can further eliminate patient HSCs and residual malignant cells and provide lasting immune surveillance against the malignancy, the Graft-versus-Leukemia (GvL) effect. However, when non-hematopoietic tissues of the patient are recognized, Graft-versus-Host-Disease (GvHD) may develop.[5,6]

Allo-immune responses are induced by presentation of antigens to functional alloreactive T cells. To become properly activated, naïve alloreactive T cells require costimulatory signals from activated professional antigen-presenting cells (APCs). Important factors influencing the balance between donor- and patient-derived allo-immunological pressure include greater genetic disparity between patient and donor encoding more antigens that can induce alloreactivity[7,8]. Activation of professional APCs by tissue damage may increase the risk of GvHD after more toxic myeloablative (MA) compared with less toxic nonmyeloablative (NMA) conditioning regimens.[9] The risk of GvHD decreases over time as the professional patient APCs are gradually replaced by donor-derived APCs.[10] Finally, the recovery of regulatory T cells and *de novo* lymphopoiesis after transplantation may lead to a state of tolerance.[11,12]

Several strategies to modulate the allo-immunological pressure after alloSCT have been developed. Most patients receive prophylactic systemic immunosuppression with or without a form of T-cell depletion (TCD) to prevent rejection and GvHD.[13] The impact of TCD on patient- and/or donor-derived T cells depends on the method (*in vivo* versus *in vitro*) and timing (before or after alloSCT).[14-18] Excessive suppression of donor-derived allo-immunological pressure against the patient immune cells, HSCs, and tumor cells favors their persistence and eventual dominance, with the risk of graft failure and/or recurrence of the malignancy.[19-21] To improve engraftment or boost the GvL effect, unmodified donor lymphocyte infusion (DLI) can be administered after alloSCT.[22-25]

Our strategy of alemtuzumab-based TCD alloSCT followed by standard prophylactic DLI[26] aims to separate the establishment of donor hematopoiesis from the introduction of donor alloreactivity. The delayed introduction of donor alloreactivity allows the induction of a GvL effect without a high risk of GvHD necessitating systemic treatment. In this context, TCD permits analysis of BM repopulation in the absence of strong allo-immunological pressure. Obviation of the need for prophylactic pharmacologic immunosuppression facilitates analysis of natural immunological recovery. DLI is administered starting 3 months after alloSCT after the competitive repopulation of the BM and early T-cell expansion have taken place. This setting offers the unique opportunity to exclusively analyze the impact of donor alloreactivity introduced by DLI on persisting patient-derived HSCs and T cells. In a cohort of 281 patients, we examined lymphohematopoietic recovery and chimerism kinetics in the BM and circulating

immune cells in relation to conditioning and GvHD after TCD alloSCT and DLI.

METHODS

Study population

This observational study included all adult patients with acute myeloid leukemia (AML), acute lymphoblastic leukemia, or myelodysplastic syndrome in complete morphologic remission after intensive induction therapy who underwent a first BM or G-CSF-mobilized peripheral blood stem cell alloSCT using a standard conditioning and TCD protocol[27-29] at Leiden University Medical Center between 2005 and 2015. Exclusion criteria were use of a haploidentical donor or prescheduled posttransplant chemotherapy. The study was approved by the Medical Ethical Committee of Leiden University Medical Center (P03.114, P03.173, and P04.003). All patients provided signed informed consent for data collection and analysis. Data were analyzed as of August 2020.

Transplantation and DLI strategy

MA conditioning consisted of cyclophosphamide (60 mg/kg i.v. for 2 days) with 9 Gy total body irradiation or busulfan (4x0.8 mg/kg i.v. for 4 days). NMA conditioning consisted of fludarabine (50 mg/m^2 orally for 6 days) and busulfan (4x0.8 mg/kg i.v. for 2 days).

Standard *in vitro* TCD was performed by adding 20 mg of alemtuzumab (Sanofi Genzyme) to the graft prior to infusion.[28] Additional *in vivo* TCD depended on donor type and conditioning regimen. MA-conditioned patients with a 10/10 HLA-matched related donor (RD) did not receive any *in vivo* TCD; all other patients received 15 mg alemtuzumab i.v. on days -6 and -5 for MA conditioning or on days -4 and -3 for NMA conditioning. NMA-conditioned patients with an unrelated donor (UD) also received rabbit anti-thymocyte globulin (Sanofi Genzyme) on day -2 (2 mg/kg until April 2010 and 1 mg/kg thereafter). Only MA-conditioned patients with an UD or a 9/10 HLA-matched RD received ciclosporin as GvHD prophylaxis, which was tapered from 1 month and stopped within 3 months post-transplantation.

Preemptive DLI was administered for increasing or persisting mixed chimerism (MC) or the presence of minimal residual disease (MRD), whereas prophylactic DLI was administered regardless of chimerism or MRD status to all patients without clinically significant GvHD indicating allo-immunological pressure. Preemptive DLI was administered at escalating doses with ≥3 months between infusions until the development of GvHD or disappearance of MRD and/or MC. Prophylactic DLI was introduced in May 2010 and administered to all patients at 6 months post-transplantation in the absence of clinically significant GvHD or relapse (3M T cells/kg for patients with RD and 1.5M T cells/kg for patients with UD). Forty-two patients at high risk of early relapse (within 6 months post-alloSCT) also received prophylactic low-dose DLI (0.3M and 0.15M T cells/kg for RD and UD, respectively) at 3 months.[30] Indications for this early prophylactic DLI were very poor risk AML or high-risk acute lymphoblastic leukemia according to the HOVON criteria[31,32], MRD positivity at time

of transplantation, incomplete pretransplant treatment, therapy-related AML with unfavorable karyotype, AML with persisting underlying disease, and acute leukemia that relapsed early after the previous curative induction chemotherapy. Along with the unmodified DLI, patients could receive modified (purified or genetically modified) T-cell products as part of several clinical trials. Interferon could be administered to patients with an increasing number of blasts in the BM (but morphologically below 5%) or with MRD not responding to DLI.

Follow-up

BM cytology, lymphocyte counts, and BM chimerism analysis were performed at least every 3 months during the first 2 years post-transplantation. In a subset of patients, chimerism was also determined in granulocytes, monocytes, CD4+ T cells, CD8+ T cells, natural killer cells, and B cells on peripheral blood (Supplemental Methods). Because we were interested in the kinetics of chimerism following TCD alloSCT and unmodified DLI, we excluded all chimerism and cell count measurements obtained 1 week or longer after infusion of a modified T-cell product. Measurements obtained after relapse, interferon administration, chemotherapy, and second alloSCT were excluded as well. Measurements used in the comparison analyses were performed at 6 weeks (actual range 5-7 weeks), 3 months (2-4 months), and 6 months (5-7 months) after alloSCT. For the clinical outcomes, all patients, including those who received a modified T-cell product, were included to assess the overall outcome of our total strategy and to prevent selection bias, as recruitment for trials with modified T-cell products started a few weeks after alloSCT.

Definitions

Neutrophil recovery was defined as the first of 3 consecutive measurements with an absolute neutrophil count $>0.5\times10^9$/l, and non-engraftment was defined as detection of <5% donor BM chimerism without prior neutrophil recovery. The date of relapse was defined as the date of the first recurrence of ≥5% blasts on cytomorphologic BM examination or ≥1% blasts in peripheral blood after alloSCT (confirmed by BM biopsy if possible). Clinically significant GvHD was defined by therapeutic systemic immunosuppression (tIS) for GvHD for at least 14 days or until death or stopped as part of palliative care due to refractory GvHD, or continued use of GvHD prophylaxis beyond 3.5 months. tIS was started for acute GvHD grade II-IV according to the modified Glucksberg criteria, for extensive chronic GvHD according to the Seattle criteria, and for mild GvHD not responding to topical treatment.[33,34] We used tIS instead of the exact grading because our strategy aims to prevent GvHD necessitating systemic treatment. MC was defined as detection of patient DNA at or above the limit of detection, and full donor chimerism (FDC) was defined as undetectable patient DNA. DLI-induced cytopenia was defined as the development of severe neutropenia ($<0.5\times10^9$/l) after DLI not caused by relapse or infection.

Study objective and endpoints

The objective of this study was to determine the relative contribution of competitive repopulation and donor-derived anti-recipient allo-immunological pressure on the establishment of full-donor lymphohematopoietic chimerism after alloSCT. DLI-

induced increase in chimerism and development of GvHD were analyzed as complementary indicators for donor-derived anti-recipient allo-immunological pressure. The primary endpoint was the level of donor BM and T-cell chimerism at 6 weeks and 3 and 6 months after alloSCT. Secondary endpoints were primary engraftment, clinically significant GvHD as a surrogate for allo-immunological pressure, and BM chimerism kinetics during the first 2 years after DLI. Other secondary endpoints were overall survival, relapse-free survival, cumulative incidence of relapse, and non-relapse mortality during the first 5 years after alloSCT.

Analyses

An algorithm was developed to assess the chimerism response after the first unmodified DLI that patients received while having mixed BM chimerism. We defined this DLI as the 'starting DLI' for this analysis (Supplemental Methods).

The probabilities of overall survival and relapse-free survival from alloSCT with 95% confidence intervals (95%-CIs) were calculated using the Kaplan-Meier method. Follow-up from alloSCT was quantified using the reverse Kaplan-Meier method.[35] Cumulative incidences of neutrophil recovery as proxy for primary engraftment and clinically significant GvHD were calculated using competing risks models (Supplemental Methods).

To evaluate the effects of donor-derived allo-immunological pressure and DLI on BM repopulation and immunological recovery, donor chimerism in the BM and T cells was evaluated at 3 and 6 months after alloSCT and compared between groups based on whether patients had developed clinically significant GvHD, had received unmodified DLI without any clinically significant GvHD, or had neither. Because chimerism levels did not follow a normal distribution, groups were compared using the Mann-Whitney U test (2 groups) or Kruskall-Wallis test followed by, if applicable, the post hoc Dunn test with Holms adjustment for multiple comparisons (>2 groups). An (adjusted) p-value <0.05 was considered significant.

Software

All analyses were performed in R version 4.0.2 using the survival, cmprsk, prodlim, rstatix, ggplot2, ggpubr, gridExtra, and ggalluvial packages.

RESULTS

Population

A total of 281 patients were included in this study. The patients' baseline characteristics are summarized in Table 1. The median follow-up was 61 months (interquartile range [IQR] 43-85 months) after alloSCT. The clinical outcomes of our total strategy of TCD alloSCT followed by DLI are presented in Supplemental Results.

Successful primary engraftment after TCD alloSCT does not depend on MA conditioning or donor-derived allo-immunological pressure

The cumulative incidence of neutrophil recovery was 91% (95%-CI 88-94) at 4 weeks

after alloSCT and increased to 99% (95%-CI 97-100) at 2.5 months (Supplemental Figure 3). One patient, who underwent transplantation after MA conditioning, failed to engraft. Two patients died, at 2 and 12 days after alloSCT, before (non-)engraftment. Successful engraftment of all 103 evaluable NMA-conditioned patients demonstrates sufficient suppression of the patient immune cells by alemtuzumab, in combination with anti-thymocyte globulin in case of an UD, to prevent graft rejection.

To evaluate whether strong donor-derived alloimmune responses after alloSCT had a profound role in the primary engraftment in this cohort, we examined the development of clinically significant GvHD before any DLI after alloSCT in the 278 engrafted patients. At 3 months after alloSCT, the cumulative incidence of clinically significant GvHD was 13% (95%-CI 9-17) in the total cohort and only 2% (95%-CI 0-5) after NMA conditioning (Supplemental Figure 4). Together with the 99% probability of

	Total (N = 281)	MA, matched RD (N = 78)	MA, mismatched RD or UD (N = 99)	NMA, matched RD (N = 41)	NMA, UD (N = 63)
Age at alloSCT (years)					
median (range)	50 (18-73)	43 (18-60)	42 (19-59)	61 (28-72)	63 (40-73)
Disease					
AML	188 (67%)	47 (60%)	56 (57%)	33 (80%)	52 (83%)
ALL	76 (27%)	26 (33%)	39 (39%)	5 (12%)	6 (10%)
MDS	17 (6%)	5 (6%)	4 (4%)	3 (7%)	5 (8%)
Conditioning regimen					
Cyclo/TBI	171 (61%)	76 (97%)	95 (96%)	0 (0%)	0 (0%)
Cyclo/Bu	6 (2%)	2 (3%)	4 (4%)	0 (0%)	0 (0%)
Flu/Bu	103 (37%)	0 (0%)	0 (0%)	41 (100%)	62 (98%)
Flu/Bu/Cyclo*	1 (0%)	0 (0%)	0 (0%)	0 (0%)	1 (2%)
Donor					
10/10 matched RD	119 (42%)	78 (100%)	0 (0%)	41 (100%)	0 (0%)
9/10 matched RD	2 (1%)	0 (0%)	2 (2%)	0 (0%)	0 (0%)
10/10 matched UD	120 (43%)	0 (0%)	63 (64%)	0 (0%)	57 (90%)
9/10 matched UD	39 (14%)	0 (0%)	33 (33%)	0 (0%)	6 (10%)
8/10 matched UD	1 (0%)	0 (0%)	1 (1%)	0 (0%)	0 (0%)
Graft source					
G-CSF-mobilized PBSC	266 (95%)	69 (88%)	94 (95%)	41 (100%)	62 (98%)
BM	15 (5%)	9 (12%)	5 (5%)	0 (0%)	1 (2%)
Period of alloSCT**					
first transplantation in cohort	2005-01-20	2005-01-20	2005-03-10	2008-08-14	2009-10-06
before May 2010	87 (31%)	35 (45%)	37 (37%)	9 (22%)	6 (10%)
since May 2010	194 (69%)	43 (55%)	62 (63%)	32 (78%)	57 (90%)

Table 1. Baseline characteristics of the total cohort and subgroups based on conditioning intensity and donor type. MA, myeloablative; NMA, nonmyeloablative; RD, related donor; UD, unrelated donor, AML, acute myeloid leukemia; ALL, acute lymphoblastic leukemia; MDS, myelodysplastic syndrome; Cyclo, cyclophosphamide; TBI, total body irradiation; Bu, busulfan; Flu, fludarabine; G-CSF, granulocyte-colony stimulation factor; PBSC, peripheral blood stem cells; BM, bone marrow; alloSCT, allogeneic stem cell transplantation. *One patient received cyclophosphamide 750 mg/m^2 i.v. for 2 days in the conditioning regimen because a second consolidation course before transplantation was not given. **Prophylactic DLI has been included in the transplantation strategy since May 2010.

engraftment, these data show that primary engraftment after alemtuzumab-based TCD alloSCT was not impaired after MA or NMA conditioning and in the absence of clinically significant GvHD.

MC is more common in the T-cell compartment than in the BM

Because engraftment does not necessarily lead to persistent complete (100%) donor-derived hematopoiesis, we investigated chimerism kinetics in the 278 engrafted patients. Among the 223 patients alive without any prior cellular intervention, chemotherapy, interferon, or relapse and with evaluable BM chimerism at 3 months after alloSCT, 59% had FDC. Within the group with MC, the middle 50% (i.e., the IQR) had donor chimerism levels between 94% and 98%. To investigate whether the circulating immune cells also were of mixed origin after TCD alloSCT, we measured the level of donor chimerism in 6 immune cell types in a subset of patients, again excluding all samples after cellular intervention, chemotherapy, interferon, and relapse (Supplemental Figure 5; Supplemental Figure 6 and Supplemental Table 2 provide the cell counts in the total cohort). At 3 months after alloSCT, 73% to 78% of the patients showed FDC in the granulocytes, monocytes, natural killer cells, and B cells. The IQR of the donor chimerism values with MC in these cells ranged between 96% and 99%. In contrast, only 22% and 28% of the patients showed FDC in the CD4+ and CD8+ T-cell populations, respectively, and the IQR of donor chimerism within T cells with MC was 7% to 92%. Even in patients with complete donor-derived hematopoiesis, circulating T cells could be predominantly of patient origin at 3 months post-alloSCT (Figure 1).

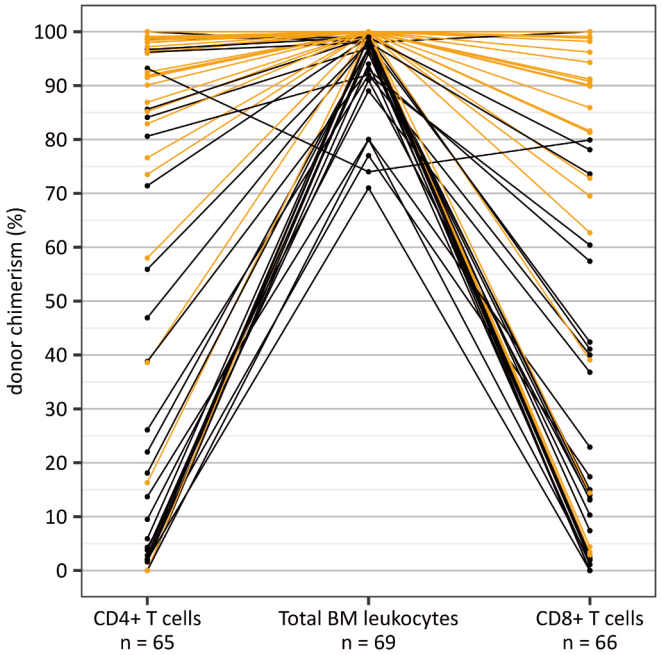

Figure 1. Donor chimerism in the BM and T cells at 3 months. Donor chimerism in the BM, CD4+ and CD8+ T cells at 3 months post-alloSCT without any prior cellular intervention, relapse, chemotherapy or interferon. Lines corresponding to patients with FDC in the BM are highlighted in orange.

Associations between conditioning intensity and clinically significant GvHD and BM and T-cell chimerism at 3 months after alloSCT

Although primary engraftment after TCD alloSCT was not affected by conditioning intensity or donor-derived allo-immunological pressure, these factors could influence the level of donor chimerism. To investigate the influence of conditioning intensity and allo-immunological pressure on the development of complete donor-derived hematopoiesis, we compared BM chimerism at 3 months after alloSCT between patient groups defined by conditioning intensity and development of clinically significant GvHD prior to measurement of chimerism (Figure 2A). In the absence of clinically significant GvHD, MA-conditioned patients had significantly higher donor BM chimerism (71% of the patients had FDC) compared to NMA-conditioned patients (32% FDC), showing that MA conditioning promoted the establishment of complete donor-derived hematopoiesis after alloSCT. To evaluate the effect of GvHD on the development of complete donor-derived hematopoiesis, we compared donor BM chimerism between MA-conditioned patients with and without clinically significant GvHD. Patients with clinically significant GvHD had higher donor BM chimerism at 3 months after alloSCT compared with those without (88% versus 71% FDC; adjusted p-value = 0.12).

To investigate the influence of conditioning intensity and clinically significant GvHD on T-cell chimerism, we compared the level of donor chimerism in CD4+ and CD8+ T cells at 3 months between the same groups for all patients with available T-cell chimerism (Figure 2B-C). In the absence of clinically significant GvHD, donor chimerism in CD4+ T cells and CD8+ T cells was significantly higher in MA-conditioned patients (33% had FDC in CD4+ T cells and 41% had FDC in CD8+ T cells) compared with NMA-conditioned patients (7% and 12%, respectively). In the MA-conditioned group, there was no significant difference in the level of donor chimerism between patients with and those without clinically significant GvHD: 43% versus 33% had FDC in CD4+ T cells (adjusted p-value 0.37) and 43% versus 41% had FDC in CD8+ T cells (adjusted p-value 0.74). Together, these data indicate that myeloablative conditioning led to higher donor T-cell chimerism after TCD alloSCT, but we did not find a significant effect of clinically significant GvHD on the level of CD4+ or CD8+ T-cell chimerism at 3 months after alloSCT. This may be explained by the immunosuppressive treatment that almost all patients with GvHD still were receiving at the time of chimerism measurement.

Donor chimerism in the BM and T cells increases after early DLI

Starting from 3 months, prophylactic and preemptive DLI was administered to induce an alloimmune response against patient-derived hematopoietic cells. To investigate the impact of this allo-immunological pressure by early DLI in the absence of GvHD, we compared donor BM chimerism between 3 and 6 months after alloSCT in patients who received unmodified DLI within 4 months after alloSCT but without any clinically significant GvHD up to 6 months, and in patients without any DLI or GvHD in this period. Of the 71 evaluable patients (51% NMA-conditioned) without any DLI or GvHD during this period, 66% showed FDC at 3 months and 61% did so at 6 months, illustrating that in absence of donor-derived allo-immunological pressure, mixed BM chimerism remained prevalent after TCD alloSCT. Thirty patients received unmodified DLI within 4 months after alloSCT, 30% after NMA conditioning. Notably, although only 38% of these patients showed FDC in the BM at 3 months after alloSCT, this

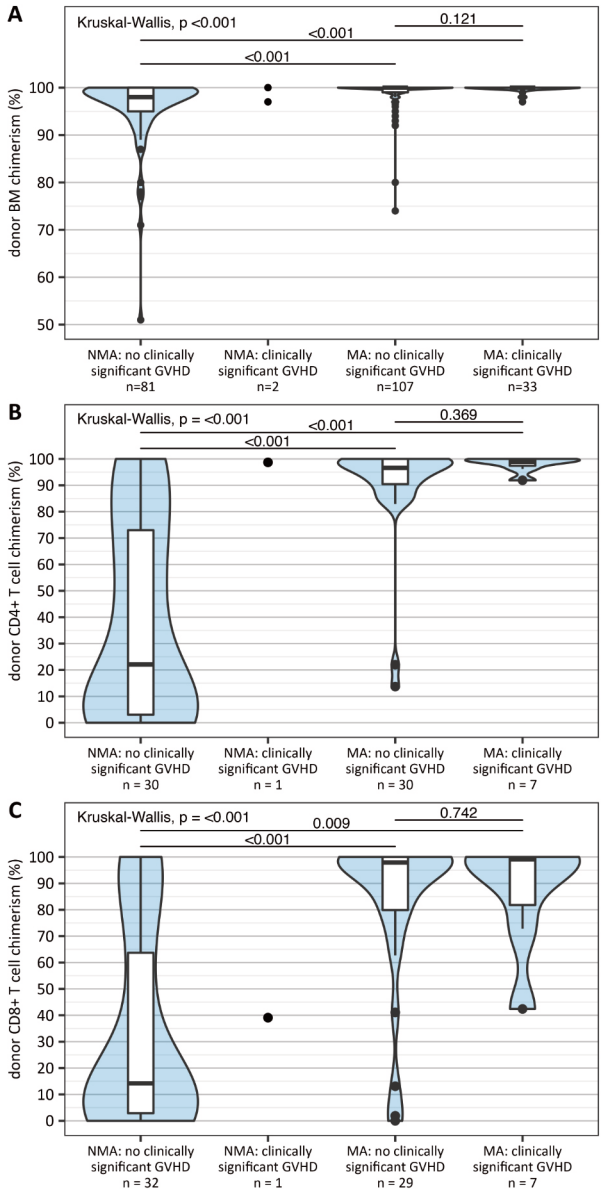

Figure 2. Donor chimerism in the BM and T cells at 3 months according to conditioning regimen intensity and the development of GvHD before the measurement. Donor chimerism in the BM (A), CD4+ T cells (B) and CD8+ T cells (C) at 3 months after TCD alloSCT without any prior cellular intervention, relapse, chemotherapy or interferon. T-cell chimerism was measured in a subset of patients. In 7 patients, either the CD4+ or the CD8+ fraction was missing. In all panels, the data are grouped based on conditioning intensity and development of clinically significant GvHD before the 3-month measurement. 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, respectively. In each panel, the level of donor chimerism was compared among 3 groups as the 2 NMA-conditioned patients with GvHD were excluded from this test. The p-values for the pairwise comparisons are adjusted for multiple comparison.

percentage increased to 63% at 6 months after alloSCT, indicating that early unmodified DLI could increase donor BM chimerism without the concomitant development of GvHD.

To evaluate the impact of early DLI on donor T-cell chimerism, we investigated the kinetics of donor T-cell chimerism during the first 6 months after alloSCT in patients without any DLI or GvHD during this period and in patients who received DLI (Figure 3). Again, the 18 evaluable patients without any DLI or GvHD showed a stable pattern of MC, and almost all patients with an early DLI without any clinically significant GvHD (n=8) showed increasing levels of donor CD4+ and CD8+ T-cell chimerism.

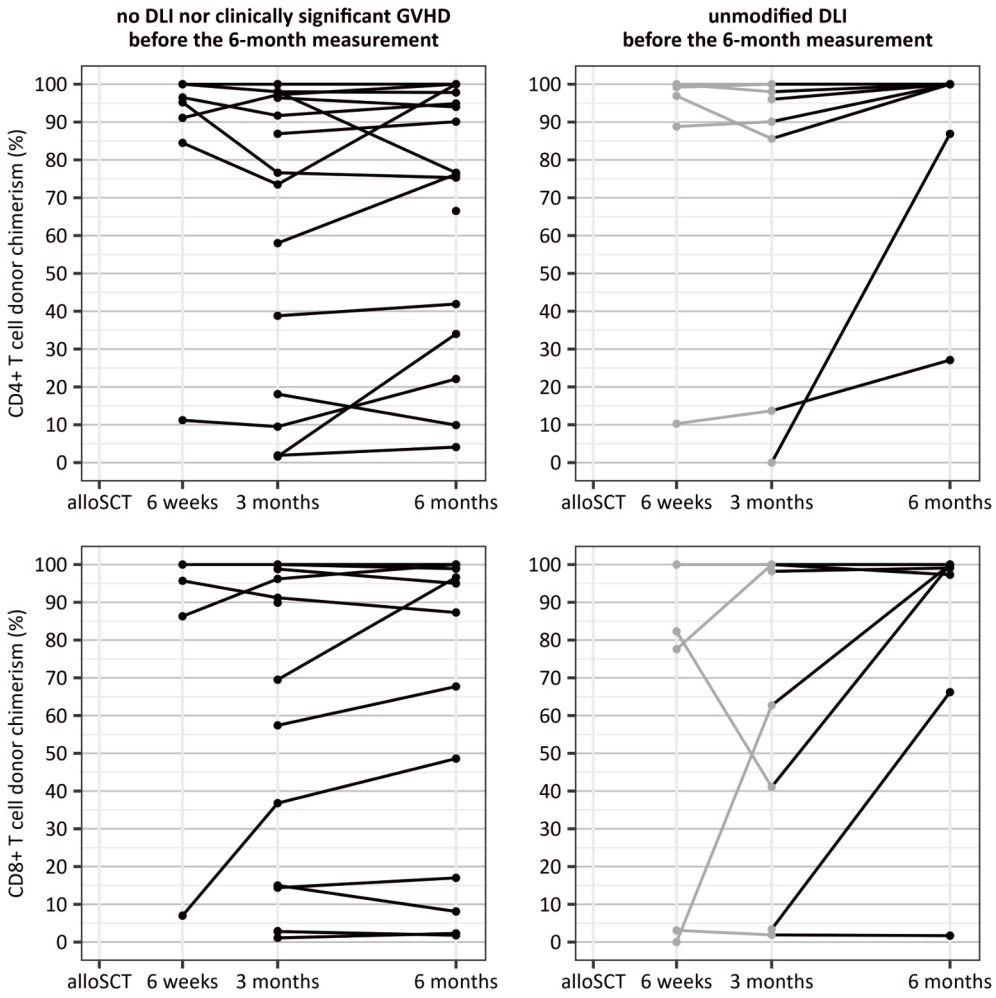

Figure 3. T-cell chimerism kinetics during the first 6 months after TCD alloSCT in patients without any clinically significant GvHD nor DLI before the 6-month measurement and in patients who received an unmodified DLI before the 6-month measurement. Patients who died, relapsed, or received chemotherapy, interferon, second alloSCT or a modified T-cell product before the 6-month measurement were excluded. In the second column, the chimerism measurements done before DLI are in grey, and the measurements done after DLI are in black, to visualize the impact of DLI on the level of donor T-cell chimerism.

To test whether these kinetics led to significant differences in the level of donor T-cell chimerism at 6 months after alloSCT and to compare the impact of DLI with the impact of clinically significant GvHD without DLI on chimerism, we compared the levels of 6-month donor chimerism in CD4+ and CD8+ T cells between these 2 patient groups, adding a third group comprising the 12 evaluable patients who developed clinically significant GvHD before the 6-month measurement, all without any prior DLI (Figure 4A-B). CD4+ T-cell donor chimerism was significantly higher in patients with DLI (75% FDC) or clinically significant GvHD (83%) compared to patients without any DLI or GvHD (24% FDC). CD8+ T-cell chimerism showed a similar trend, with 33% of the

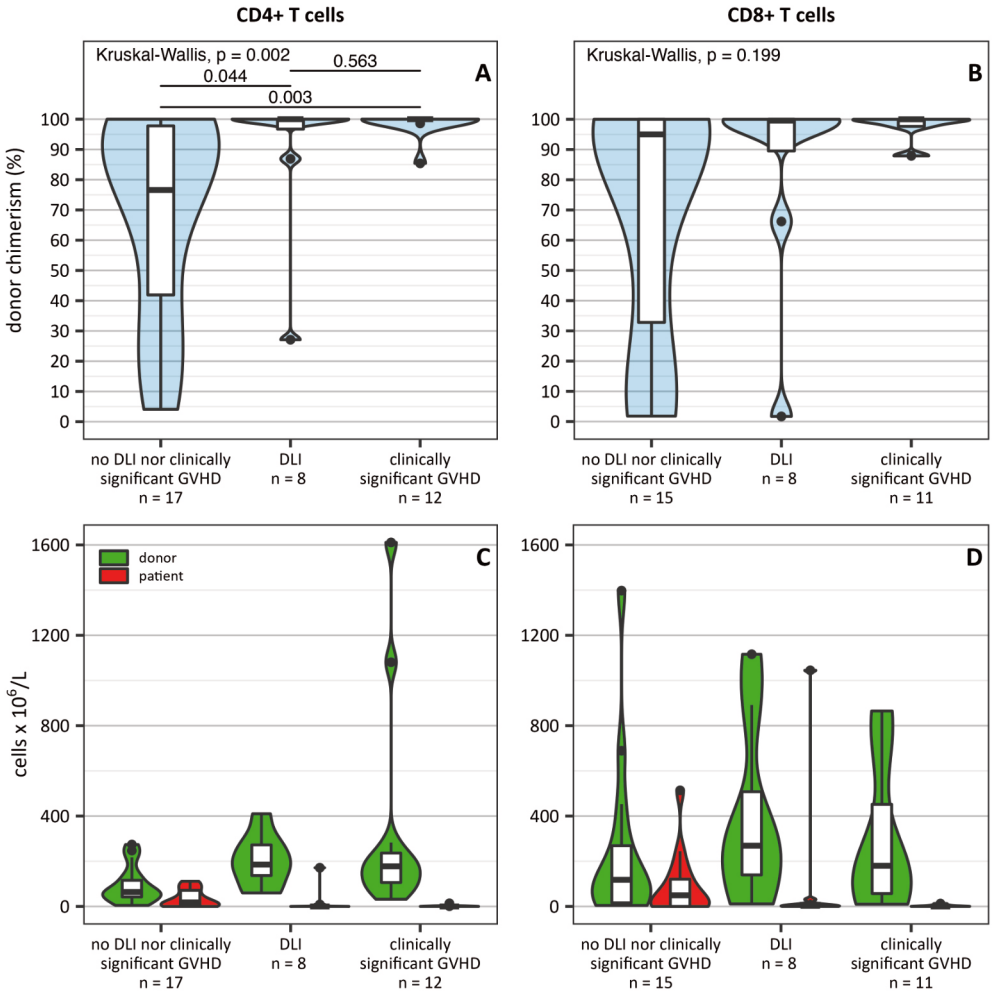

Figure 4. T-cell chimerism and patient/donor-specific counts at 6 months in patients with DLI, GvHD or neither. CD4+ and CD8+ T-cell chimerism (A and B) and patient/donor-specific counts (C and D) at 6 months after alloSCT in patients without prior DLI or clinically significant GvHD, patients with DLI before this measurement, and patients who had developed clinically significant GvHD after alloSCT without prior DLI. Patients who relapsed, or received chemotherapy, interferon, second alloSCT or a modified T-cell product before the 6-month measurement were excluded. The p-values for the pairwise comparisons in (A) are adjusted for multiple comparison.

patients without any DLI or GvHD having FDC, compared to 50% of those with DLI and 55% of those with clinically significant GvHD. Patients with DLI or clinically significant GvHD had both a lower number of circulating patient-derived T cells and a higher number of donor-derived T cells (Figure 4C-D). Together, these data show that 6-month donor CD4+ T-cell chimerism is significantly higher in patients with than in those without GvHD, and that early DLI can increase the level of donor T-cell chimerism in the absence of GvHD.

Strategy of dose-escalating DLIs can convert mixed hematopoiesis to durable complete donor-derived hematopoiesis without necessarily inducing clinically significant GvHD

To investigate the allo-immunological effects of our total DLI strategy, we developed an algorithm to quantify BM chimerism responses in the 65 patients with mixed hematopoiesis receiving unmodified DLI without any prior relapse (Methods). Clinical outcomes of all patients who received an unmodified DLI are presented in the Supplemental Results. The median level of donor BM chimerism in these patients at time of the starting DLI was 98% (IQR 94-99, Figure 5A). In 7 patients, the BM chimerism response could not be evaluated because of early death from severe GvHD after DLI (n=4) or early relapse (n=3) (Figure 5B, Supplemental Table 3). Within 2 months after starting DLI, 15 of the 65 patients (23%) converted to FDC and 9 (14%) showed a partial response with decreasing patient chimerism. Over time and with our dose-escalating DLI protocol, the numbers of patients with a response increased: 38 (58%) showed a response within 4 months and 46 (71%) did so within 7 months after starting DLI. At 25 months, 47 patients (72%) had converted to FDC, including 35 after 1 DLI, and 5 (8%) had shown a partial response. Six patients (9%) with available BM chimerism measurements after DLI did not show any response within this period, of whom 3 relapsed and 2 died within 25 months after the starting DLI. Only 1 patient completed the 25-month follow-up period without showing any chimerism response. Notably, this patient eventually converted to FDC in the BM at 29 months after the starting DLI, 6 months after the fourth DLI. After complete conversion, 4 of the 47 patients died and 4 relapsed within 25 months after the starting DLI. The other 39 (83%) patients with complete conversion were still alive and in complete remission at 25 months after DLI. Only 1 patient occasionally had some detectable patient DNA (Supplemental Figure 7).

To study whether GvHD is required for conversion to complete donor-derived hematopoiesis after DLI, we evaluated the development of clinically significant GvHD in the 47 patients with conversion from MC to FDC and found that 16 (34%) developed clinically significant GvHD within 25 months after starting DLI and 31 (66%) did not (Figure 5C). Together, these data show that our DLI strategy led to durable complete donor-derived hematopoiesis in the majority of the patients with mixed hematopoiesis receiving DLI after TCD alloSCT, without necessarily inducing clinically significant GvHD.

DISCUSSION

In this study, we leveraged a strategy of TCD alloSCT followed by standard prophylactic DLI to investigate how competitive repopulation and allo-immunological pressure influence the lymphohematopoietic recovery after alloSCT. The sequential introduction of donor hematopoiesis and alloreactivity enabled us to study these mechanisms

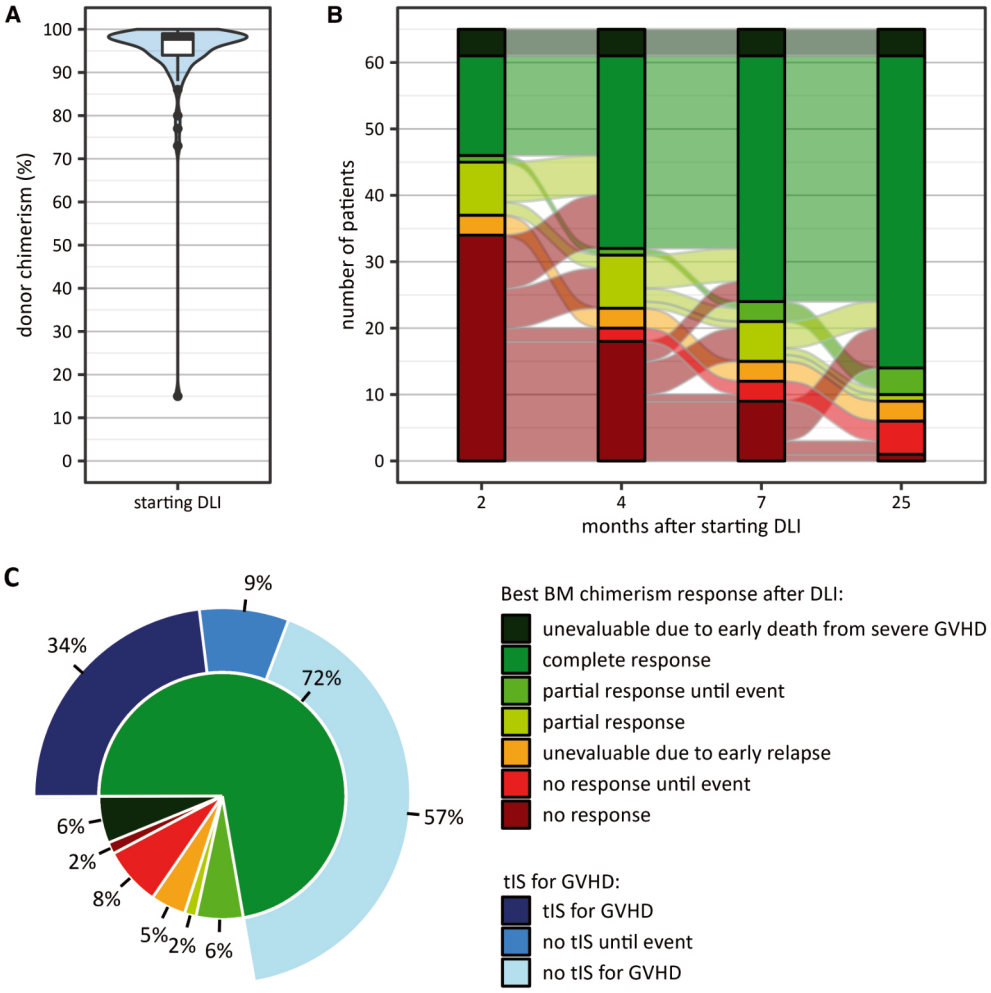

Figure 5. Alloimmune responses in the 65 patients with mixed hematopoiesis receiving unmodified DLI. Patients who relapsed or received chemotherapy, a second alloSCT, or a modified T-cell product before DLI or who continued GvHD prophylaxis after DLI were excluded. (A) The level of donor BM chimerism at time of DLI initiation. (B) The best BM chimerism response achieved by different time points after the first unmodified DLI. The events that terminated the evaluation period (death, relapse, chemotherapy, interferon or second alloSCT) are described in Supplemental Table 3. Note that recurrence of MC or terminating events occurring after a response are not shown in this plot. The current response, which considers these possibilities, is shown in Supplemental Figure 7. (C) Distribution of the best BM chimerism responses at 25 months after initiation of DLI (inner circle) and the use of tIS for GvHD by the converted patients during this period (outer ring). Five of these patients did not start tIS for GvHD but relapsed (n = 4) or died (n = 1) during this period.

separately, which is not possible in T-cell replete alloSCT or TCD alloSCT without standard DLI. Effective suppression of the patient-derived alloimmunity by the conditioning regimens sufficed for sustained engraftment without the need for myeloablative conditioning or evident donor-derived allo-immunological pressure. However, the development of complete donor-derived hematopoiesis depended on both competitive repopulation and allo-immunological pressure; the proportion of patients with FD BM chimerism at 3 months was lowest in the NMA-conditioned patients without any GvHD (32%), higher in the MA-conditioned patients without any GvHD (71%), and highest in the MA-conditioned patients who had developed GvHD (88%). In patients without GvHD, an alloimmune response against patient-derived hematopoietic cells could be efficiently induced by DLI even in the absence of concomitant GvHD. Following our total strategy of dose-escalating DLIs, 72% of the patients with mixed BM chimerism at time of DLI converted to complete donor-derived hematopoiesis. Only 34% of converting patients developed clinically significant GvHD after DLI, illustrating that the GvL effect can be separated from GvHD. For the establishment of a completely donor-derived T-cell compartment, some allo-immunological pressure seemed to be required.

Although the level of donor chimerism in CD4+ and CD8+ T cells at 3 months was higher after MA than NMA conditioning, only 33% and 41% of the MA-conditioned patients without any GvHD had FDC in these subsets, respectively. However, 83% of the patients who had developed GvHD, and 75% of the patients who had received an early DLI without developing any GvHD, had FDC in the CD4+ T cells at 6 months after alloSCT, compared to 24% of the patients without prior DLI or GvHD, showing that DLI also could convert mixed T-cell chimerism to FDC in the absence of GvHD. Together, these data indicate that the establishment of complete donor-derived hematopoiesis can be the result of competitive repopulation, but that donor-derived allo-immunological pressure is needed for the development of FD T-cell chimerism.

Because competitive repopulation can be sufficient to induce FD BM chimerism, the presence of FDC itself does not prove occurrence of an alloimmune response against patient hematopoietic cells or achievement of a meaningful GvL effect. This conclusion can explain why the value of FDC in predicting relapse remains controversial in different settings of alloSCT. For instance, Konuma et al[36] did not observe any association between FDC in the BM and relapse after MA single-unit umbilical cord blood transplantation. Owing to the MA conditioning and the relatively low allo-immunological pressure after cord blood transplantation[37], the achieved FDC might have been mainly the result of competitive repopulation. In contrast, Koreth et al[38] showed that having <90% donor chimerism in the BM or peripheral blood increased the risk of relapse after T-cell replete alloSCT following NMA conditioning. As in this case, the competitive repopulation probably played a more limited role, FDC was more likely a result from donor-derived allo-immunological pressure. The chimerism kinetics also can indicate whether alloreactivity played a role. Although FDC early after transplantation in the absence of GvHD may reflect the outcome of competitive repopulation, conversion from stable MC to FDC is most likely the result of an alloimmune response, leading to low relapse rates after chimerism conversion from MC to FDC, as observed in this study and as reported by others.[39-41] Therefore, not only the level of donor chimerism, but also the clinical setting and the chimerism kinetics, should be considered

when using chimerism to monitor the presence of donor-derived allo-immunological pressure and thereby the GvL effect in patients.

Because a conversion to FDC can be observed only in patients who have MC to start with, the ideal cell lineage for monitoring alloreactivity needs to show stable levels of MC after alloSCT in the majority of patients. Owing to the persistence of long-living patient-derived T cells, mixed T-cell chimerism is common after TCD alloSCT, and can exist in patients with a completely donor-derived hematopoiesis.[41-44] Therefore, changes in T-cell chimerism potentially could be used as marker for alloreactivity more often than BM chimerism. Applicability depends on the transplantation strategy. For instance, mixed T-cell chimerism is common after CD34+-selected alloSCT[46], whereas Carnevale-Schianca et al[45] observed 97% FDC at 28 days after MA alloSCT with posttransplant cyclophosphamide.

An important question is whether DLI can induce a sufficient GvL effect without needing to induce clinically significant GvHD as well. In concordance with our data, others have shown that conversion from MC to FDC can occur in the absence of GvHD after DLI, and that this conversion significantly decreases the risk of relapse.[24,41,47,48] The accumulating evidence that DLI can be effective in preventing relapse even without the induction of GvHD encourages further investigation into how the risk of GvHD after DLI can be decreased without losing the beneficial GvL effect. Several DLI modification strategies are being investigated that either remove cell subsets that are important for the development of GvHD (e.g., depletion of CD8+ T cells) or select only immune cells that target hematopoietic cells.[49] The toxicity of unmodified DLI can be reduced by administering prophylactic immunosuppression around DLI or by decreasing the initial DLI dose for patients with a higher risk of severe GvHD.[50]

In conclusion, we examined how the fundamental processes of BM repopulation and allo-immunological pressure shape the lymphohematopoietic recovery after TCD alloSCT and DLI. The suppression of the patient-derived allo-immunological pressure by the conditioning suffices for sustained engraftment without requiring intensive myeloablation or donor-derived allo-immunological pressure. We show that competitive repopulation can be sufficient to reach complete donor-derived hematopoiesis, but that some allo-immunological pressure is needed for the establishment of a completely donor-derived T-cell compartment, either by the development of GvHD or by administration of DLI. We illustrate that it is possible to separate GvL from GvHD, as conversion to durable complete donor-derived hematopoiesis following DLI did not require the induction of clinically significant GvHD.

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SUPPLEMENTAL METHODS

Methodology for measuring chimerism and circulating immune cell counts

BM chimerism was determined on unfractionated BM samples by short-tandem-repeat (STR) PCR. For some patients, transplanted before 2007 with a sex-mismatched donor, unfractionated BM chimerism was determined 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. In a subset of patients, chimerism was also determined in granulocytes, monocytes, CD4+ T cells, CD8+ T cells, NK cells and B cells on peripheral blood by STR PCR. For this analysis, 50,000 cells per population were sorted by flow cytometry (Supplemental Table 1). Absolute numbers of circulating CD4+ T cells, CD8+ T cells, B cells and NK cells were measured routinely on anticoagulated fresh venous blood by flow cytometry with bead calibration (Trucount tubes, Becton Dickinson, Breda, The Netherlands) at a detection limit of $0.5x10^6$ cells/l.

Quantification of donor-derived alloimmune responses after DLI in patients with mixed chimerism

To evaluate whether DLIs can induce BM chimerism conversion from MC to FDC or improve the level of donor chimerism, we developed an algorithm to assess the best chimerism response after the first unmodified DLI that patients received while having MC in the BM. We defined this DLI as the 'starting DLI' for this analysis. Patients could enter the analysis only once: the analysis was not restarted if a patient received another DLI after recurrence of MC. The algorithm considered all BM chimerism measurements from 1 week until 25 months after the starting DLI, including measurements after successive DLIs and excluding measurements taken after relapse or administration of interferon, chemotherapy, or second alloSCT. Patients receiving DLI during continued GVHD prophylaxis or after a relapse, administration of chemotherapy, second alloSCT, or modified T cell product were excluded from this analysis. A complete donor-derived alloimmune response was defined as conversion to FDC. Partial donor-derived alloimmune response was defined as a relative decrease in patient chimerism of 50% or an absolute decrease of 20% when starting patient chimerism was at least 50%, 10% when starting patient chimerism was between 20% and 50%, or 5% when patient chimerism was <20%. These values were chosen to prevent that minor fluctuations in patient chimerism were defined as a response. Patients with evaluable BM chimerism measurements after DLI who failed to show a complete or partial response were considered to have no donor-derived alloimmune response. For partial and non-responders a distinction was made between patients who had completed the required follow up period and patients who had died, relapsed, or received chemotherapy, interferon, or second alloSCT within this period. For the evaluation of the durability of the chimerism responses we also considered loss of response, defined as the recurrence of patient chimerism after a complete response or an increase in patient chimerism (using the same cut-offs as described above) after a partial response. The number of DLIs before achieving the best response (until conversion to FDC for complete responders or until the start of decreasing patient chimerism for partial responders) or during the total evaluable follow-up (for non-responders) were recorded.

To evaluate whether clinically significant GVHD is required for chimerism conversion, we examined how many of the complete responders started tIS for GVHD within 25 months after the starting DLI.

Competing risks models

The cumulative incidence of neutrophil recovery as proxy for primary engraftment was calculated in a competing risks model starting at alloSCT and with non-engraftment and death as competing events. The cumulative incidence of clinically significant GVHD after TCD alloSCT was calculated with cellular intervention ((un)modified DLI, stem cell boost or second alloSCT), relapse, start of chemotherapy or interferon, and death as competing events. The cumulative incidences of clinically significant GVHD and of the development of acute GVHD grade II-IV or extensive chronic GVHD were calculated in separate competing risks models with relapse and death as competing events.

SUPPLEMENTAL RESULTS

Clinical outcomes of the total strategy

The 5-year overall and relapse-free survival were 49% (95%-CI 43-55) and 46% (95%-CI 40-52), respectively. The cumulative incidence of relapse was 24% (95%-CI 19-29) at this time, while the non-relapse mortality was 30% (95%-CI 24-35). The outcomes per conditioning and donor type are shown in Supplemental Figure 1. The 1-year cumulative incidence of clinically significant GVHD was 37% (95%-CI 32-43; see Supplemental Figure 2 for comparison with the overall grading of GVHD).

Development of GVHD after DLI and DLI-induced cytopenia

In total, 131 patients received an unmodified DLI after alloSCT without any prior relapse, chemotherapy or other cellular intervention or ongoing prophylactic immunosuppression. Of these patients, 65 had mixed BM chimerism at time of DLI, 59 FDC and for 7 the level of BM chimerism was unknown. 24 (37%) of the 65 patients with MC at time of DLI developed clinically significant GVHD compared to 9 (15%) of the 59 FDC patients. Of these 33 patients with GVHD after DLI, 14 died during GVHD (2 had FDC at time of DLI), while only one relapsed. Three patients, all with mixed BM chimerism (80-98% donor), showed DLI-induced cytopenia, all just before or at time of the start of GVHD.

SUPPLEMENTAL TABLES

Marker	Panel 1 Tube 1	Tube 2	Tube 3 (only used for sorting)	Panel 2 Tube 1
CD3	FITC	APC	-	APC
CD4	-	FITC	-	PB
CD8	-	PE	-	FITC
CD14	-	-	PE	APC-H7
CD16	PE	-	-	PE
CD19	APC	-	-	PE-Cy7
CD45	PerCP	PerCP	PerCP	PerCP
CD56	PE	-	-	PE

Supplemental Table 1. Fluorescence panels used for sorting and counting of the immune cells. APC, allophycocyanin; FITC, fluorescein isothiocyanate; PB, PacificBlue; PE, phycoerythrin; PerCP, peridinin-chlorophyll protein. All fluorochromes were from BD, Becton Dickinson, Breda, The Netherlands. The CD45intSSChiCD14- gate was used to identify and sort granulocytes.

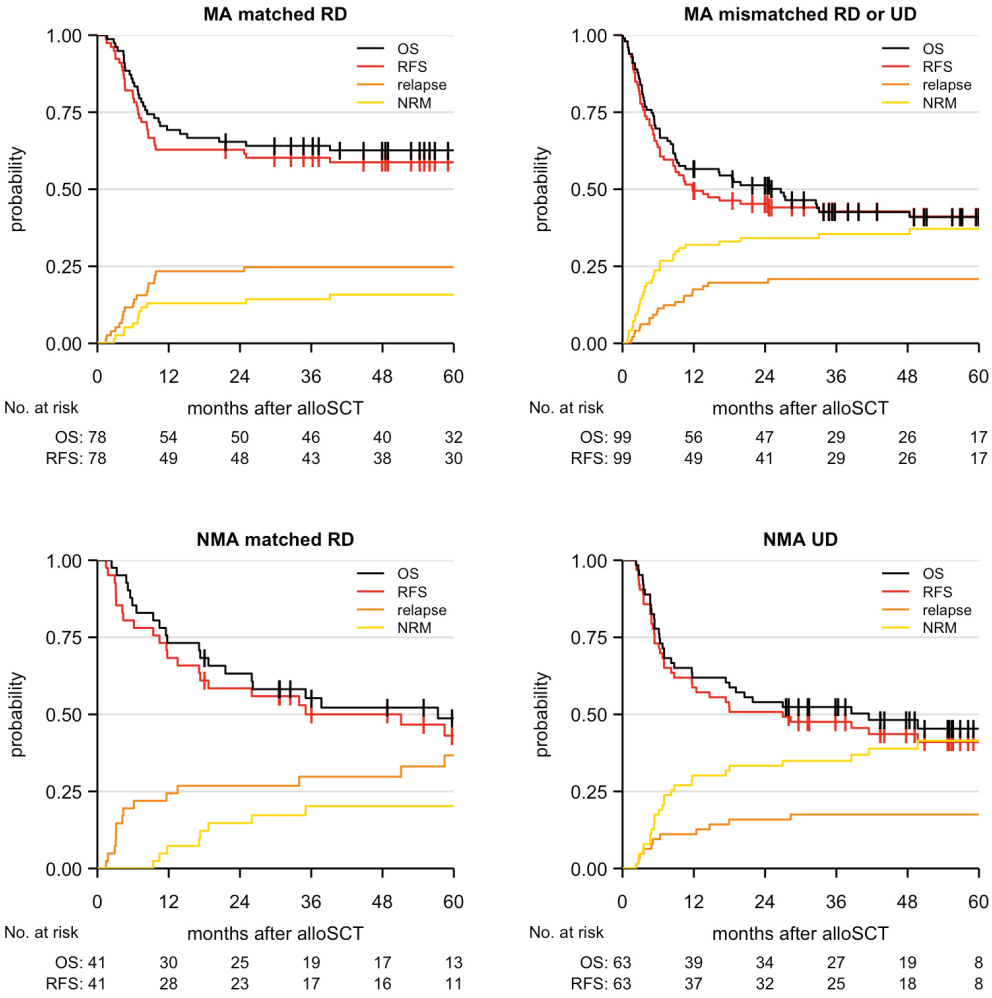
	Granulocytes	Monocytes	NK cells	B cells	CD4+ T cells	CD8+ T cells
Lower limit of reference range (cells/l)	1500	100	40	60	560	260
Time after alloSCT						
6 weeks	76%	90%	90%	24%	3%	23%
3 months	75%	95%	97%	61%	12%	32%
6 months	79%	97%	96%	79%	8%	43%

Supplemental Table 2. Recovery of immune cell subset counts after TCD alloSCT. Percentages of patients having immune cell counts of at least the lower limit of the reference range. Measurements after relapse or administration of chemotherapy, interferon, modified T cell product or second alloSCT were excluded.

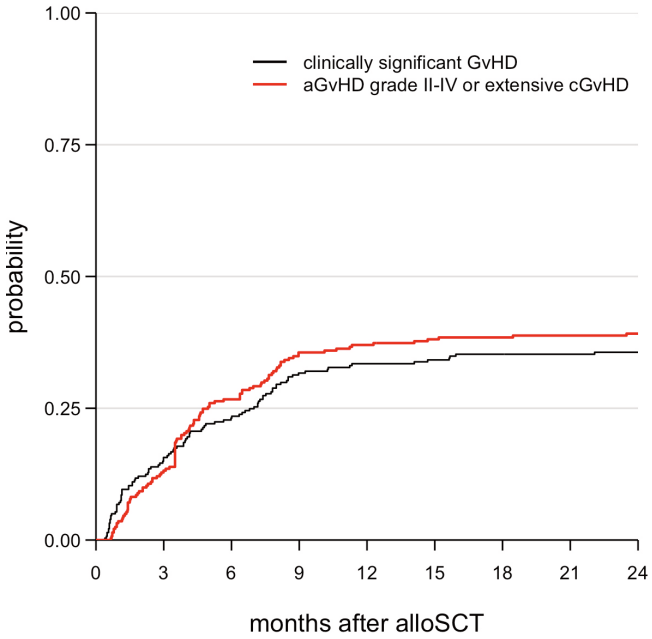
	Evaluable period from starting DLI			
Best response	**2 months**	**4 months**	**7 months**	**25 months**
Unevaluable due to early death from GvHD	4 (6%)	4 (6%)	4 (6%)	4 (6%)
Unevaluable due to early relapse	3 (5%)	3 (5%)	3 (5%)	3 (5%)
No response				
No event within period	34 (52%)	18 (28%)	9 (14%)	1 (2%)
Relapse, chemotherapy or interferon within period	0	2 (3%)	2 (3%)	3 (5%)
Death within period	0	0	1 (2%)	2 (3%)
Partial response				
No event within period	8 (12%)	8 (12%)	6 (9%)	1 (2%)
Relapse, chemotherapy or interferon within period	0	0	0	1 (2%)
Death within period	1 (2%)	1 (2%)	3 (5%)	3 (5%)
Complete response	15 (23%)	29 (45%)	37 (57%)	47 (72%)

Supplemental Table 3. Best BM chimerism response after DLI. Details regarding the best BM response and events that terminated the evaluation period.

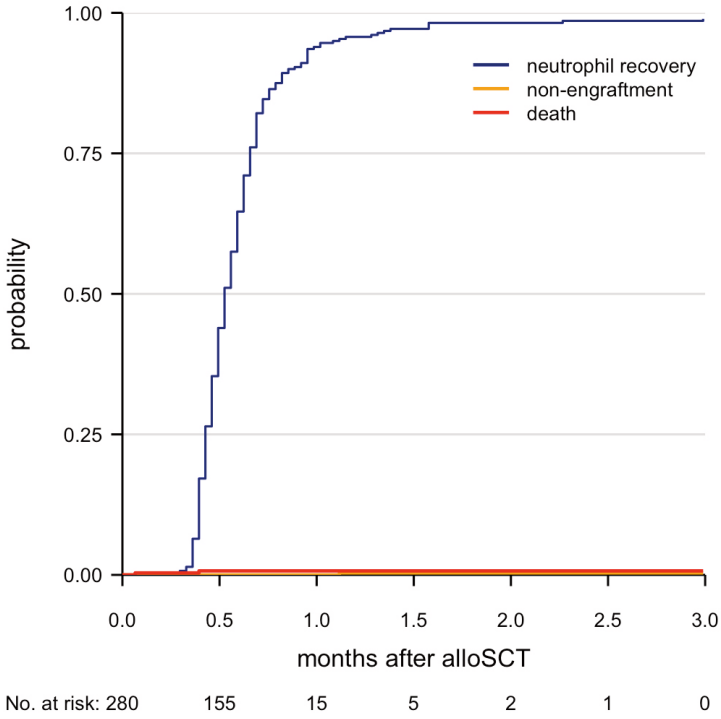
SUPPLEMENTAL FIGURES



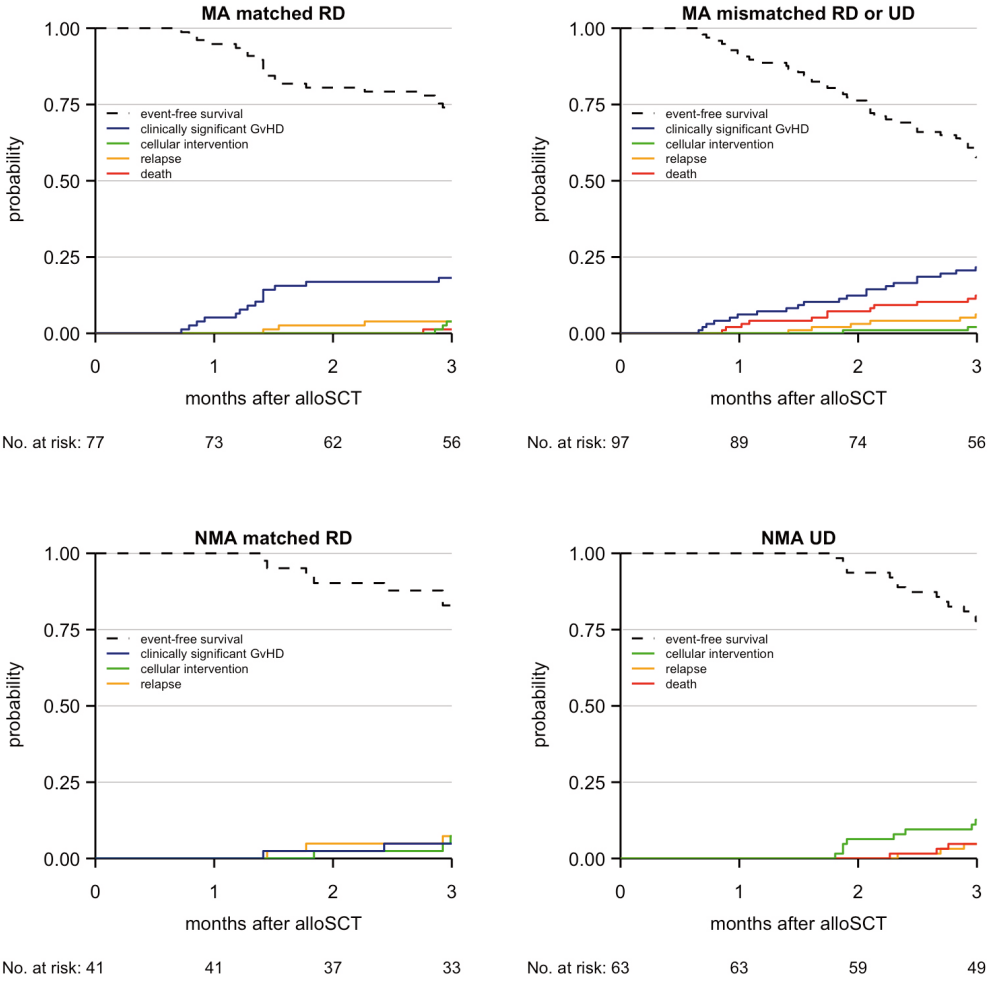
Supplemental Figure 1. Overall and relapse-free survival, relapse and non-relapse mortality. Kaplan-Meier curves for overall and relapse-free survival and cumulative incidence curves for relapse and non-relapse mortality per cohort. MA, myeloablative conditioned; NMA, nonmyeloablative conditioned; RD, related donor; UD, unrelated donor; OS, overall survival; RFS, relapse-free survival; NRM, non-relapse mortality. | indicates censoring times.



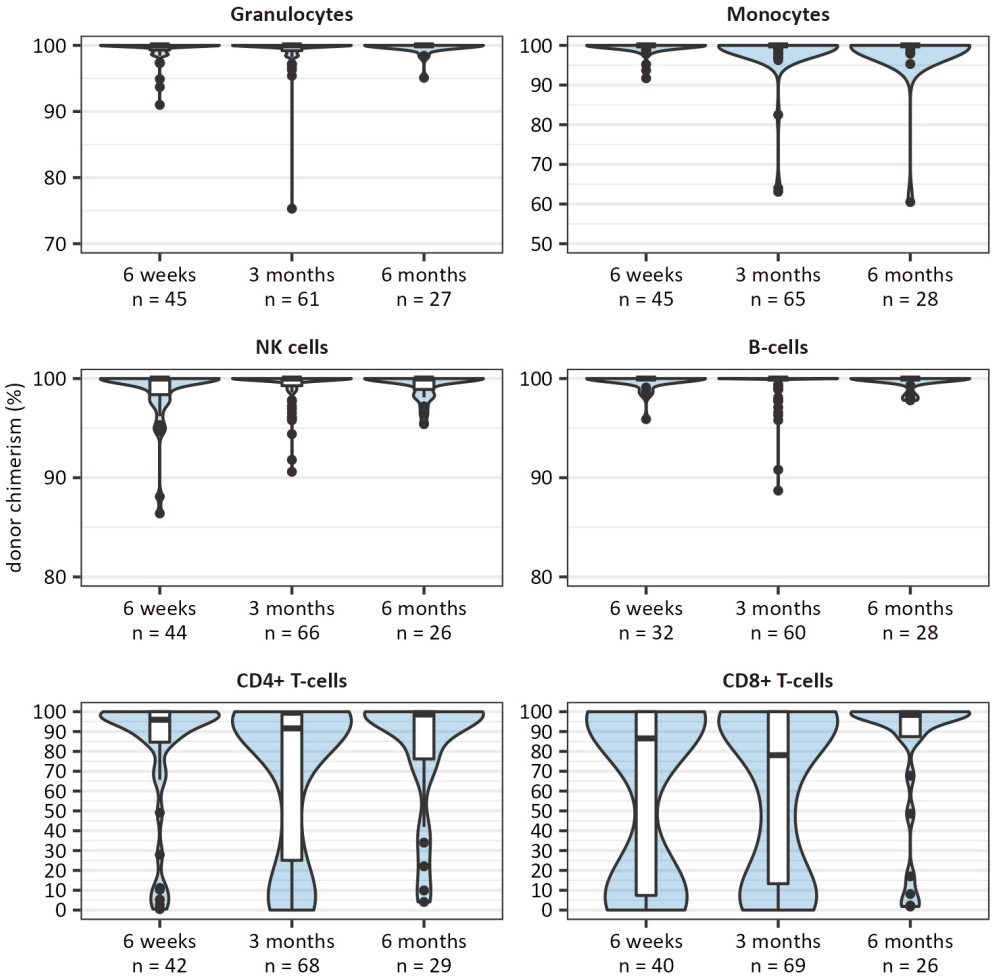
Supplemental Figure 2. Cumulative incidence of clinically significant GVHD. Cumulative incidence curves of clinically significant GVHD (GVHD requiring systemic treatment) and a combined curve of acute GVHD grade II-IV and extensive chronic GVHD. The cumulative incidences were calculated in separate competing risks models with relapse and death as competing events. The difference between the lines is caused by patients receiving tIS for lower grade GVHD not responding to topical treatment, patients with higher grade GVHD responding rapidly to topical treatment or requiring less than 14 days tIS, and patients with tIS for GVHD not proven by histology.



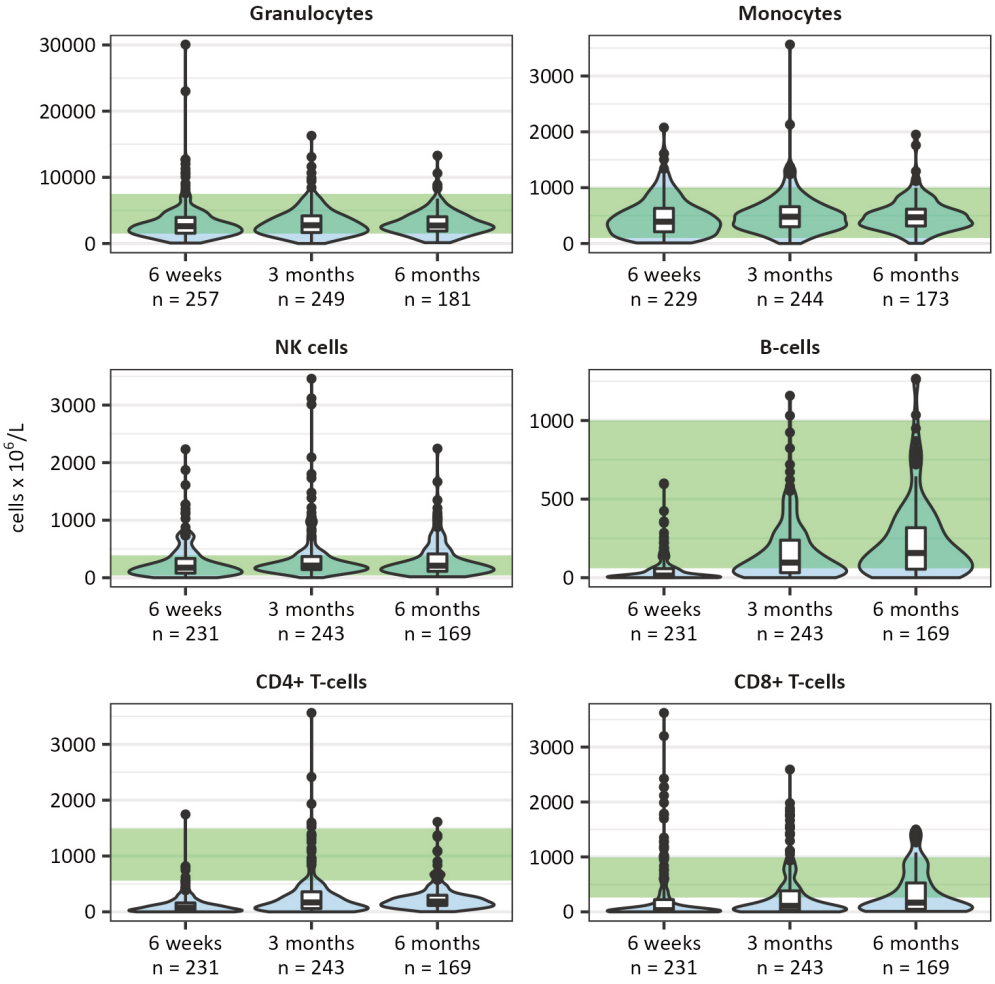
Supplemental Figure 3. Primary engraftment. Cumulative incidence curves of the competing events neutrophil recovery as a proxy for engraftment, non-engraftment and death. One patient never had neutrophils below 0.5×10^9/l and was excluded from this analysis.



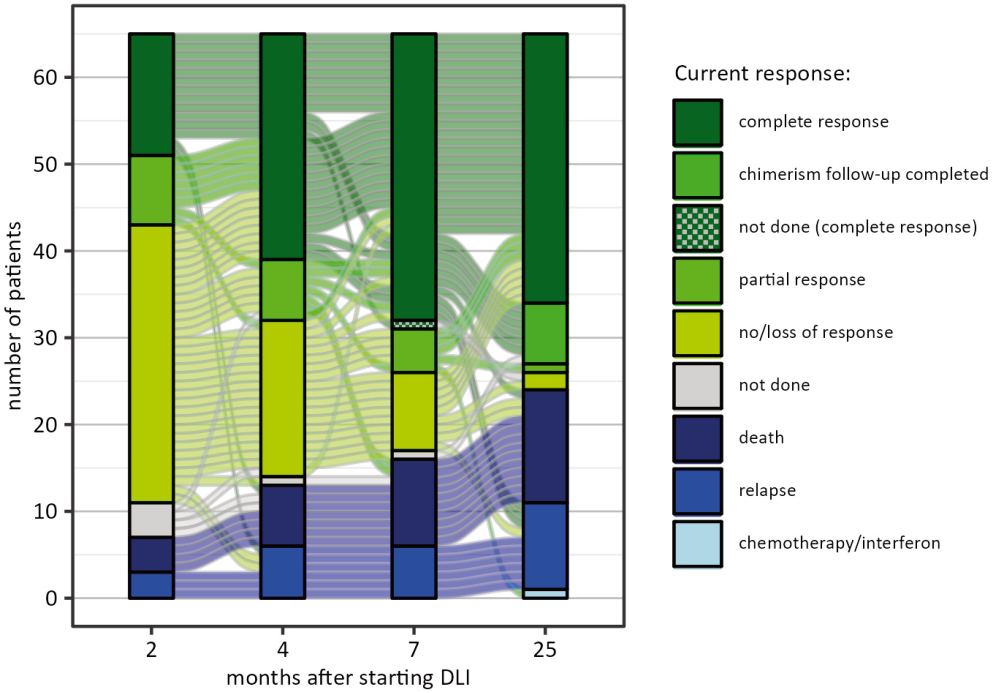
Supplemental Figure 4. Clinically significant GVHD after TCD alloSCT without DLI. Cumulative incidence curves of the competing events clinically significant GVHD, cellular intervention, relapse, start of chemotherapy or interferon, and death. Per plot only the curves corresponding to events observed in the subgroup are shown. The event-free survival was defined as the time from alloSCT until the occurrence of one of these events. MA, myeloablative; NMA, nonmyeloablative; RD, related donor; UD, unrelated donor; GVHD, graft-versus-host disease.



Supplemental Figure 5. Donor chimerism in immune cell subsets. Donor chimerism in immune cell subsets at 6 weeks, 3 months and 6 months after TCD alloSCT without any prior cellular intervention, relapse, chemotherapy or interferon. The lymphocyte counts of the total cohort, also including measurements after unmodified DLI, are shown in Supplemental Figure 6 and summarized in Supplemental Table 2.



Supplemental Figure 6. Recovery of absolute numbers of immune cell subsets after TCD alloSCT. Measurements after relapse or administration of chemotherapy, interferon, modified T cell product or second alloSCT were excluded. The green areas represent the reference ranges used in our laboratory.



Supplemental Figure 7. Current BM chimerism responses after unmodified DLI. Current BM chimerism responses in the 65 patients with mixed hematopoiesis receiving unmodified DLI. This plot provides insight in the durability of the achieved responses as patients with a response may lose their response or may for instance relapse after chimerism conversion. Per patient only the first terminating event (relapse, death, chemotherapy, interferon or second alloSCT) is considered: patients cannot move on to 'death' after a relapse. All patients in the green and grey areas were alive without any relapse, chemotherapy or interferon at the corresponding timepoint after their starting DLI. Per protocol, BM biopsies are stopped at 2 years after alloSCT if the patient has complete donor-derived hematopoiesis. These patients move to 'chimerism follow-up completed' in the plot. All other patients without any BM chimerism measurement within 2 months before the respective timepoint move to the 'not done' areas. Of these patients, those who showed a complete response in their previous measurement are shaded with green.