T-control: T-cell therapy in the context of allogeneic stem cell transplantation

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CHAPTER 2

EFFECT OF ALEMTUZUMAB-BASED T-CELL DEPLETION ON GRAFT COMPOSITIONAL CHANGE IN VITRO AND IMMUNE RECONSTITUTION EARLY AFTER ALLOGENEIC STEM CELL TRANSPLANTATION

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ABSTRACT

BACKGROUND

To reduce the risk on graft-versus-host disease (GVHD) after allogeneic stem cell transplantation (alloSCT), T-cell depletion (TCD) of grafts can be performed by the addition of alemtuzumab (ALT) 'to the bag' (*in vitro*) before transplantation. In this prospective study, we analyzed the effect of *in vitro* incubation with 20 mg ALT on the composition of grafts prior to graft infusion. Furthermore, we assessed whether graft composition at the moment of infusion was predictive for T-cell reconstitution and development of GVHD early after TCD-alloSCT.

METHODS

Sixty G-CSF-mobilized stem cell grafts were obtained from ≥9/10 HLA-matched related and unrelated donors. The composition of the grafts was analyzed by flow cytometry before and after the *in vitro* incubation with ALT. T-cell reconstitution and the incidence of severe GVHD were monitored until 12 weeks after transplantation.

RESULTS

In vitro incubation of grafts with 20 mg ALT resulted in an initial median depletion efficiency of T-cell receptor (TCR) α/β T cells of 96.7% (range 63.5-99.8%) followed by subsequent depletion *in vivo*. Graft volumes or the absolute leukocyte counts of grafts before the addition of ALT were not predictive for the efficiency of TCR α/β T-cell depletion. $CD4^{pos}$ T cells were depleted more efficient than $CD8^{pos}$ T cells, and naïve and regulatory T cells were depleted more efficient than memory and effector T cells. This differential depletion of T-cell subsets was in line with their reported differential CD52-expression. *In vitro* depletion efficiencies or absolute numbers of (naïve) TCR α/β T cells in the grafts after ALT incubation were not predictive for T-cell reconstitution or the development of GVHD post-alloSCT.

CONCLUSIONS

We conclude that the addition of ALT 'to the bag' is an easy, fast and general applicable strategy to prevent GVHD in patients receiving alloSCT after myeloablative or non-myeloablative conditioning, due to the efficient differential depletion of donor-derived lymphocytes and T cells.

INTRODUCTION

Allogeneic stem cell transplantation (alloSCT) is a potentially curative treatment for patients with a variety of malignant and non-malignant hematologic diseases.[1,2] The therapeutic effect of alloSCT is mediated by alloreactive donor T-cell responses directed against (malignant) hematopoietic cells of the patient.[3,4] However, donor-derived T cells can also elicit immune responses directed against other healthy cells in tissues and organs of the patient, causing detrimental acute or chronic graft-versus-host disease (GVHD).[5] Although long-term immunosuppressive treatment post-transplantation can strongly reduce the risk of GVHD, this strategy also suppresses disease- and pathogen-specific immune responses. To control the T-cell compartment of the graft, *in vitro* T-cell depletion (TCD) can be achieved. By controlling only the number of T cells in the grafts, such as through physical purification of $CD34^{pos}$ stem cells (positive selection), or selective depletion of total T-cell receptor (TCR) alpha/beta (α/β) T cells (negative selection) using antibody-coated magnetic beads and magnetic separation, no clear discrimination between these opposed T-cell effects can be achieved.[6-11] Therefore, several strategies have been explored to manipulate stem cell grafts in a way that potentially harmful alloreactive T cells are depleted, while beneficial T cells are preserved. Pre-clinical models have demonstrated that donor-derived naïve TCR α/β T cells are the major inducers of GVHD.[12,13] For direct protection against viral complications after alloSCT, peripheral expansion of memory TCR α/β T cells derived from seropositive donors is important,[14] whereas α/β-expressing regulatory T cells are similarly instrumental for the maintenance of tolerance and suppression of alloreactive T-cell responses.[15] In this regard, selective depletion of naïve TCR α/β T cells from grafts have been explored.[16,17] Furthermore, the use of alemtuzumab (ALT, Campath-1H) is so far widely known for both *in vitro* and/or *in vivo* aspecific TCD,[18-20] but might differentially target TCR α/β-expressing T-cell subsets more than other T-cell subsets.

ALT is a humanized monoclonal IgG1 antibody targeting the glycophosphatidylinositol (GPI)-anchored protein CD52, which is expressed on the surface of mature lymphocytes but not (or only marginally) on hematopoietic stem cells.[20-23] Since CD52 is not homogenously expressed on lymphocyte subsets, its susceptibility to ALT-induced TCD may vary.[21] *In vivo* application of ALT as part of the pre-transplant conditioning regimen aims to prevent graft rejection by elimination of patient T cells before graft infusion and to prevent the development of GVHD by depletion of donor T cells after graft infusion.[24-26] ALT can also be added directly to the stem cell graft - known as *in vitro* TCD or 'ALT to the bag'. This fast and easy applicable strategy has been shown to be effective in the prevention of GVHD when combined with both myeloablative (MA) and non-myeloablative (NMA) conditioning regimens.[27-31] Although TCD results in delayed immune reconstitution post-transplant, the incidence of cytomegalovirus (CMV) disease is not increased after *in vitro* ALT-based TCD alloSCT compared with non-TCD alloSCT strategies, suggesting that protective immunity is at least partly conserved.[29,32-34] Soon after ALT-based TCD alloSCT, expansion

of CD52^{neg} T cells that are insensitive to ALT has been observed.[14,35-40]

In this study, we investigated the effect of *in vitro* ALT addition to grafts on depletion efficiencies of lymphocyte subsets and changes in graft composition before infusion into patients. Furthermore, we analyzed whether graft composition was predictive for T-cell reconstitution and development of GVHD soon after ALT-based TCD alloSCT.

MATERIALS AND METHODS

PATIENTS AND TRANSPLANTATION PROTOCOLS

In this prospective study, 60 patients treated with an ALT-based TCD alloSCT for a hematologic disease at Leiden University Medical Center (LUMC, Leiden, The Netherlands) were included. Patient characteristics are provided in Table 1. Granulocyte colony-stimulation factor (G-CSF)-mobilized peripheral blood stem cell (PBSC) grafts were obtained from ≥9/10 HLA-matched related and unrelated donors. Informed consent was obtained in accordance with the Declaration of Helsinki. Patients received myeloablative or non-myeloablative conditioning according to the protocols indicated in Supplementary Table 1. The applied form of *in vivo* T-cell depletion differed slightly depending on the conditioning regimen and donor source. In the case of NMA conditioning and/or an unrelated donor, patients received 15 mg ALT (MabCampath, Sanofi Genzyme, Naarden, The Netherlands) intravenously (iv) twice the week before transplantation. Because patients receiving only ALT-based NMA conditioning and a graft from an unrelated donor still had an relatively high risk of GVHD, in addition to ALT, this group also received 1 mg/kg rabbit-derived anti-thymocyte globulin (ATG; Sanofi Genzyme, Naarden, The Netherlands) for additional T-cell depletion. Prophylactic immune suppression using cyclosporine A was given only temporarily to patients receiving MA conditioning and a graft from an unrelated donor. Irrespective of the conditioning regimen, 20 mg ALT was added to each graft for *in vitro* T-cell depletion. After 30 minutes of incubation on a roller bank at room temperature, the graft was immediately infused into the patient.

PROCESSING AND ANALYSIS OF GRAFT SAMPLES

Stem cell graft volumes were determined by weighing (1 mg = 1 mL). Samples were taken of each graft prior to and 30 minutes after the addition of 20 mg ALT. The concentration of leukocytes in these samples was determined using a Sysmex KX-21N (Sysmex, Etten-Leur, The Netherlands). Samples were centrifuged, and cells were resuspended in red blood cell lysis buffer (8.4g/L NH_4Cl and 1 g/L $KHCO_3$, pH 7.4; LUMC Pharmacy) and incubated for 10 minutes at 4°C. After centrifugation, cells were resuspended in Iscove's Modified Dulbecco's Medium (Lonza, Basel, Switzerland) containing 10% heat-inactivated human serum, 3 mM L-glutamine (Lonza) and 100 U/ml penicillin/

streptomycin (Lonza) at a concentration of 1-2*10^6 cells/ml and stored overnight at 4°C. The next day, the concentration and percentage of viable cells was determined using a hemocytometer and eosin. The total numbers of viable cells in the grafts were calculated by multiplying the leukocyte concentrations of the grafts by the graft volumes, followed by multiplication by the percentages of viable cells after overnight storage. The percentages of TCR α/β T cells ($CD3^+α/β^+$), TCR gamma/delta (γ/δ) T cells ($CD3^+γ/δ^+$), B cells ($CD19^+$), NK cells ($CD56^+$), $CD4^{pos}$ and $CD8^{pos}$ naïve T cells ($CD3^+CD4^+CD27^+CD45RA^+$/$CD3^+CD8^+CD27^+CD45RA^+$), $CD4^{pos}$ and $CD8^{pos}$ memory T cells ($CD3^+CD4^+CD45RO^+CD45RA^-$/$CD3^+CD8^+CD45RO^+CD45RA^-$), $CD4^{pos}$ and $CD8^{pos}$ effector T cells ($CD3^+CD4^+CD27^-CD45RA^+$/$CD3^+CD8^+CD27^-CD45RA^+$), and regulatory T cells ($CD3^+CD4^+CD25^+CD127^-$ $FoxP3^+$) in the graft samples before and after ALT incubation were analyzed by flow cytometry. Cells were stained with Alexa Fluor 647-labelled TCR α/β antibodies (ITK Diagnostics, Uithoorn, The Netherlands), Alexa Fluor 700-labelled CD45RO antibodies (ITK Diagnostics), allophycocyanin (APC)-H7-labelled CD3 antibodies (Beckton Dickinson (BD) Biosciences, San Diego/San Jose, USA), fluorescein isothiocyanate (FITC)-labelled CD27 and TCR γ/δ antibodies (BD Biosciences), phycoerythrin (PE)-labelled CD25 antibodies (BD Biosciences), phycoerythrin-cyanine7 (PeCy7)-labelled CD56 (BD Biosciences) and CD127 antibodies (Invitrogen, Waltham, MA, USA), PE-Texas-Red-labelled CD19 and CD45RA antibodies (Invitrogen), peridinin-chlorophyll-protein complex (PerCP)-labelled CD4 antibodies (BD Biosciences), V450-labelled CD8 antibodies (BD Biosciences) or V500-labelled CD4 antibodies (BD Biosciences). Intracellular FoxP3 staining was performed using the FoxP3 staining kit (FoxP3-APC monoclonal antibody, Invitrogen) according to the manufacturer's instructions. Cells were measured on an LSR II (BD Biosciences) and analyzed using Diva Software (BD Biosciences). Peripheral blood mononuclear cells (PBMC) of a healthy control were regularly measured as normal control. The absolute numbers of lymphocyte and T-cell subsets in the grafts were calculated by multiplying the absolute numbers of leukocytes in the grafts with the percentages of the cell subsets analyzed by flow cytometry. Depletion efficiencies (%) of specific cell populations were calculated as (1-(number of viable cells in the graft after ALT incubation / number of viable cells in the grafts before ALT incubation)) x 100%.

GVHD AND IMMUNE RECONSTITUTION AFTER alloSCT

Severe acute GVHD was defined as acute GHVD requiring (additional) systemic immunosuppressive therapy within a follow-up period of 12 weeks after TCD alloSCT. To investigate early immune reconstitution, peripheral blood (PB) samples were collected at 3 and 6 weeks after transplantation. PB samples for assessment of immune reconstitution collected between day 11 and day 31 were categorized as 3 weeks post-alloSCT and those collected between day 32 and day 52 as 6 weeks post-alloSCT. Absolute numbers of circulating T cells ($CD45^+CD3^+$) were determined as part of routine clinical evaluation on fresh blood samples using TruCount Tubes (BD Biosciences), following the manufacturer's instructions. Samples were stained with APC-labelled CD3 (BD Biosciences) antibodies and V500-conjugated CD45 (BD Biosciences) antibodies.

The percentages of $CD52^{pos}$ ($CD45^{+}CD3^{+}CD52^{+}FLAER^{+}$) and $CD52^{neg}$ ($CD45^{+}CD3^{+}CD52^{-}FLAER^{-}$) T cells were determined by staining follow-up samples with Alexa Fluor 488-labelled GPI-anchor-specific inactivated toxin pro-aerolysin (FLAER-AF488, SanBio, Uden, The Netherlands), APC-labelled CD52 antibodies (ITK Diagnostics), APC-H7-labelled CD3 antibodies (BD Biosciences) and V500-labelled CD45 antibodies (BD Biosciences). Cells were measured on an LSR II (BD Biosciences) and analyzed using Diva Software (BD Biosciences). PBMC of a healthy control were regularly measured as normal control. Absolute numbers of $CD52^{pos}$ and $CD52^{neg}$ T cells were calculated by multiplying the percentages of these cells within the $CD3^{pos}$ cell populations by the absolute T-cell counts obtained via measurements on fresh blood samples using TruCount Tubes (BD Biosciences).

STATISTICAL ANALYSIS

Absolute lymphocyte and T-cell counts (Table 2) and percentages of lymphocytes (Supplementary Table 2) in grafts before and after *in vivo* ALT incubation were compared using Wilcoxon matched-pairs signed-rank test. The Friedman test was used to detect a significant difference between depletion efficiencies of the different lymphocyte subsets (Figure 1A) and T-cell subsets (Figure 3A) in the total study cohort. For this analysis, the alpha level was corrected using the Bonferroni method (alpha = 0.05 / 6 = 0.008). As post hoc analysis, the Wilcoxon matched-pairs signed-rank test was used to calculate the difference between depletion efficiencies of two specific cell subsets in Figures 1A and 3A. The Mann-Whitney test was used to analyze the differences in absolute numbers and depletion efficiencies of naïve and regulatory T cells and the ratio between the numbers of naïve and regulatory T cells between patients who did or did not experienced GVHD early after transplantation (Figures 5A-E). Level of significance was <0.05 (2-sided). Statistical analysis were performed using Prism 8.0 (GraphPad, La Jolla, CA, USA).

RESULTS

IN VITRO ALT-BASED T-CELL DEPLETION OF STEM CELL GRAFTS LEADS TO DIFFERENTIAL DEPLETION OF LYMPHOCYTE SUBSETS

In vitro TCD of allogeneic stem cell grafts for the prevention of GVHD was applied by the addition of 20 mg ALT to the bag, followed by an incubation of 30 minutes and direct subsequent infusion into patients. Lysis of part of the lymphocytes in the grafts was expected to take place directly *in vitro*, followed by anticipated ongoing lysis of both donor and patient lymphocytes *in vivo* after infusion of the graft into the patient. To measure the magnitude of the direct *in vitro* effect of ALT on the number and distribution of lymphocyte subsets in the grafts, we analyzed the composition of 60 G-CSF mobilized PBSC grafts before and after *in vitro* incubation with 20 mg ALT. Stem cell grafts were obtained from 20 related and 40 unrelated donors for patients undergoing TCD alloSCT for a variety of hematologic diseases (Table 1).

Table 1. Patient and transplantation characteristics of study cohort. MA, myeloablative; neg, negative; NMA, non-myeloablative.

Median age, years (range)	60 (20-73)
Gender, *n* (%)	
Male	36 (60)
Female	24 (40)
Diagnosis, *n* (%)	
Acute myeloid leukemia	28 (47)
Acute lymphoblastic leukemia	7 (12)
Multiple myeloma	10 (17)
Myelodysplastic syndrome	4 (7)
Hodgkin lymphoma	1 (2)
B-cell lymphoma	3 (5)
T-cell lymphoma	2 (3)
Myeloproliferative syndrome	4 (7)
Severe aplastic anemia	1 (2)
Conditioning regimen and donor type (HLA-matching), *n* (%)	
MA related (12/12)	6 (10)
MA unrelated (≥9/10)	14 (23)
NMA related (12/12)	14 (23)
NMA unrelated (≥9/10)	26 (43)

Graft composition was analyzed 0 days (*n* = 5), 1 day (*n* = 48), or 2 days (*n* = 7) after the donor leukapheresis procedure, depending on logistics. Before the addition of ALT, the grafts had a median volume of 379 mL (range, 75-789 mL) and contained a median of $70*10^9$ leukocytes (range, 15.1-$147.7*10^9$). Subset analysis of lymphocytes showed that before ALT addition, grafts were dominated by TCR α/β T cells, followed by B cells, NK cells and TCR γ/δ T cells (Supplementary Figure 1A). After incubation with ALT, the absolute numbers of all these lymphocyte subsets in the grafts significantly decreased (Table 2 and Supplementary Figure 1B). TCR α/β T cells were depleted significantly more efficient than TCR γ/δ T cells, B cells and NK cells (TCR α/β T cells versus TCR γ/δ T cells, B cells or NK cells: all $p<0.001$), as illustrated by median depletion efficiencies of 96.7% (range, 63.5-99.8%), 86.9% (range, 8.7-98.7%), 88.6% (range, 0-99%) and 72.5% (range, 14.3-93.6%), respectively (Figure 1A). In 7 grafts, the depletion efficiencies of TCR α/β T cells were below 90% (grafts 5, 19, 20, 35, 38, 44, and 46, indicated by green symbols in all figures). These grafts were among the grafts with the highest absolute numbers of TCR α/β T cells at the moment of infusion into the patient (Supplementary Figure 1B). The differential depletion of lymphocyte subsets resulted in significant compositional changes of grafts. Before incubation with ALT, a median of 71.9% (range, 55.8-85.5%) of lymphocytes consisted of TCR α/β T cells (Figure 1B), whereas after incubation with ALT this decreased to a median of 33.7% (range, 4.8-81.0%; $p<0.001$) (Figure 1C and Supplementary Table 2). Since TCR γ/δ T cells, B and NK cells were depleted less efficiently compared to TCR α/β T cells, these cell types comprised a significantly larger proportion of cells in the graft after ALT incubation than before ALT incubation (Figure 1C

and Supplementary Table 2). No differences in depletion efficiencies or graft compositions were observed between grafts obtained from related or unrelated donors (Supplementary Figure 2A) or between grafts that were analyzed on the same day or 1 or 2 days after the donor leukapheresis procedure (Supplementary Figure 2B), suggesting no selective loss of lymphocytes during transport or *in vitro* depletion.

Next, we investigated whether the magnitude of direct *in vitro* depletion of TCR α/β T cells in the grafts could be predicted based on graft characteristics before the addition of ALT. A trend was observed between the levels of direct *in vitro* depletion efficiency of TCR α/β T cells and leukocyte concentrations in the grafts before ALT addition (R^2 = 0.059, p = 0.060) (Figure 2A). However, no correlation was found between the levels of direct *in vitro* depletion of TCR α/β T cells and absolute leukocyte counts in the grafts before ALT addition (R^2 = 0.005, p = 0.579, data not shown) or the volumes of the grafts (R^2 = 0.013, p = 0.385) (Figure 2B). Interestingly, if only samples with TCR α/β T-cell depletion efficiencies higher than 90% (excluding the green outliers) are considered in the analysis, significant correlations with levels of *in vitro* depletion of TCR α/β T cells are seen for both leukocyte concentrations in the grafts (R^2=0.1405; p=0.0057) and graft volumes (R^2=0.1084; p=0.0161) (data not shown). However, we do not have an indication as to the underlying cause of the outliers.

These observations illustrate that lymphocyte subsets were unequally depleted from the grafts during the 30 minutes *in vitro* incubation with ALT, resulting in a major compositional change of the grafts already before infusion into the patient. Of lymphocyte subsets, TCR α/β T cells were the most efficiently depleted *in vitro*. The extent of direct *in vitro* TCR α/β TCD was not predictable based on graft volume or absolute leukocyte count in the graft before ALT addition.

EFFICIENT *IN VITRO* DEPLETION OF NAÏVE AND REGULATORY T CELLS BY THE ADDITION OF ALT TO STEM CELL GRAFTS

Since donor-derived memory and effector T cells from the graft are mainly expected to contribute to (early) protective immunity against pathogens post-transplantation, whereas the presence of naïve T cells and absence of regulatory T cells derived from the donor have been associated with the development of GVHD post-transplantation, we investigated the effect of ALT on the *in vitro* depletion of different T-cell subsets. T cells with a naïve, memory and effector phenotype within the $CD8^{pos}$ and $CD4^{pos}$ TCR α/β T-cell compartments and $CD4^{pos}$ regulatory T cells were quantified before and 30 minutes after addition of ALT to the grafts.

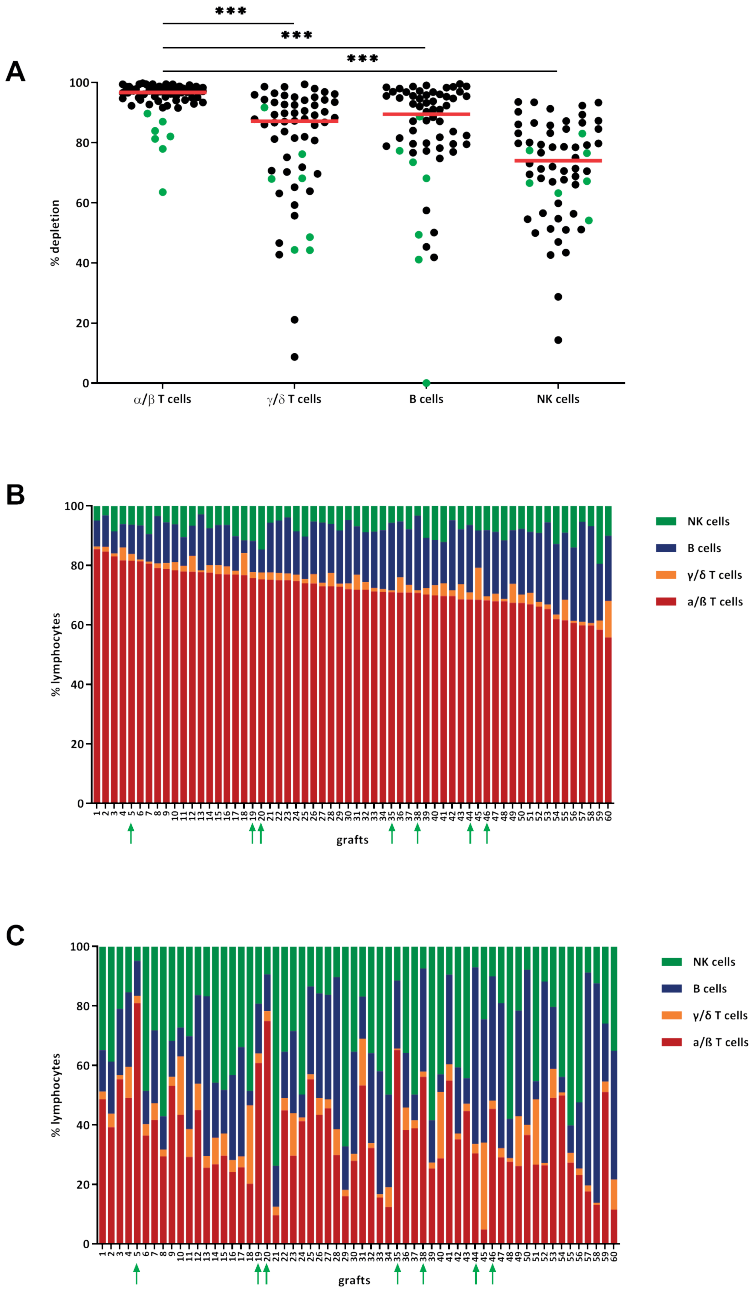

Figure 1. Lymphocytes in alloSCT grafts before and after *in vitro* ALT incubation (n=60). The seven grafts with TCR α/β T-cell depletion efficiencies below 90% are indicated by green symbols (dots in A, arrows in B and C). **A**, Depletion efficiencies (%) of lymphocytes after in vitro ALT incubation. The red solid lines indicate medians. The Wilcoxon matched-pairs signed-rank test was used for statistical analysis. *** means p<0.001. **B**, Compositions of the lymphocyte compartment in the grafts before *in vitro* ALT incubation. **C**, Compositions of the lymphocyte compartment in the grafts after *in vitro* ALT incubation.

Table 2. Absolute numbers of lymphocytes in the grafts before and after *in vitro* incubation with ALT.

		Before ALT addition		After ALT addition		Significance of differences
		Median	*Range*	*Median*	*Range*	*P value**
TCR α/β T cells (*10^9)		12.4	3.67 – 41.0	0.45	0.03 – 5.13	<0.001
$CD8^{pos}$ (*10^7)	Naïve T cells	118	12.3 – 758	6.62	0.74 – 411	<0.001
	Memory T cells	56.5	1.00 – 260	6.62	0.16 – 37.3	<0.001
	Effector T cells	24.8	1.04 – 182	29.4	1.40 – 245	0.098
$CD4^{pos}$ (*10^7)	Naïve T cells	540	62.5 – 2350	4.73	0.07 – 426	<0.001
	Memory T cells	484	100 – 1230	2.59	0.08 – 34.0	<0.001
	Effector T cells	6.69	0.28 – 60.9	0.61	0.00 – 6.05	<0.001
	Regulatory T cells	20	0.00 – 122	0.25	0.00 – 5.77	<0.001
TCR γ/δ T cells (*10^9)		0.34	0.05 – 2.18	0.04	0.003 – 0.61	<0.001
B cells (*10^9)		2.84	7.21 – 9.06	0.23	0.02 – 10.0	<0.001
NK cells (*10^9)		1.16	0.42 – 3.06	0.30	0.04 – 1.18	<0.001

** Wilcoxon matched-pairs, signed-rank test*

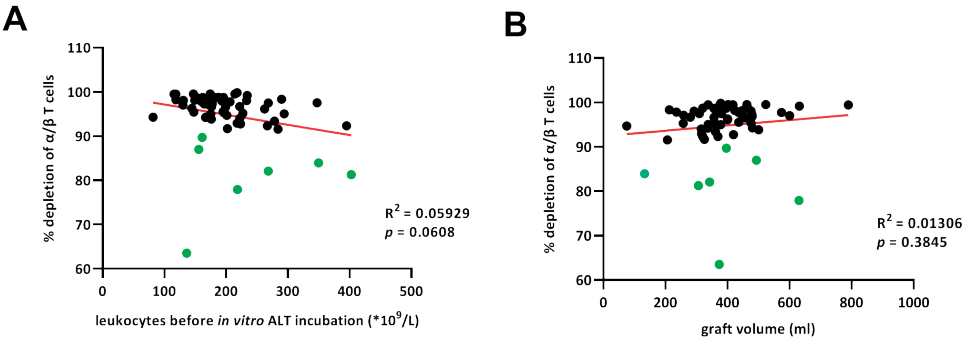

Figure 2. Correlations between the efficiencies of α/β T-cell depletion and graft characteristics (*n*=60). The 7 grafts with TCR α/β T-cell depletion efficiencies below 90% are indicated by green symbols. The solid lines indicate the linear regression analysis. **A**, Relation between the TCR α/β T-cell depletion efficiencies (%) and the leukocyte counts in the grafts before ALT addition. **B**, Relation between the TCR α/β T-cell depletion efficiencies (%) and the graft volumes.

The absolute numbers of all TCR α/β T-cell subsets, except for $CD8^{pos}$ effector T cells, decreased significantly after ALT incubation *in vitro* (Table 2 and Supplementary Figure 3). Depletion efficiencies revealed that $CD4^{pos}$ T cells were more efficiently depleted than $CD8^{pos}$ T cells (Figure 3A). Within $CD4^{pos}$ T cells, the median depletion efficiencies of naïve, memory, effector and regulatory T cells were 99.4% (range, 72.8-100%), 99.5% (range, 94.6-100%), 92.8% (range, 0-100%), and 98.6% (range, 0-100%), respectively, showing that $CD4^{pos}$ effector T cells had a significantly lower depletion efficiency compared with the other $CD4^{pos}$ T-cell subsets (p<0.0001).

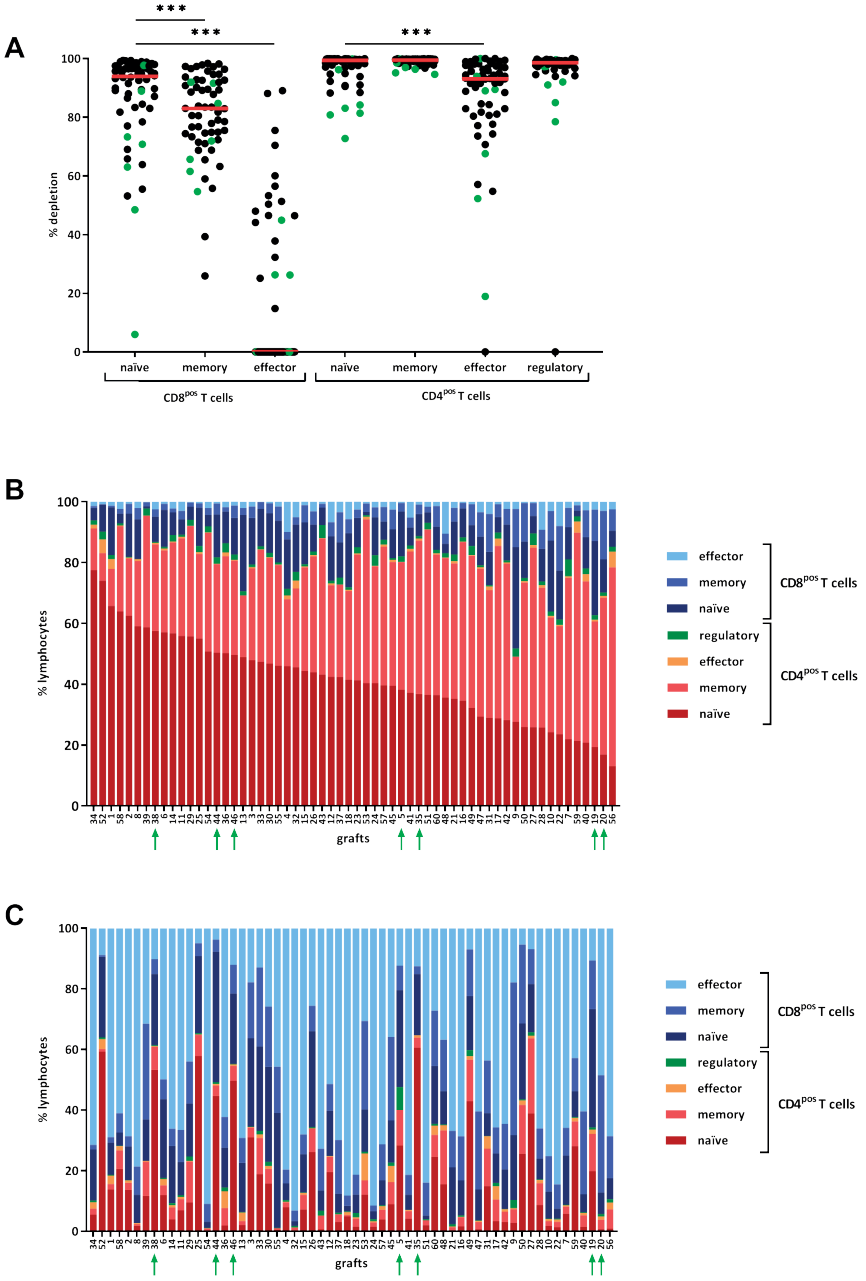

Figure 3. T cells in alloSCT grafts before and after *in vitro* ALT incubation (n=60). The 7 grafts with α/β T-cell depletion efficiencies below 90% are indicated by green symbols (dots in A, arrows in B and C). **A**, Depletion efficiencies (%) of T cells after *in vitro* ALT incubation. The red solid lines indicate medians. The Wilcoxon matched-pairs signed-rank test was used for statistical analysis. *** means p<0.001. **B**, Compositions of the T-cell compartment in the grafts before *in vitro* ALT incubation. **C**, Compositions of the T-cell compartment in the grafts after *in vitro* ALT incubation.

In the CD8^{pos} T-cell compartment, the depletion effect of ALT on naïve T cells in the grafts was significantly stronger than the effect on memory (naïve versus memory CD8^{pos} T cells, p=0.0003) and effector T cells (naïve versus effector CD8^{pos} T cells, p<0.0001), as illustrated by median depletion efficiencies of 93.6% (range, 6.0-99.4%), 82.3% (range, 25.9-98.4%), and 0% (range, 0-89.1%), respectively. Directly after *in vitro* ALT incubation, the compositions of the T-cell compartments in the grafts changed substantially. Hence, although before ALT addition the T-cell compartments were dominated by naïve and memory CD4^{pos} T cells in the majority of grafts, after ALT incubation the majority of grafts contained mainly effector and memory CD8^{pos} T cells (Figures 3B and C, Supplementary Table 2).

These results illustrate that during the *in vitro* incubation of stem cell grafts with ALT, the differential depletion of T-cell subsets resulted in an alteration of the T-cell compartment of the grafts. The most efficient depletion was observed for CD4^{pos} naïve, memory, and regulatory T cells, whereas no or only a marginal depletion of CD8^{pos} effector T cells was achieved. Although the ALT-based TCD of the grafts was already substantial after *in vitro* incubation, we cannot rule out that the process continues *in vivo* after graft infusion into the patients, thereby further influencing the graft composition. However, this effect can only be assessed indirectly by measuring immune cell reconstitution in patients.

THE ABSOLUTE NUMBER OF T CELLS IN THE GRAFTS AT THE MOMENT OF TRANSPLANTATION IS NOT PREDICTIVE FOR T-CELL RECONSTITUTION AT 3 OR 6 WEEKS POST-alloSCT

To investigate whether the graft compositions after *in vitro* ALT incubation were predictive for T-cell reconstitution after graft infusion, we determined the absolute numbers of circulating T cells in PB at 3 and 6 weeks post-transplantation. Because T-cell reconstitution can also be influenced by the pre-transplant conditioning, patients were analyzed per conditioning regimen as indicated in Supplementary Table 1. The six patients who received MA conditioning and a related donor graft did not receive *in vivo* ALT iv in the week before transplantation as part of the conditioning regimen, whereas the remaining 54 patients did receive *in vivo* ALT iv. Patients who underwent NMA conditioning and had an unrelated donor graft also received ATG iv. Patients with MA conditioning and a graft from an unrelated donor received cyclosporine A in the first weeks post-transplantation.

Follow-up samples were available for 54 and 57 patients at 3 and 6 weeks, respectively, after alloSCT. Correlation analysis per conditioning regimen revealed that the absolute numbers of T cells in the grafts at the moment of infusion in the patients were not predictive for the numbers of circulating T cells in peripheral blood at 3 weeks (data not shown) or 6 weeks after transplantation (Figure 4A). The three patients with relatively high numbers of T cells 6 weeks after transplantation suffered from an active viral reactivation (Epstein Barr virus reactivation, CMV reactivation, or

combined CMV and varicella zoster virus reactivation in patients receiving grafts 18, 39 and 41, respectively, indicated by red symbols). The patients who received grafts that showed *in vitro* TCR α/β T-cell depletion efficiencies below 90% (green symbols) had no advantage in T-cell reconstitution post-transplantation.

As ALT targets only $CD52^{pos}$ T cells, T cells that have lost membrane expression of CD52 are insensitive to ALT. We and others have previously shown that after ALT-based TCD alloSCT, reconstitution of T cells is partly due to the expansion of $CD52^{neg}$ T cells.[35,36] Figure 4B illustrates that both $CD52^{pos}$ and $CD52^{neg}$ T cells contributed to T-cell reconstitution soon after TCD alloSCT, although the reconstitution was variable among conditioning regimens. In 23 of 24 evaluable patients who received NMA conditioning and a related donor graft, MA conditioning and a related donor graft or MA conditioning and an unrelated donor graft, $CD52^{neg}$ T cells at a concentration of $>10^6$/L were detectable in the PB at 6 weeks after TCD alloSCT. This level of $CD52^{neg}$ T-cell reconstitution was observed in only nine of 22 evaluable patients that received NMA conditioning and an unrelated donor graft and also received ATG as part of the conditioning regimen.

Since the T-cell compartments of the grafts directly after *in vitro* ALT incubation were dominated by effector T cells, whereas memory T cells are especially expected to contribute to T-cell reconstitution in the weeks after transplantation due to their expansion capacity, we evaluated whether the absolute numbers of memory T cells in the grafts at the moment of infusion correlated with reconstitution of $CD52^{pos}$ T cells 3 and 6 weeks after transplantation. For all conditioning regimens, the absolute numbers of memory T cells in the grafts were not predictive for the reconstitution of $CD52^{pos}$ T cells at 3 weeks (data not shown) or 6 weeks after transplantation (Figure 4C).

These results indicate that T-cell reconstitution early after TCD alloSCT cannot be predicted based on the composition of the graft at the moment of infusion into the patient.

OCCURRENCE OF EARLY ACUTE GVHD CANNOT BE PREDICTED BASED ON GRAFT COMPOSITION AFTER *IN VITRO* ALT INCUBATION

The ultimate aim of *in vitro* ALT-based TCD is to reduce the risk of GVHD post-alloSCT. Hence, we determined the incidence of early acute GVHD requiring immunosuppressive therapy in the study cohort. Since donor-derived naïve T cells are thought to be the main initiators of GVHD and regulatory T cells the main suppressors of alloreactive immune responses, an imbalance between these cell populations in the infused grafts might affect the chance of developing GVHD after transplantation. Therefore, we compared the absolute numbers, depletion efficiencies and ratio between absolute numbers of naïve and regulatory T cells in the grafts at the moment of infusion between patients who did or did not develop GVHD.[41]

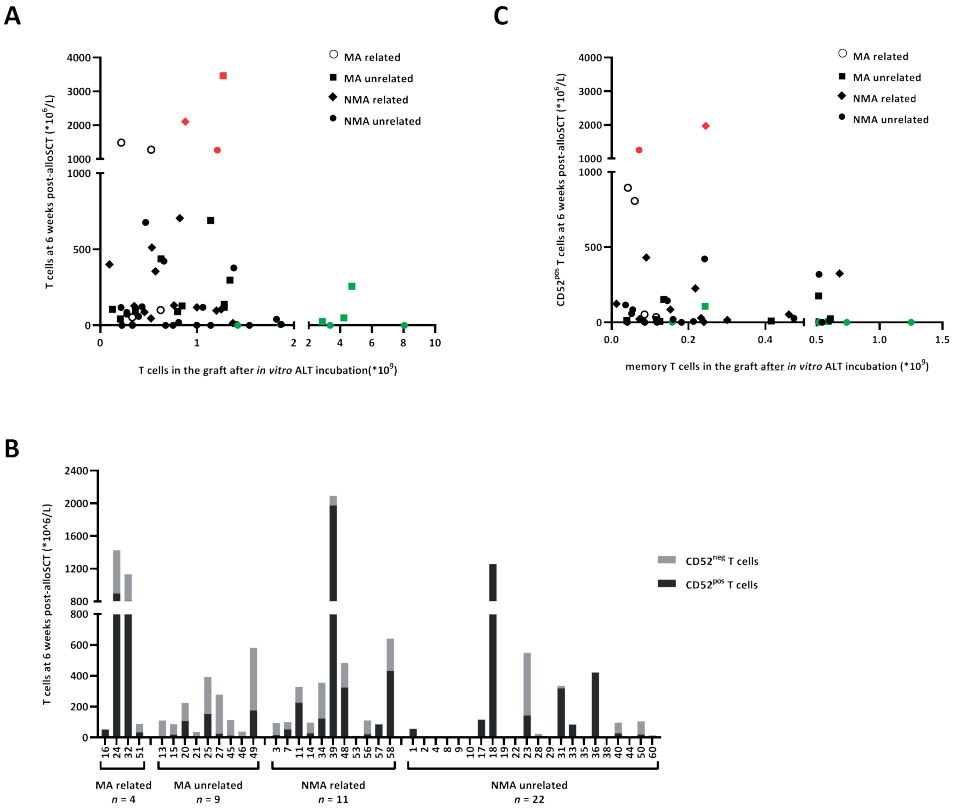

Figure 4. Correlations between the absolute numbers of T cells in the grafts after *in vitro* ALT incubation and T-cell reconstitution at 6 weeks after TCD alloSCT. Squares represent patients that received MA conditioning and a graft from an unrelated donor. Dots represent patients that received NMA conditioning and a graft from an unrelated donor. Diamonds represent patients that received NMA conditioning and a graft from a related donor. **A**, Relation between the absolute numbers of total T cells in the grafts after *in vitro* ALT incubation and the concentrations of total T cells in the peripheral blood at 6 weeks post-transplantation (*n* = 57). The 7 grafts with TCR α/β T-cell depletion efficiencies below 90% are indicated by green symbols. The 3 red symbols indicate patients with viral reactivations. **B**, Concentrations of $CD52^{neg}$ and $CD52^{pos}$ T cells in the peripheral blood at 6 weeks after alloSCT (*n* = 46). **C**, Relation between the absolute numbers of memory T cells in the grafts after *in vitro* ALT incubation and the concentrations of $CD52^{pos}$ T cells in the peripheral blood at 6 weeks post-transplantation (*n* = 42). The 6 grafts with TCR α/β T-cell depletion efficiencies below 90% are indicated by green symbols. The 2 red signs indicate patients with viral reactivations.

Within our cohort of 60 patients, eight patients required start of (additional) systemic immunosuppressive therapy due to the development of acute GVHD at a median of 29 days (range, 22-70 days) after transplantation. Of these eight patients, five patients received MA conditioning and an unrelated donor graft (grafts 5, 20, 21, 25, and 49), two patients received NMA conditioning and an unrelated donor graft (grafts 40 and 43), and one patient received

MA conditioning and a related donor graft (graft 51). All eight patients suffered from skin GVHD; patient 43 also developed liver GVHD. Figures 5A and 5B illustrate that no significant differences were observed between patients who did or did not develop GVHD regarding the absolute counts of naïve and regulatory T cells in the grafts at the moment of infusion into the patients (p=0.362 and p=0.217, respectively). As the ALT-based lytic effect is expected to continue *in vivo* after graft infusion, we hypothesized that the depletion efficiency of naïve and regulatory T cells *in vitro* might be used as an indication whether naïve and regulatory T cells were sensitive to ALT-based depletion. However, no differences in *in vitro* depletion efficiencies of these two T-cell subsets were observed between the two groups of patients (p=0.871 and p=0.613, respectively) (Figures 5C and 5D). Interestingly, the ratio between the numbers of naïve and regulatory T cells in the grafts before infusion was significantly higher in patients without GVHD after TCD alloSCT compared with patients who developed GHVD soon after TCD alloSCT (p=0.016) (Figure 5E). Furthermore, no significant differences were found in concentrations of circulating total or $CD52^{pos}$ T cells in the PB of patients with versus without development of GVHD 3 weeks post-transplantation (data not shown).

The reported low incidence of severe acute GVHD shows that the risk of developing acute (skin) GVHD requiring (additional) systemic immunosuppressive therapy was very limited after the described conditioning regimens and ALT-based *in vitro* TCD strategy of grafts. Because of this low incidence, the few patients who did develop early acute GVHD post-alloSCT could not be identified based on the *in vitro* depletion efficiency or absolute cell count of naïve or regulatory T cells in their graft at the moment of transplantation.

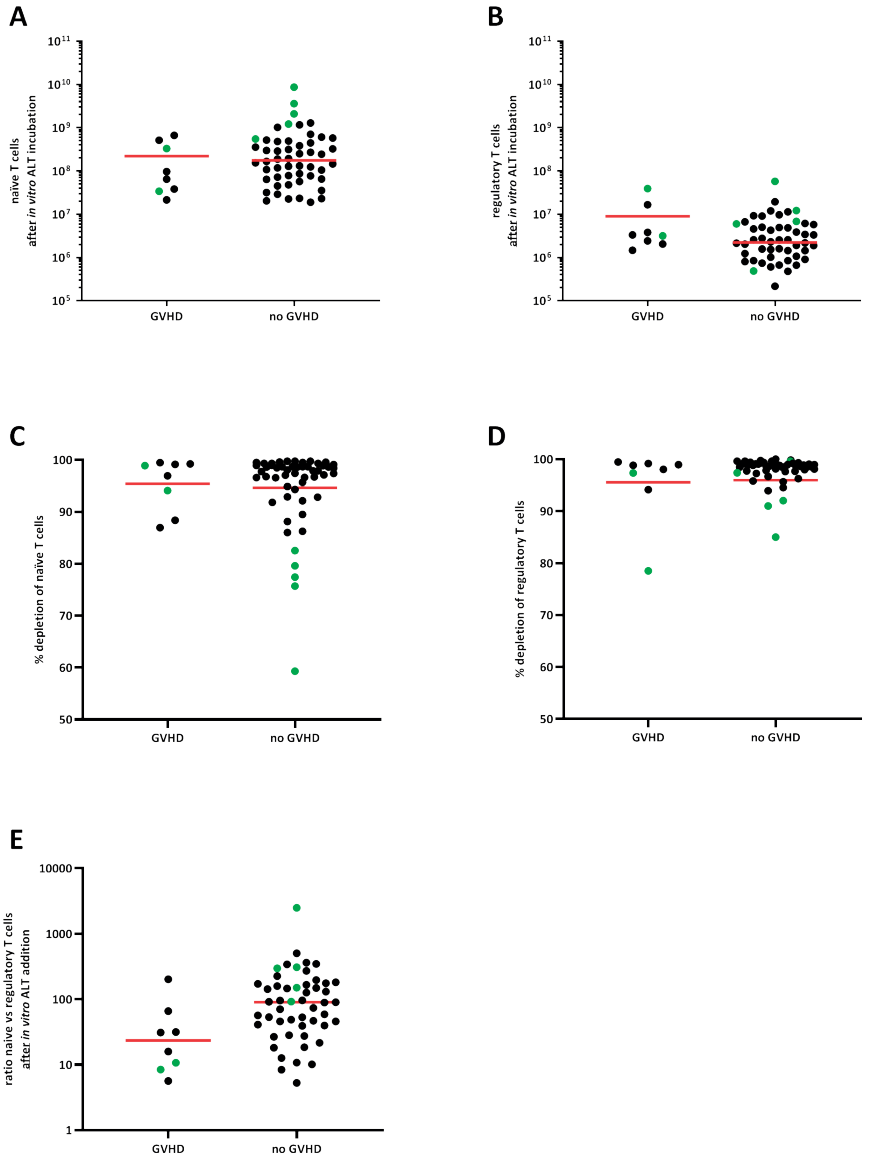

Figure 5. The absolute amounts nor the depletion efficiencies of naïve or regulatory T cells in the grafts can identify patients at risk of developing acute GVHD early after TCD alloSCT. After alloSCT, 8 of 60 patients developed acute GVHD requiring start of or change in systemic immunosuppressive therapy within 12 weeks post-alloSCT. The 7 grafts with α/β T-cell depletion efficiencies below 90% are indicated by green symbols. The Mann-Whitney test was used for statistical analysis. **A**, The absolute numbers of naïve T cells and **B**, regulatory T cells in the grafts after the incubation with ALT in patients with or without GVHD. The median is indicated by the red line. **C**, The depletion efficiencies of naïve T cells and **D**, regulatory T cells in the grafts due to the *in vitro* incubation with ALT in patients with or without GVHD. The median is indicated by the red line. **E**, Ratio between the numbers of naïve and regulatory T cells in the grafts after the incubation with ALT.

DISCUSSION

In this study, we show that the *in vitro* incubation of allogeneic stem cell grafts with ALT resulted in a differential depletion of lymphocytes, leading to a significant compositional change in the grafts before infusion into the patient. However, notwithstanding graft variability, no predictive parameters for reconstitution of T cells at 3 or 6 weeks or GVHD development after transplantation could be defined. This can be explained by continuation of TCD *in vivo* after graft infusion, whereas the low reported incidence of acute GVHD within 12 weeks after transplantation implies that the described conditioning regimens containing *in vitro* ALT-based TCD of grafts resulted in efficient GVHD prophylaxis.

In our ALT 'to the bag' protocol, 20 mg of ALT was added *in vitro* to every graft irrespective of graft characteristics. This resulted in a significant reduction in lymphocytes after 30 minutes of *in vitro* incubation. Among the lymphocyte subsets, TCR α/β T cells were depleted the most efficiently compared with TCR γ/δ T cells, B cells and NK cells. Among the TCR α/β T-cell subsets, $CD4^{pos}$ T cells in the grafts were depleted more efficiently than $CD8^{pos}$ T cells, and naïve and regulatory T cells were depleted more efficiently than effector T cells. The median absolute number of effector T cells seemed to increase slightly after *in vitro* incubation with ALT, which is most likely within the measurement's margin of error. The differences in observed depletion efficiencies of lymphocyte and T-cell subsets were in line with their reported CD52-expression levels and resulted in major compositional changes in the grafts.[21] The T-cell compartment in the grafts consisted of mainly $CD4^{pos}$ T cells with a naïve or memory phenotype before ALT addition, whereas the remaining T cells in the grafts at the moment of infusion were in the majority of patients dominated by $CD8^{pos}$ T cells with an effector or memory phenotype. Although the absolute numbers of T cells in the grafts were significantly reduced by the direct effect of ALT *in vitro*, the grafts were not completely depleted of T cells at the moment of graft infusion. Furthermore, the absolute numbers of infused TCR α/β T cells or memory T cells were not predictive for immune reconstitution 3 or 6 weeks after transplantation.

We have previously demonstrated that in the majority of patients no circulating T cells are found immediately after or in the days following infusion of stem cell grafts pre-incubated with ALT, suggesting that the process of ALT-based TCD most likely continues *in vivo* after graft infusion.[42] Accordingly, reliable measurements of the absolute numbers of lymphocytes and T cells that are actually depleted by our ALT-based TCD cannot be assessed, but the reported depletion efficiencies do give insight in the proportions of depletion among cell subsets. The efficiency of TCD of the ALT-based TCD strategy is therefore difficult to compare with other *ex vivo* TCD strategies, such as physical isolation of $CD34^{pos}$ cells or selective depletion of TCR α/β T cells using antibody-coated magnetic beads and magnetic separation, since the complete effect of these TCD strategies can be evaluated before graft infusion.[6,8]

The composition of the grafts varied widely in quantity and quality of T cells already before ALT addition. This might be due to differences in lymphocyte composition among donors as well as technique and duration of leukapheresis.[43,44] Graft characteristics, such as absolute leukocyte counts and leukocyte concentrations of the grafts before ALT addition, and graft volumes did not predict depletion efficiency of TCR α/β T cells. Furthermore, graft source (related versus unrelated) or the time interval between the donor leukapheresis procedure and actual graft infusion into the patient did not influence *in vitro* depletion efficiency or graft compositional change of lymphocytes or T-cell subsets. These observations illustrate that our *in vitro* TCD strategy works equally well for grafts obtained from national and international donor centers with variable volumes and leukocyte counts.

Analysis of grafts before and after the addition of ALT *in vitro* showed that naïve T cells and regulatory T cells were depleted in similar proportions. Previous studies using selective naïve T-cell depletion have shown that an efficient depletion of naïve T cells is associated with a decreased risk of acute GVHD post-transplantation.[11] By contrast, efficient depletion of regulatory T cells from the graft might reverse this effect, whereas high frequencies of regulatory T cells in stem cell grafts are associated with a decreased chance of developing GVHD.[45-47] However, in our cohort, only eight of 60 patients developed acute GVHD requiring (additional) systemic immunosuppressive therapy within 12 weeks after transplantation. This low incidence of mainly limited acute GVHD suggests that, in the majority of patients, alloreactive T cells were efficiently depleted from donor stem cell grafts and/or that remaining alloreactive T cells were adequately suppressed after transplantation. The observed balanced depletion of both naïve and regulatory T cells from grafts might therefore contribute to GVHD prevention. It has been suggested that the ratio of regulatory T cells to total $CD4^{pos}$ T cells at 2 weeks after alloSCT is an indicator for the development of GVHD in patients after HLA-mismatched, non-TCD alloSCT.[48] With regard to naïve or regulatory T cells, we were not able to find a difference in *in vitro* depletion efficiency or absolute cell counts after *in vitro* ALT incubation between patients who did or did not develop GVHD post-transplantation. This might be explained by the low incidence of GVHD in our cohort. However, the ratio between the numbers of naïve and regulatory T cells was significantly higher in patients without GVHD compared to patients who developed GHVD soon after TCD alloSCT. This unexpected result is difficult to interpret because of the low number of patients who experienced GVHD. In addition, the absolute numbers of naïve and regulatory T cells were very low after *in vitro* ALT incubation, and small differences in depletion efficiency between naïve and regulatory T cell subsets may result in large differences in the ratio. Based on the results, it was not possible to predict beforehand which patients were at risk of developing GVHD based on graft composition after *in vitro* ALT incubation.

We have shown that 6 weeks after TCD alloSCT, T-cell reconstitution was mediated by both $CD52^{pos}$ and $CD52^{neg}$ T cells. In almost all patients, only ALT was used as T-cell depleting agent, and reconstitution of $CD52^{neg}$ T cells was observed. As these $CD52^{neg}$ T cells can give adequate protection

against viral reactivations, delayed reconstitution of CD52^{pos} T cells in this group of patients seems not to be problematic.[35] Importantly, patients who receive NMA conditioning and an unrelated donor graft have a relatively high risk of GVHD development, and therefore received both ALT and ATG as T-cell depleting agents. As CD52^{neg} T cells are sensitive to ATG, CD52^{neg} T-cell reconstitution was not predominant over CD52^{pos} T-cell reconstitution in these patients. Theoretically, an excess of unbound ALT infused with the graft, in combination with ongoing lytic levels of ATG in PB could result in a further delay in T-cell reconstitution. In the patients who received both ALT and ATG, T-cell reconstitution was indeed delayed compared with T-cell reconstitution in patients receiving only ALT. Based on these observations, we conclude that the addition of 20 mg ALT to stem cell grafts *in vitro* as TCD strategy for patients receiving MA or NMA conditioning is sufficient to prevent GVHD without extreme delays in protective T-cell repopulation. Furthermore, since long-term immunosuppression is not indicated after *in vitro* ALT-based TCD, this alloSCT protocol is a suitable platform for application of post-transplant cellular therapies such as donor lymphocyte infusion or adoptive transfer of *in vitro* selected T-cell populations to specifically boost the graft-versus-leukemia effect or pathogen-specific immunity.[49-54]

In conclusion, we have shown that the *in vitro* addition of 20 mg ALT to allogeneic stem cell grafts is an easy, fast and generally applicable method for the efficient depletion of donor-derived T cells from allogeneic stem cell grafts. The heterogenous expression of CD52 results in the differential depletion of lymphocyte and T-cell subsets, leading to a major compositional change in the graft before infusion into the patient. The continuation of TCD *in vivo* results in a limited incidence of GVHD, which can be explained by the balanced depletion of naïve and regulatory T cells by ALT.

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SUPPLEMENTARY MATERIAL

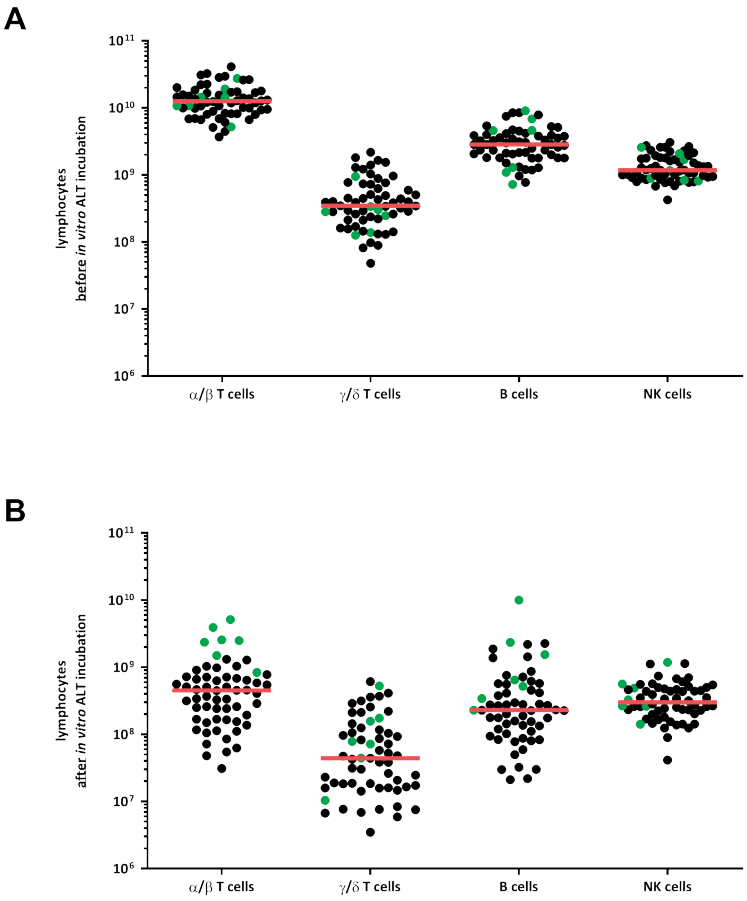
Supplementary Table 1. ALT-based TCD conditioning regimens for G-CSF mobilized peripheral blood allogeneic stem cell transplantations. T-cell depleting agents are underlined. iv, intravenously; MA, myeloablative; NMA, non-myeloablative; TBI, total body irradiation.

Donor	Conditioning intensity	Agents	Dose	Administration days related to transplantation (day 0)
Related	MA *n* = 6	Cyclophosphamide	60 mg/kg iv	-6,-5
		Mesna	60 mg/kg iv	-6,-5,-4
		TBI	9 Gy	-1
		Alemtuzumab added to the graft	20 mg 'in the bag'	0
		or		
		Busulfan	3.2 mg/kg iv	-9 until-6
		Cyclophosphamide	60 mg/kg iv	-4,-3
		Mesna	60 mg/kg iv	-4,-3,-2
		Alemtuzumab added to the graft	20 mg 'in the bag'	0
	NMA *n* = 14	Fludarabine	50 mg/m2 oral	-10 until-5
		Busulfan	3.2 mg/kg iv	-7,-6
		Alemtuzumab	15 mg iv	-4,-3
		Alemtuzumab added to the graft	20 mg 'in the bag'	0
		or		
		Fludarabine	30 mg/m2 iv	-14 until-10
		Cytarabine	2000 mg/m2 iv	-13 until-10
		Amsacrine	100 mg/m2 iv	-13 until-10
		Busulfan	3.2 mg/kg iv	-6,-5
		Alemtuzumab	15 mg iv	-4,-3
		Alemtuzumab added to the graft	20 mg 'in the bag'	0
Unrelated	MA *n* = 14	TBI	9 Gy	-8 or-7
		Cyclophosphamide	60 mg/kg iv	-6,-5
		Mesna	60 mg/kg iv	-6,-5,-4
		Alemtuzumab	15 mg iv	-6,-5
		Cyclosporine A	3 mg/kg iv	-1 until +30, then tapered off
		Alemtuzumab added to the graft	20 mg 'in the bag'	0
		or		
		Busulfan	3.2 mg/kg iv	-9 until-6
		Cyclophosphamide	60 mg/kg iv	-4,-3
		Mesna	60 mg/kg iv	-4,-3,-2
		Alemtuzumab	15 mg iv	-6,-5
		Cyclosporine A	3 mg/kg iv	-1 until +30, then tapered off
		Alemtuzumab added to the graft	20 mg 'in the bag'	0
	NMA *n* = 26	Fludarabine	50 mg/ m2 oral	-10 until-5
		Busulfan	3.2 mg/kg iv	-7,-6
		Alemtuzumab	15 mg iv	-4,-3
		Anti-thymocyte globulin	1 mg/kg iv	-2
		Alemtuzumab added to the graft	20 mg 'in the bag'	0
		Prednisolone	1 mg/kg iv or oral	1 until 10, then tapered off
		or		
		Fludarabine	30 mg/m2 iv	-14 until-10
		Cytarabine	2000 mg/m2 iv	-13 until-10
		Amsacrine	100 mg/m2 iv	-13 until-10
		Busulfan	3.2 mg/kg iv	-6,-5
		Alemtuzumab	15 mg iv	-4,-3
		Anti-thymocyte globulin	1 mg/kg iv	-2
		Alemtuzumab added to the graft	20 mg 'in the bag'	0
		Prednisolone	1 mg/kg iv or oral	1 until 10, then tapered off

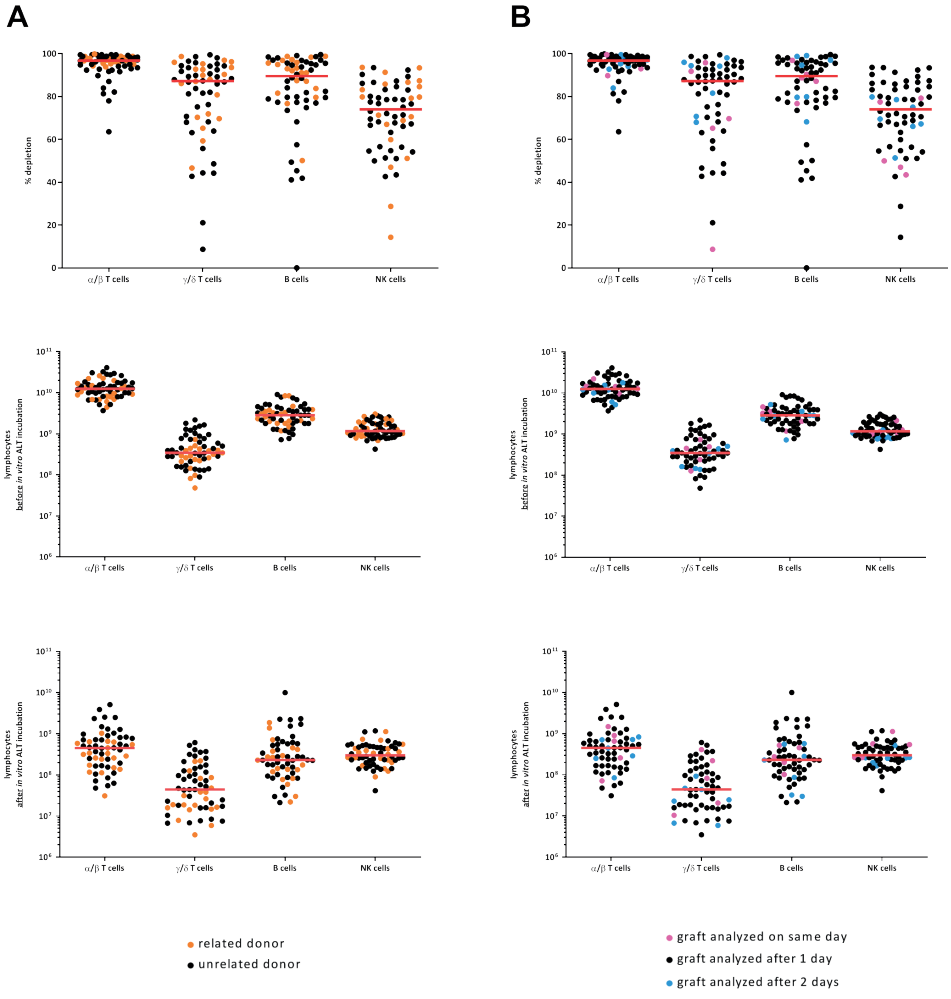
Supplementary Table 2. Percentages of lymphocyte subsets in the grafts before and after the *in vitro* incubation with ALT. The significance of differences in percentages of lymphocytes in grafts before and after *in vitro* ALT incubation was calculated.

		Before ALT addition		After ALT addition		Significance of differences
		Median	*Range*	*Median*	*Range*	*P value**
TCR α/β T cells (%)		71.9	55.8 – 85.5	33.7	4.8 – 81.0	<0.001
$CD8^{pos}$ (%)	Naïve T cells	10.5	0.8 – 33.3	12.8	1.1 – 43.1	0.003
	Memory T cells	3.3	0.1 – 15.9	10.0	0.6 – 50.4	<0.001
	Effector T cells	2.0	0.1 – 10.5	60.8	3.8 – 93.2	<0.001
$CD4^{pos}$ (%)	Naïve T cells	40.9	13.1 – 77.5	7.6	0.2 – 60.5	<0.001
	Memory T cells	37.9	9.0 – 68.4	3.7	0.5 – 24.7	<0.001
	Effector T cells	0.5	0.0 – 5.1	0.8	0.0 – 8.9	0.019
	Regulatory T cells	1.5	0.0 – 5.6	0.4	0.0 – 7.6	<0.001
TCR γ/δ T cells (%)		2.1	0.6 – 12.3	3.2	0.5 – 29.2	<0.001
B cells (%)		17.3	4.3 – 33.7	22.3	4.8 – 73.8	<0.001
NK cells (%)		7.6	2.8 – 19.5	29.3	4.9 – 73.8	<0.001

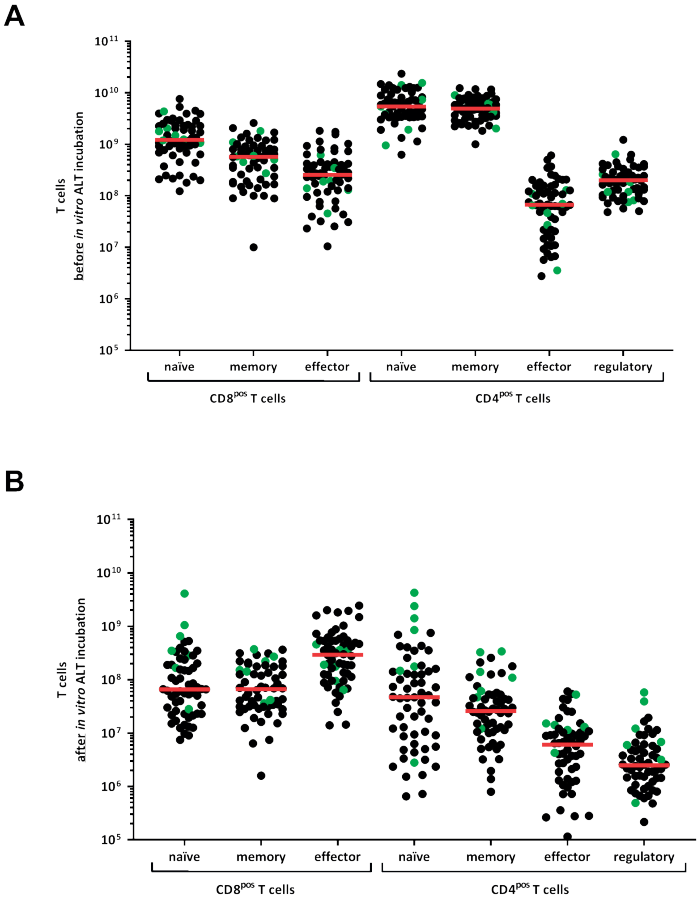
** Wilcoxon matched-pairs, signed-rank test*



Supplementary Figure 1. Absolute numbers of lymphocytes in grafts (n = 60). The 7 grafts with TCR α/β T-cell depletion efficiencies below 90% are indicated by green symbols. The red solid lines indicate medians. **A**, Before *in vitro* ALT incubation. **B**, After *in vitro* ALT incubation.



Supplementary Figure 2. Depletion efficiencies (%) and absolute numbers of lymphocytes in grafts before and after *in vitro* ALT incubation (n = 60). **A**, grafts of related donors are indicated by orange symbols, grafts of unrelated donors are indicated by black symbols. **B**, grafts analyzed 0, 1 or 2 days after the donor leukapheresis procedure are indicated by pink, black and blue symbols, respectively.



Supplementary Figure 3. Absolute numbers of T cells in grafts (n = 60). The 7 grafts with TCR α/β T-cell depletion efficiencies below 90% are indicated by green symbols. The red solid lines indicate medians. **A**, Before *in vitro* ALT incubation. **B**, After *in vitro* ALT incubation.