The association of CD25 expression on donor CD8+ and CD4+ T cells with graft-versus-host disease after donor lymphocyte infusions

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Background and Objectives. Graft-versus-host disease (GvHD) remains a major complication of allogeneic stem cell transplantation (SCT) and donor lymphocyte infusions (DLI). CD25-expressing donor T cells may be involved in the prevention or induction of GvHD as these cells comprise both $CD4^+CD25^+$ regulatory T (Treg) cells and preactivated $CD4^+$ or $CD8^+$ conventional T cells. Therefore, we evaluated the relationship between CD25-expressing $CD4^+$ and $CD8^+$ donor T cells and the clinical outcome of DLI.

Design and Methods. We retrospectively studied the DLI products of 47 HLA-identical DLI recipients by FACS analyses. As Treg cells are often identified within the $CD4^+CD25^{hi}CD45RB^{low}$ subset, we determined the frequencies of $CD4^+$ and $CD8^+$ T cells with different expression levels of CD25 and CD45RB.

Results. The frequencies or infused doses of donor $CD4^+CD25^+$ T cells, regardless of their CD25 and CD45RB expression levels, showed no correlation with the clinical outcome in univariate and multivariate analyses. In contrast, elevated frequencies of donor $CD8^+CD25^+$ T cells showed significant correlations with grade II-IV acute GvHD. Patients with GvHD also appeared to have received higher doses of $CD8^+CD25^+$ cells. Increased frequencies of $CD8^+CD25^+$ cells in the DLI products and the infused-doses of these cells also correlated with complete remissions accompanying GvHD. However, there was no correlation between increased levels of $CD8^+CD25^+$ cells with complete remissions achieved in the absence of a clinically apparent acute GvHD.

Interpretations and Conclusions. Donor $CD8^+CD25^+$ T cells appear to represent a risk factor for GvHD in the DLI setting. Selective depletion of these cells from DLI products may attenuate GvHD without significantly compromising anti-tumor efficacy.

Key words: donor lymphocyte infusions, graft-versus-host disease, donor $CD8^+CD25^+$ T cells; donor $CD4^+CD25^+$ T cells.

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Allogeneic stem cell transplantation is the preferred approach for the treatment of several hematologic malignancies as this therapy is associated with a curative graft-versus-tumor effect mediated by donor T cells.[1,2] This immunotherapeutic effect is also clearly illustrated by the successful application of donor lymphocyte infusions (DLI) in the treatment of leukemia relapses following stem cell transplantation.[3] Unfortunately, both stem cell transplantation and DLI are associated with graft-versus-host disease (GvHD), a major cause of treatment-related morbidity and mortality. To date, effective prevention or treatment of GvHD is not possible without abrogating the graft-versus-tumor effect. In search of novel strategies for GvHD prevention, attention has recently been focused on CD25-expressing T cells, particularly naturally occurring regulatory T (Treg) cells, a subset of $CD4^+$ T cells that constitutively express the CD25 molecule.[4] Studies in murine bone marrow transplantation models demonstrated that GvHD could be improved or even prevented by co-administration of donor $CD4^+CD25^+$ Treg cells with marrow grafts.[5-8] Moreover, in a number of studies GvHD was prevented without abrogating the graft-versus-tumor effect.[9-11] In humans, the role of CD25-expressing donor T cells in the outcome of stem cell transplantation and DLI may be more complex, since human $CD25^+$ T cells contain not only regulatory T cells but also pre-activated $CD4^+$ and $CD8^+$ conventional T cells. In fact, a recent study of peripheral blood stem cell transplants showed that increased levels of both $CD4^+CD25^+$ and $CD8^+CD25^+$ T cells in the grafts are significant risk factors for acute GvHD.[12]

Here, we aimed to evaluate the role of CD25-expressing donor $CD4^+$ and $CD8^+$ T

cells on the clinical outcome of DLI. To this end, we measured the frequencies of CD25-expressing T-cell subsets in the DLI products from 47 HLA-matched DLI recipients. Human Treg cells are usually identified within the $CD4^+CD25^{hi}$ population and, similar to their murine counterparts,[13,14] were shown to express low levels of CD45RB.[15-19] Therefore, in our analyses we used CD45RB as an additional marker and determined the frequencies of $CD4^+$ and CD8 T cells with different expression levels of CD25 and CD45RB. Subsequently we sought correlations between these donor cell frequencies and the clinical outcome in univariate and multivariate statistical analyses.

Design and Methods

Patients and donors

The study cohort included 47 randomly selected patients and their HLA-matched stem cell donors who donated buffy-coats for DLI between 1989 and 2002. The demographic data of the patients and donors are shown in Table 1. Forty-five patients received partially T-cell-depleted grafts (1×10^5 T cells/kg) after myeloablative conditioning, consisting of either cyclophosphamide (120 mg/kg) plus total body irradiation (6×2Gy) (n=35) or antithymocyte globulin plus cyclophosphamide plus total body irradiation (n=9) or busulfan (120 mg/m^2) plus cyclosporine (n=1). Two patients received unmodified stem cells after non-myeloablative conditioning with antithymocyte globulin plus fludarabine (90 mg/m^2) plus low dose total body irradiation (2Gy). Clinically significant acute GvHD (grade II) was observed in only three of the transplant recipients. All patients received a single infusion of unmanipulated donor lymphocytes after discontinuation of all immunosuppressive medication. At the time of DLI, three patients were in remission and were therefore included only in the analyses related to the development of GvHD. Acute GvHD was graded as grade 0 to grade IV until 100 days after DLI using the standard Glucksberg-Seattle criteria.[20] Chronic GvHD was defined as none, limited, or extensive in patients who survived more than 100 days.[21] The mean follow-up time after DLI was 1097 days, (range 126-3513, median: 676 days). Clinical remission was diagnosed by physical examination, marrow examination, or radiology at any time after DLI. All analyses were performed after obtaining informed consent from donors and patients.

Immunophenotyping

Three-color FACS analyses were performed on cryopreserved peripheral blood mononuclear cells isolated from the remainders of the buffy-coats infused into the recipients. Thawed cells were first incubated with Fc-receptor blocking reagent (Miltenyi Biotech; Bergisch Gladbach, Germany) in 20 µL phosphate-buffered saline plus 2% fetal calf serum for 10 minutes at 4°C. The cells were then labeled with fluoroscein isiothiocyanate (CD4/CD8), PE(CD25) and pCy5 (CD45RB) conjugated antibodies (Becton Dickinson, St. Josè, CA, USA) for 30 min at 4°C and analyzed using a FACS Calibur flow cytometer (Becton Dickinson). The FACS-data were analyzed using the WinMDI software (J. Trotter, The Scripps Research Institute, La Jolla, CA, USA). A representative FACS staining and the gating strategies are depicted in Figure 1. The expression of CD25 on $CD4^+$ cells was heterogeneous with no clear demarca-

Table 1. Recipient-donor characteristics and the clinical outcome of DLI.

	Number (range)
Recipient median age, years	41 (19-58)
Donor median age, years	43 (22-56)
Recipient diagnosis	
acute lymphocytic leukemia	4
acute myeloid leukemia	8
chronic myeloid leukemia	16
lymphoma	3
myeloma	8
other	8[a]
Transplant conditioning regimen:	
Myeloablative[b]	
TBI+CS	35
TBI+ATG+CS	9
CS+BS	1
Non myeloablative[c]	
TBI+Flu+ATG	2
Stem cell source	
T-cell reduced peripheral blood	45
Unmodified peripheral blood	2
Recipient-donor relation	
HLA-identical sibling	39
HLA-matched unrelated	8
Recipient sex: male/female	24/23
Donor sex: male/female	28/19
Sex-mismatch (female to male)	6
DLI median lymphocyte dose/kg:	1×10^7 (5×10^5-2×10^8)
Acute GvHD: grade 0-I/grade II-IV	32 15
Chronic GvHD: no/limited/extensive	32/3/9
Remission: No/Partial/Complete	21/1/22

[a]other diagnoses were myelodysplastic syndrome (n=4), myelofibrosis (n=1), plasmacytoma (n=1), EBV-lymphoma after SCT for severe aplastic anemia (n=2); [b]Myeloablative conditioning TBI: total body irradiation (6×2Gy); CS: cyclophosphamide (120 mg/kg) ATG=anti-thymocyte globulin; BS: busulfan (120 mg/m^2); [c]TBI: 2gy; Flu: fludarabine (90 mg/m^2).

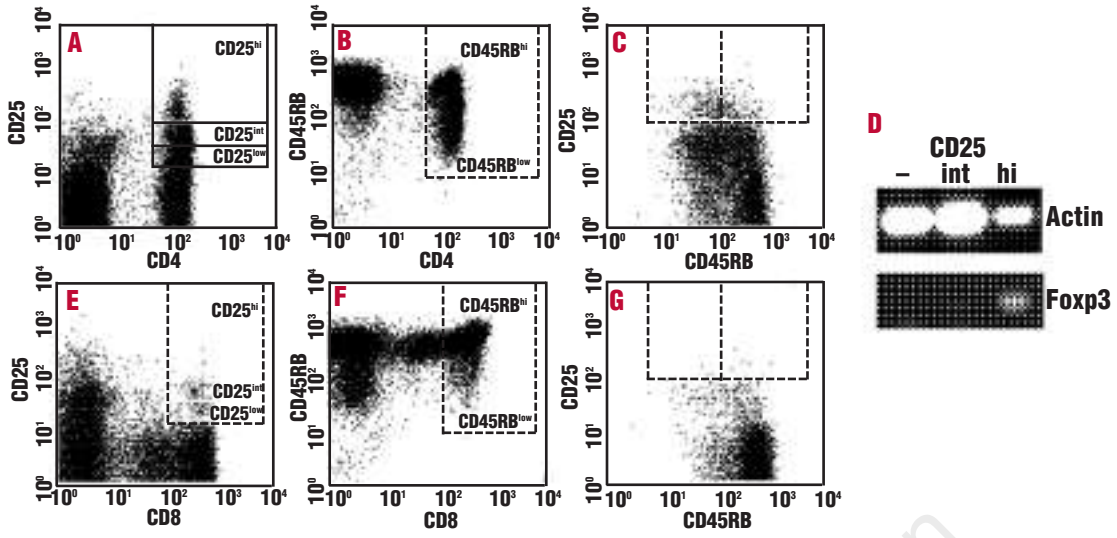

Figure 1. Immunophenotyping strategy for peripheral blood mononuclear cells obtained from DLI-products. Thawed peripheral blood mononuclear cells isolated from DLI-material were stained with the indicated antibodies as described in the Design and Methods. Appropriate gates were set on viable lymphocytes according to their typical forward and side scatter profiles. As the CD25 expression (left panels) showed no clear demarcations on CD4 (A) and CD8 subsets (E) the indicated arbitrary regions were set to determine cells with low, intermediate (int) and high (hi) CD25 expression. Foxp3 RNA transcripts were determined in $CD4^+CD25^-$, $CD4^+CD25^{int}$ CD^+CD254^{hi} subsets as described in the Design and Methods (D). The frequencies of $CD45RB^{hi}$ and $CD45RB^{low}$ cells on $CD25^{hi}$ cells were determined after setting appropriate gates on $CD4^+$ (C) and $CD8^+$ (G) cells.

tions. Therefore three arbitrary regions were set to determine the cells with low, intermediate (int) and high (hi) CD25 expression. Accordingly, $CD25^{hi}$ cells were mainly located within the $CD4^+$ cell subset (Figure 1A). These $CD4^+CD25^{hi}$ cells strongly expressed RNA transcripts for foxp3, a Treg-cell associated transcription factor (Figure 1D). Foxp3 expression was weak in $CD4^+CD25^{int}$ cells and was not detectable in $CD4^+CD2^-$ cells (Figure 1D). CD25 expression on $CD8^+$ cells was generally low or intermediate. Only a small fraction of $CD8^+$ cells expressed $CD25^{hi}$ (mean 0.4%; range 0-2.1%) (Figure 1E). Therefore, the $CD8^+$ cells were first analyzed by adding the frequencies of $CD25^{low}$ $CD25^{int}$ and $CD25^{hi}$ cells. Subsequently separate analyses were performed on $CD8^+$ $CD25^{hi}$ cells.

Within the $CD4^+$ population, $CD45RB^{hi}$ and $CD45R^{low}$ cells appeared to be evenly distributed (Figure 1B). In contrast, $CD8^+$ cells contained significantly more $CD45RB^{hi}$ cells (Figure 1F). Interestingly, while $CD4^+$ $CD25^{hi}$ cells comprised both $CD45RB^{hi}$ and $CD45RB^{low}$ cells (Figure 1C) there were almost no $CD8^+CD25^{hi}$ cells with low CD45RB expression (Figure 1G).

Detection of Foxp3 gene transcripts

$CD4^+CD25^-$, $CD4^+CD25^{int}$ and $CD4^+CD25^{hi}$ cells were sorted using a FACS Vantage cell sorter. cDNA from each population were amplified by polymerase chain reaction using a β-actin specific primer set (5'-GTG CTA TCC CTG TAC GCC TCT-3'; 5'-AGG ACT CCA TGC CCA GGA AG G-3') and a Foxp3-specific primer set previously described by Miura *et al.*[22] The expression of Foxp3 gene was confirmed using two other primer sets.

Statistical analyses

Univariate analyses were conducted using GraphPadPrism (version 4.0; *www.graphpad.com*). Multivariate binominal regression analyses were performed using the SPSS software (version 11.5). In these analyses, the frequencies of different T-cell subsets were stratified according to the severity of acute GvHD (grade 0-I versus grade II-IV), chronic GvHD (none or limited versus extensive) and for clinical responses (no or partial remission versus complete remission). Differences between groups were tested with two tailed *p* values using non-paired t tests and were considered statistically significant if the *p* value was ≤0.05.

Results

Correlation between $CD4^+CD25^+$ T cells and the clinical outcome of DLI

To assess the relationship between clinical outcome and the analyzed cell subsets, we first stratified the frequencies of different $CD4^+$ T-cell subsets according to the clinical outcome (acute GvHD, chronic GvHD, clinical response). As illustrated in Figure 2A, recipients who developed acute GvHD grade 0-I and those who developed acute GvHD grade II-IV had been infused with donor lymphocytes containing similar frequencies of $CD4^+CD25^{low}$, $CD4^+CD25^{int}$ or $CD4^+CD25^{hi}$ T

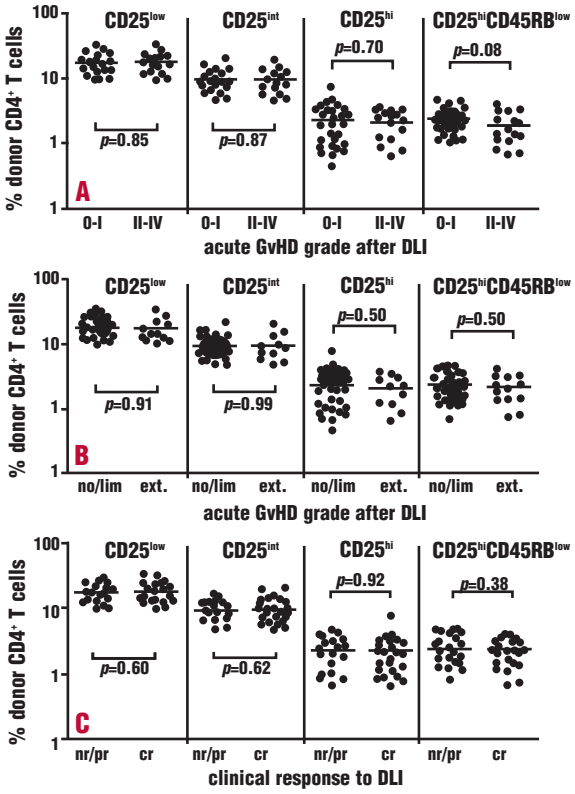

Figure 2. Correlation between the clinical outcome of DLI and the frequencies of donor CD4+ T cells expressing different levels of CD25 and CD45RB. The frequencies of indicated cell subsets in the DLI-products were stratified according to A) acute GvHD (grade 0-I vs. grade II-IV); B) chronic GvHD (none or limited (no/lim) vs. extensive (ext); and C) clinical response (nr or partial response (no/pr) vs. complete remission (cr). Differences between groups were tested with two-tailed *p* values using a non-paired t test.

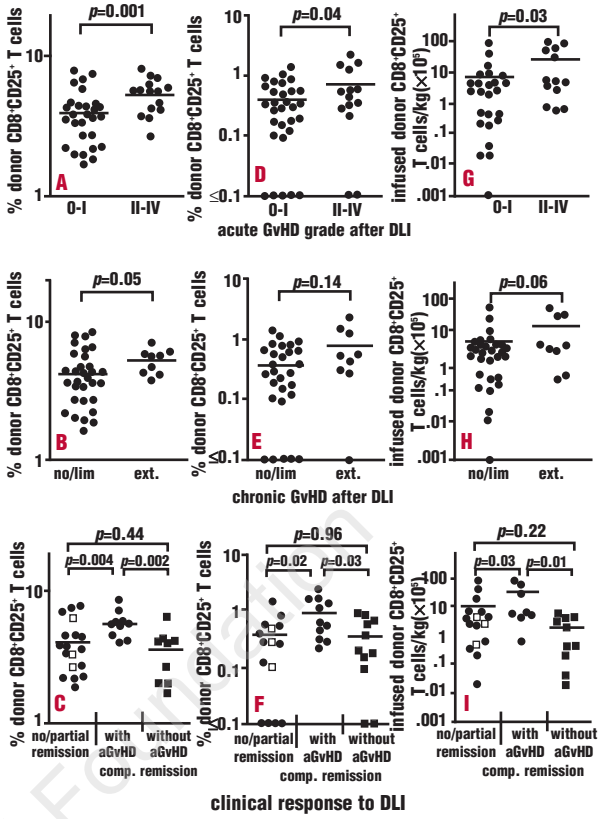

Figure 3. Correlation between the donor CD8+CD25+ T cells and the clinical outcome of DLI. The frequencies of the indicated cell subsets in the DLI products (A-F) or the infused-doses of CD8+CD25+cells (G-I) were stratified according to acute GvHD (grade 0-I vs. grade II-IV) (A,D,G); chronic GvHD (none or limited (no/lim) vs. extensive (ext) (B, E, H); and clinical response (no or partial response vs. complete (comp.) remission (cr) (C, F, I). In Figures C, F, and I, the recipients who showed no or partial clinical responses despite development of acute GvHD grade II are indicated with open squares. There were no statistical differences between this latter group of recipients and those who showed no or partial responses without GvHD. Differences between groups were tested with two tailed *p* values using a non-paired t test.

cells. When the CD45RB marker was taken into account, it appeared that recipients who developed acute GvHD grade 0-I had been infused with somewhat, but statistically not significant, higher frequencies of $CD4^+CD25^{hi}$ $CD45RB^{low}$ T cells as compared to the recipients who developed acute GvHD grade II-IV (Figure 2A). Similar statistical analyses revealed no significant associations between the frequencies of donor $CD4^+CD25^+$ cells and the development of chronic GvHD or achievement of complete remissions (Figure 2B,2C). There was also no correlation between the infused doses of $CD4^+CD25^+$ cells and the clinical outcome (*data not shown*). Multivariate analyses, whereby adjustments were made for donor and recipient age, diagnosis, DLI-dose, sex mismatches and patient donor relation also revealed no associations between donor $CD4^+CD25^+$ T cells and the clinical outcome of DLI (*data not shown*).

Correlation between donor $CD8^+CD25^+$ cells and the clinical outcome after DLI

Statistical analyses performed on $CD8^+$ T cells revealed significant associations between the frequencies of donor $CD8^+$ $CD25^+$ T cells and the clinical outcome: recipients who developed acute GvHD grades II-IV appeared to have been treated with donor lymphocytes containing significantly higher frequencies of $CD8^+CD25^+$ T cells as compared to the recipients who developed acute GvHD grades 0-I (Figure 3A). A similar correlation was found between the frequencies of $CD8^+CD25^{hi}$ cells and acute GvHD (Figure 3D). Furthermore, it appeared that recipients who developed acute GvHD grade II-IV were infused with higher doses of $CD8^+CD25^+$ T cells (Figure 3G). Finally, in a multivariate binominal regression analysis, the frequencies of $CD8^+CD25^+$ cells appeared a significant risk factor for grade II-IV GvHD with an

Table 2. Relation between donor CD8+CD25+ cell frequencies and acute GvHD grade II-IV in a multivariate binominal regression analysis.

	odds ratio	*95% CI*	*p*[a]
Donor relation[b]	0.889	0.123-6.449	0.907
Sex mismatch[c]	0.099	0.006-1.372	0.083
DLI dose	0.999	0.983-1.016	0.935
Recipient age	0.968	0.884-1.061	0.493
Donor age	0.985	0.888-1.091	0.767
Diagnosis		0.46	
Acute leukemia	1	NA	NA
Chronic myeloid leukemia	2.251	0.291-17.427	0.437
Others	0.507	0.055-4.659	0.548
% CD8+CD25+ T cells[d]	1.805	1.046-3.115	0.034[e]

[a]values are derived from Wald's test; [b]siblings vs matched unrelated donors [c]recipient male, donor female; [d]variable entered in step 1; [e]p value was 0.021 in a likelihood test.

odds ratio of 1.8 after adjusting for patient and donor age, diagnosis, sex-mismatches, DLI-dose and donor relation (Table 2).

Relatively higher frequencies of CD8+CD25+ T cells in the DLI-products was also associated with the development of extensive chronic GvHD in univariate analyses (Figure 3B). A similar trend was observed when the CD8+CD25hi cells were analyzed (Figure 3E) However, these associations disappeared after adjustment for other factors in multivariate analyses (*data not shown*).

Elevated frequencies of donor CD8+CD25+, and CD8+CD25hi T cells in the DLI products were also associated with the achievement of complete remissions that accompanied acute GvHD grade II-IV (Figure 3C, F, I). Interestingly, however, complete remissions, which were achieved in the absence of severe acute GvHD (11 out of 22 complete remissions) showed no correlation with increased frequencies of donor CD8+CD25+ T cells (Figure 3C). These results suggest that selective graft-versus-leukemic responses after DLI can be mediated by donor lymphocytes with no increased levels of pre-activated CD8vCD25+ T cells.

Discussion

Donor T cells are key players in GvHD and the graft-versus-tumor effect after stem cell transplantation and DLI. The development of novel strategies for effective prediction and prevention of GvHD is therefore largely dependent on the identification of relevant donor T-cell subsets that mediate or regulate GvHD and the graft-versus-tumor effect. Here we show that donor CD8+ T cells co-expressing the interleukin-2 receptor α-chain, CD25, may represent such a subset with significant influences on the clinical outcome of DLI. In 47 DLI-products infused into HLA-identical recipients, elevated frequencies of CD8+CD25+ T cells and the infused-doses of these cells showed significant associations with the development of acute GvHD grade II-IV and with complete remissions achieved in the presence of acute GvHD. Our study did not, however, reveal any obvious associations between the CD25-expressing CD4+ T cells and the clinical outcome of DLI.

Our results concerning CD4+CD25+ T cells are apparently contradictory to those of a recent study by Stanzani *et al*. who observed significant correlations between increased levels of CD4+CD25+ and CD8+CD25+ T cells in the peripheral blood stem cell grafts and acute GvHD.[12] While this discrepancy may be related to the differences between DLI and peripheral blood stem cell transplants, we agree with Stanzani *et al*. that the possible presence of pre-activated conventional T cells within the CD4+ CD25+ cell fractions makes it very difficult to enumerate human Treg cells by surface markers, even when using additional markers such as CD45RB. Although the preferential expression of the foxp3 gene in the CD4+ CD25hi subset suggested that most, if not all, Treg cells resided within the CD4+ CD25hi subset, the fact that the CD4+ CD25hi fraction contained both CD45RBhi and CD45RBlow cells suggested that this subset comprises not only Treg cells but also non-regulatory T cells. Thus, the use of CD45RB may represent an improvement in the identification of Treg cells. Nonetheless, we acknowledge that we currently do not know whether all CD4+CD25hiCD4RBlow cells are Treg cells. It was not possible to address this issue in the current study as such an analysis requires functional studies of high numbers of individual T-cell clones derived from CD4+CD25hiCD4RBlow fractions. Thus, the lack of a correlation between CD4+CD25hiCD45RBlow cells and the clinical outcome may not necessarily reflect a null-association between Treg cells and clinical outcome. Nonetheless, we also do not exclude the possibility that the relatively low frequencies and infused numbers of donor Treg cells may not be sufficient to influence the development of GvHD and the graft-versus-tumor effect after DLI. In this respect, it is important to note that in murine studies an effector-cell to Treg-cell ratio of 1:1 is usually necessary to prevent or improve GvHD.[8] Alternatively, the regulation of GvHD by Treg cells could be dependent on their *in vivo* development, expansion and interaction with other cells. Indeed, this latter notion is supported by recent reports showing an inverse correlation between GvHD and the *de novo* development of foxp3 expressing T cells,[22] and the activation of interleukin-10 and transforming growth factor-β producing regulatory T cells by CD4CD25+ Tregs.[23] Unraveling the role of Treg cells in stem cell trans-

plants and DLI appears to require further analyses using blood and tissue samples of recipients after DLI. Unlike $CD4^+CD25^+$ cells, $CD8^+CD25^+$ cells showed a clear association with GvHD, which was also shown by Stanzani *et al.*[12] As expected, the frequencies and infused doses of $CD8^+CD25^+$ cells not only correlated with acute GvHD but also with the graft-versus-tumor effect accompanying GvHD. Interestingly however, 50% of the recipients (11/22) achieved complete remissions without severe GvHD and these clinical responses were not associated with increased frequencies or infused doses of $CD8^+CD25^+$ T cells. It may be possible that, due to the low numbers of pre-activated $CD8^+CD25^+$ T cells in the donor blood, the development of anti-host responses in these recipients did not result in a devastating cytokine storm, which appears to be an essential feature of acute GvHD.[24] These results also suggest that lowering the frequencies of $CD8^+CD25^+$ T cells in donor blood may not be detrimental for the development of graft-versus-leukemia responses, but can ameliorate GvHD. In fact, previous studies have shown that GvHD can be attenuated by *in vivo* depletion of host reactive T cells with anti-CD25 antibodies infused after transplantation.[25,26] It has also been shown that *ex vivo* depletion of $CD25^+$ donor T cells after activation by host alloantigens can diminish GvHD-associated alloreactivity, while retaining tumor reactivity of donor T cells.[27,28] Furthermore the number of $CD8^+$ T cells infused was shown to be associated with acute GvHD.

In particular, a recent study by Mohty *et al.*[29] demonstrated that $CD8^+$ T-cell dose affects development of acute GvHD after reduced-intensity conditioning peripheral blood stem cell transplantation. Also, in a number of studies depletion of $CD8^+$ T cells from DLI products appeared to be successful in attenuating GvHD.[30,31] In the light of these studies and of our results, we speculate that the frequencies and infused numbers of $CD8^+CD25^+$ cells in DLI may represent a predictive indicator of acute GvHD. Based on our results presented in Figure 3C, we could speculate that depletion of $CD8^+CD25^+$ cells from DLI products containing 5% or more $CD8^+CD25^+$ cells prior to DLI may improve GvHD without significantly compromising the graft-versus-tumor effect. It will be interesting to explore the feasibility and safety of such a strategy in appropriate *in vivo* models.

TM and LFV were responsible for the design, analysis an interpretation of data. TAR performed all in vitro experiments. She was also involved in analysis of the FACS data. LFV collected, validated and interpreted the clinical data; TM drafted the paper and created the tables and figures and he is primarily responsible for the paper. All authors approved the final version to be published and also declare they have no potential conflict of interest.

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