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

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^hiCD45RB^lo 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. 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. 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. 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. Moreover, in a number of studies GvHD was prevented without abrogating the graft-versus-tumor effect. 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.

Here, we aimed to evaluate the role of CD25-expressing donor CD4^+ and CD8^+ T cells
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\(^7\) 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 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 fluorescein isothiocyanate (CD4/CD8), PE(CD25) and pCy5 (CD45RB) conjugated antibodies (Becton Dickinson, St. Josè, CA, USA) for 50 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-
T cells with GvHD

The frequencies of CD45RB+ cells (Figure 1F). Interestingly, CD4+ and CD25+ cells comprised both CD45RB+ cells was generally low or intermediate. Only a small fraction of CD8+ cells expressed CD25+ (mean 0.4%; range 0-2.1%) (Figure 1E). Therefore, the CD8+ cells were first analyzed by adding the frequencies of CD25low CD25int and CD25hi cells. Subsequently separate analyses were performed on CD8+ CD25hi cells.

Within the CD4+ population, CD45RBhi and CD45Rblo cells appeared to be evenly distributed (Figure 1B). In contrast, CD8+ cells contained significantly more CD45RBhi cells (Figure 1F). Interestingly, while CD4+ CD25hi cells comprised both CD45RBhi and CD45Rblo cells (Figure 1C) there were almost no CD8+CD25hi cells with low CD45RB expression (Figure 1G).

Detection of Foxp3 gene transcripts

CD4CD25−, CD4CD25hi and CD4CD25lo 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 binomial 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 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 CD4CD25low, CD4CD25hi or CD4CD25lo T cells. However, the frequency of CD4CD25+ cells was significantly higher in recipients who developed acute GvHD grade II-IV than in those who developed acute GvHD grade 0-I.
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 CD45RB+ 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 (nr/pr) vs. complete remission (cr). Differences between groups were tested with two-tailed p values using a non-paired t test.

**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 (nr/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); 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.

**Correlation between donor CD8+CD25+ T 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 higher doses of CD8+CD25+ T cells (Figure 3G). A similar correlation was found between the frequencies of CD8+CD25+ 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...
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.

Discussing the role of Treg cells in stem cell transplantation, 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+CD25lo fraction contained both CD45RB+ and CD45RB– 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+CD25hiCD45RB– 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+CD25hiCD45RB– fractions. Thus, the lack of a correlation between CD4+CD25hiCD45RB– 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." 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 CD4+CD25hiTregs.23 Unraveling the role of Treg cells in stem cell transplantation...
plants and DLI appears to require further analyses using blood and tissue samples of recipients after DLI. Unlike CD4<sup>+</sup>CD25<sup>+</sup> cells, CD8<sup>+</sup>CD25<sup>+</sup> cells showed a clear association with GVHD, which was also shown by Stanzani <em>et al</em>.<sup>13</sup> As expected, the frequencies and infused doses of CD8<sup>+</sup>CD25<sup>+</sup> 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<sup>+</sup>CD25<sup>+</sup> T cells. It may be possible that, due to the low numbers of pre-activated CD8<sup>+</sup>CD25<sup>+</sup> 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.<sup>14</sup> These results also suggest that lowering the frequencies of CD8<sup>+</sup>CD25<sup>+</sup> 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 <em>in vivo</em> depletion of host reactive T cells with anti-CD25 antibodies infused after transplantation.<sup>15,16</sup> It has also been shown that <em>ex vivo</em> depletion of CD25<sup>+</sup> donor T cells after activation by host alloantigens can diminish GVHD-associated alloreactivity, while retaining tumor reactivity of donor T cells.<sup>17,18</sup> Furthermore, the number of CD8<sup>+</sup> T cells infused was shown to be associated with acute GVHD.

In particular, a recent study by Mohty <em>et al</em> demonstrated that CD8<sup>+</sup> 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<sup>+</sup> T cells from DLI products appeared to be successful in attenuating GVHD.<sup>19,20</sup> In the light of these studies and of our results, we speculate that the frequencies and infused numbers of CD8<sup>+</sup>CD25<sup>+</sup> 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<sup>+</sup>CD25<sup>+</sup> cells from DLI products containing 5% or more CD8<sup>+</sup>CD25<sup>+</sup> 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 <em>in vivo</em> 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 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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