Reconstitution of FOXP3\(^+\) regulatory T cells (T\(_{\text{regs}}\)) after CD25-depleted allotransplantation in elderly patients and association with acute graft-versus-host disease

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Selectivedepletion (SD) of host-reactive donor T cells from allogeneic stem-cell transplants (SCTs) using an anti-CD25 immunotoxin (IT) is a strategy to prevent acute graft-versus-host disease (aGvHD). There is concern that concurrent removal of regulatory T cells (T\(_{\text{regs}}\)) with incomplete removal of alloactivated CD25\(^+\) T cells could increase the risk of aGvHD. We therefore measured T\(_{\text{regs}}\) in the blood of 16 patients receiving a T-cell–depleted allograft together with anti–CD25-IT–treated SD lymphocytes, in 13 of their HLA-identical donors, and in 10 SD products. T\(_{\text{regs}}\) were characterized by intracellular staining for forkhead box protein 3 (FOXP3) and by quantitative reverse-transcription–polymerase chain reaction (qRT-PCR) for FOXP3 gene in CD4\(^+\) cells. Patients received a median of 1.0 \(\times 10^8\)/kg SD T cells and a stem cell product containing a median of 0.25 \(\times 10^{10}\)/kg residual T cells. T\(_{\text{regs}}\) reconstituted promptly after SCT and underwent further expansion. Of the CD4\(^+\) T cells in SD products, 1.5% to 4.8% were CD25\(^-\) T\(_{\text{regs}}\). Acute GvHD (grade II) was restricted to 5 patients whose donors had significantly (\(P = .019\)) fewer T\(_{\text{regs}}\) compared with those without clinically significant aGvHD. These results suggest that rapid T\(_{\text{reg}}\) reconstitution can occur following SD allografts, either from CD25\(^-\) T\(_{\text{regs}}\) escaping depletion, or from residual CD25\(^-\) and CD25\(^+\) T\(_{\text{regs}}\) contained in the stem-cell product that expand after transplantation and may confer additional protection against GvHD. (Blood. 2007;110:1689-1697)

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Introduction

Severe graft-versus-host disease (GvHD) in the early posttransplantation period limits the success of allogeneic stem-cell transplantation (SCT) and requires powerful immunosuppression that deprives the allotransplant of its full graft-versus-leukemia (GvL) potential.\(^1\) Selective depletion (SD) of host-reactive donor T cells from lymphocyte products is a promising strategy to prevent severe, acute GvHD (aGvHD) while maintaining useful donor-derived immune functions such as GvL effects and antiviral immunity.\(^2\) A variety of experimental approaches for the ex vivo removal of alloreacting lymphocytes has been proposed targeting surface expression of activation-associated molecules such as CD25,\(^3\) CD69,\(^9\) CD71,\(^12\) HLA-DR,\(^12\) and CD137,\(^14\) alterations in the expression of activation-associated molecules such as CD25,\(^3\) CD69,\(^9,10,12,13\) CD71,\(^12\) HLA-DR,\(^12\) and CD137,\(^14\) alterations in the multidrug resistance pump p-glycoprotein,\(^15,16\) or proliferation.\(^17,18\) Targeted T cells can be removed or eliminated by using immunomagnets,\(^3,9,12,14\) immunotoxins,\(^3,7,11,19\) flow sorting,\(^17,18\) induction of apoptosis,\(^19,20\) or photodepletion techniques.\(^15,16\) Most clinical experience concerns SD techniques using an anti-CD25 immunotoxin (CD25-IT) to target the \(\alpha\)-chain of the interleukin-2 (IL-2) receptor (CD25) to eliminate ex vivo–activated donor lymphocytes.\(^21,22\) We recently reported a 46% grade II to IV and 12% grade III to IV aGvHD incidence in 16 elderly patients with advanced hematologic malignancies who were treated with selectively CD25-depleted allografts from HLA-matched siblings.\(^23\) Because CD25 is not exclusively expressed on effector T cells (T\(_{\text{effs}}\)), but also on a subset of CD4\(^+\) regulatory T cells (T\(_{\text{regs}}\)) that suppress alloresponses and protect against GvHD,\(^24-32\) there is a concern that the concurrent removal of T\(_{\text{regs}}\) could have increased the risk of GvHD in our series.

While the phenotypic distinction between T\(_{\text{regs}}\) and T\(_{\text{effs}}\) has been challenging in the past, especially in humans when based on CD25 alone, nowadays availability of monoclonal antibodies directed against the forhead box protein 3 (FOXP3) make it possible to identify a subset of CD25\(^+\) FOXP3\(^+\) T\(_{\text{regs}}\) from CD25\(^+\) FOXP3\(^-\) T\(_{\text{effs}}\).\(^33-36\) FOXP3 encodes a forkhead/winged helix transcription factor and was identified as a key regulator required for the development and functional activity of T\(_{\text{regs}}\).\(^33-35\) To characterize T\(_{\text{reg}}\) recovery after SD transplantation, we therefore retrospectively measured T\(_{\text{regs}}\) in transplant products and in 16 of our patients receiving SD transplants in the first 3 months after SCT.\(^23\) We found that T\(_{\text{reg}}\) recovery was prompt and may in part be derived from a CD25\(^-\) T\(_{\text{reg}}\) content persisting in the SD product.

Patients, materials, and methods

Study design

Patients and their HLA-identical sibling donors were treated on the National Institutes of Health protocol 01-H-0162, approved by the National...
Peripheral mononuclear cells (PBMCs) were separated using Ficoll-Hypaque density gradient centrifugation (Organon Teknika, Durham, NC) and subsequently frozen in RPMI 1640 complete medium (CM: Life Technologies, Gaithersburg, MD) supplemented with 20% heat-inactivated fetal calf serum (FCS) and 10% dimethyl sulfoxide (DMSO) according to standard protocols. Before use, frozen cells were thawed, washed, and suspended in RPMI 1640 (Mediatech, Herndon, VA) supplemented with Hepes buffer, gentamicin, and 10% pooled AB serum (Sigma Chemical, St Louis, MO). CD4+ cell populations were purified with immunomagnetic beads (Dynal Biotech, Oslo, Norway), which were detached from isolated cells by using DetachaBead (Dynal Biotech).

RNA isolation was performed using RNeasy mini kits (Qiagen, Valencia, CA). Total RNA was eluted with water and stored at −80°C. For reverse transcription of mRNA and cDNA synthesis, 1 μg total RNA was reverse transcribed and stored at −20°C until PCR analysis.

**qRT-PCR**

Gene expression was measured using an ABI Prism 7900 Sequence Detection System (Applied Biosystems, Foster City, CA) as described previously.27 Threshold cycle (CT) during the exponential phase of amplification was determined by real-time monitoring of fluorescent emission after cleavage of sequence-specific probes by nuclease activity of Taq polymerase. The internal control gene for mRNA expression was β-actin. Primer and probe sequences for β-actin were as follows: 5'-GGCACCACGGACCAATGGAAG (forward), 5'-GGCCGATCCAGCGAG-GTTT (reverse), FAM-TATATTCTCTGATGCG-TAMRA (probe). To detect FOXP3, Assays-on-Demand Gene Expression probes for FOXP3 (HS 00239589; Applied Biosystems) were used according to the manufacturer’s guidelines. To create a standard curve, β-actin and FOXP3 cDNA were amplified by PCR using the same primers designed for qRT-PCR, purified, and quantified by UV spectrophotometry. The number of cDNA copies was calculated by using the molecular weight of each gene amplicon. Serial dilutions of the amplified genes at known concentrations were tested by qRT-PCR. qRT-PCR reactions of cDNA specimens, cDNA standards, and water as negative control (NTC) were conducted in a total volume of 20 μL with TaqMan MasterMix (Applied Biosystems), 400 nM primers, and 200 nM probe. Thermal cycler parameters included 10 minutes at 95°C and 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Standard curve extrapolation of copy number was performed for both FOXP3 and β-actin. Sample data were normalized by dividing the number of copies of FOXP3 transcripts by the number of copies of β-actin transcripts. All PCR assays were performed in duplicates and reported as mean.

**Determination of donor-recipient chimerism**

As previously described,23 a quantitative polymerase chain reaction (PCR)-based analysis of short tandem repeats (STRs) was used to measure donor-recipient chimerism separately in lymphoid and myeloid lineages.

**Statistical analysis**

Graphs and statistical analyses were performed with the use of Prism 4.00 for Windows software (GraphPad Software, San Diego, CA). P values less than or equal to .05 were considered significant.

**Results**

**Study population and samples**

Peripheral blood samples of 16 patients (median age, 65 years) were analyzed for \( T_{reg} \) before transplantation and/or during the first 90 days after transplantation. \( T_{reg} \) were also analyzed in 13 of their

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**Transplantation approach**

The transplantation approach and the selective depletion procedure have been described previously.5,23 Figure 1 provides a schematic overview. Briefly, older patients with advanced hematologic malignancies and a suitable HLA-matched sibling stem cell donor were eligible for the study. All patients received a preparative regimen consisting of fludarabine (125-180 mg/m^2^) and one of the following alkylating agents: cyclophosphamide (120 mg/kg), melphalan (140 mg/m^2^), or infusional busulfan (6-8 mg/kg). Donors received granulocyte colony-stimulating factor (G-CSF) 10 μg/kg per day subcutaneously. Mobilized peripheral blood stem cells (PBSCs) were collected by leukapheresis on day 5, and again on days 6 and 7 if necessary, to obtain a target dose of at least 3 × 10^6 CD34+ cells/kg. The Isolex 300i immunomagnetic system (Nexell Therapeutics, Irvine, CA) was used to perform a combined positive CD34 selection of T cells according to the manufacturer’s instructions, which results in a PBSC product with approximately 5 logs of T-cell depletion.

The unadsorbed, T-cell–rich fraction remaining after CD34 selection was the source of donor lymphocytes for the SD procedure. Recipient stimulator cells were generated from immunomagnetically selected and expanded T lymphocytes. Stimulator cells were thawed, gamma irradiated (2500 cGy), and added to responder cells at a ratio of 1 to 1 to a final concentration of 5 × 10^6 cells/mL. Responder and irradiated stimulator cells were cocultured for 72 hours at 37°C in 7% CO2. Alloreactive responder cells expressing the activation marker, CD25, were targeted by the addition of the anti-CD25 immunotoxin, RFT5-SMPT-dgA, which consists of the anti-CD25 murine MAb, RFT5 (IgG1), coupled to the deglycosylated ricin A chain (dgA) via the heterobifunctional cross-linker SMPT (Pierce, Rockford, IL). RFT5-SMPT-dgA was added to the culture at 24 (2 μg/mL) and 48 (1 μg/mL) hours, along with the enhancing agent, ammonium chloride (0 mM; Sigma, St Louis, MO). At the completion of the 72-hour coculture, cells were harvested and cryopreserved. On the day of transplantation (day 0), both products (stem cells and SD T cells) were thawed and infused consecutively. Patients received cyclosporine alone for GvHD prophylaxis starting on day 100. As previously described,23 a quantitative polymerase chain reaction (PCR)-based analysis of short tandem repeats (STRs) was used to measure donor-recipient chimerism separately in lymphoid and myeloid lineages.

**Flow cytometry**

Cells were phenotypically analyzed by 5-color flow cytometry using CD3-PE-Cy7 (clone SK7), CD4-PerCP (clone SK3), CD25-PE (clone MA251), and CD27-FITC (clone L128) antibodies for surface staining (all from BD Biosciences, San Diego, CA). Intracellular staining of FOXP3 (eBioscience, San Diego, CA) was performed after surface staining, fixation, and permeabilization according to the manufacturer’s recommendation using an APC-conjugated antibody (clone PCH 101) or an APC-conjugated isotype control (rat IgG2A). Flow cytometry analysis was performed on the LSR II flow cytometer (BD Biosciences) using BD FacsDivA software (BD Biosciences). Flow-Jo software (Trestar, Ashland, OR) was used solely for graphic presentation in this publication. A minimum of 250 000 total cells was acquired. Lymphocytes were gated by forward-sideward scatter, followed by gates for CD3+ and subsequently for CD4+ cells. CD25+ and FOXP3+ cells were analyzed and described as their respective subpopulations within CD4+ cells.
Table 1. Patient characteristics and treatment delivery

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Blood samples (PBMCs, apheresis products, processed lymphocyte products) were studied in 16 patients (before and after transplantation [day 30 to day 90]), 13 of their donors, and 10 selectively CD25-depleted lymphocyte products (SD) based on their availability by PCR (P) and/or flow cytometry (F). As far as DLIs were given or patients received CD25 antibodies for GvHD treatment only samples taken before these events were studied.

UPN indicates unique patient number; aGVHD, acute graft-versus-host disease; cGVHD, chronic graft-versus-host disease; MDS, myelodysplastic syndrome; AML, acute myeloid leukemia; Flu, fludarabine; Bu, busulfan; NA, not assessable; Cy, cyclophosphamide; Mel, melphalan; ALL, acute lymphoblastic leukemia; SLL, small lymphocytic lymphoma; CLL, chronic lymphocytic leukemia; CMMoL, chronic myeloblastic leukemia; MCL, mantle-cell lymphoma; X, sample available for analysis.
Patient characteristics are displayed in Table 1. Six patients. Median follow-up time was 243 (range, 67-1624) days. Five patients had grade II aGvHD and one developed grade IV aGvHD of the gut. Six patients developed limited and one...

A high-risk group of elderly patients underwent matched-sibling transplantation and received a CD25-allodepleted lymphocyte product (SD) together with a purified stem-cell product following reduced-intensity conditioning with fludarabine (125-180 mg/m²) and cyclophosphamide (140 mg/m²) or infusional busulfan (6.4 mg/kg). Both products were infused at day 0. Patients received cyclosporine and CD25 immunotoxin. Flu indicates fludarabine; mel, melphalan; bu, busulfan; and Cy, cyclophosphamide. Donor-derived lymphocytes with an anti-CD25 immunotoxin. Flu indicates fludarabine; mel, melphalan; bu, busulfan; and Cy, cyclophosphamide.

Donor T-cell chimerism was measured by flow cytometry for CD3+CD8+ cells and a subset of CD3+CD4+ T cells and a stem-cell product containing a median of 1.0 x 10^6/kg CD34+ cells: d30 (n = 13), d60, and d90 (n = 8), and 90 (n = 5) days after transplantation exceeded pretransplantation levels and approached those of the donors. Median donor T-cell chimerism was 97% at day 30, 94% at day 60, and 91% at day 90 (Figure 2F). The gray-shaded area represents the interquartile (25%-75%) range of cell counts in donors. In panels A through E, ALC indicates absolute lymphocyte count (as determined by the department of laboratory medicine by flow analysis and lymphocyte gating, which ranges for CD3+ and CD3+CD8+ T cells and a subset of CD3+CD4+ T cells and a stem-cell product containing a median of 1.0 x 10^6/kg CD34+ cells: d30 (n = 13), d60, and d90 (n = 8), and 90 (n = 5) days after transplantation exceeded pretransplantation levels and approached those of the donors. Median donor T-cell chimerism was 97% at day 30, 94% at day 60, and 91% at day 90 (Figure 2F).

Treg recovery

Treg were measured either by flow cytometry or by qRT-PCR. Figure 3 displays the results of flow cytometry for CD25 and FOXP3 and the qRT-PCR analysis for FOXP3 mRNA in CD4+ T-cell populations. In the representative patient (UPN 301), all subsets of samples were available for the donor, the CD25-depleted SD product, and the patient before and 30, 60, and 90 days after transplantation. Donor T-cell chimerism was already 97% at day 30
after transplantation and slightly decreased to 94% at day 60 and to 91% at day 90. This patient did not develop aGvHD. The donor cells contained 2.3% CD25+ FOXP3+ cells (Figure 3A). As expected, the SD product was devoid of CD25+ FOXP3+ cells (Figure 3A). This patient had 4.0% CD25+ FOXP3+ cells before transplantation and recovered as early as day 30 1.5% CD25+ FOXP3+ cells increasing to 4.6% and 5.3% at days 60 and 90 (Figure 3A). With regard to all CD4+ cells, this posttransplantation trend could be observed by both flow cytometry (Figure 3B) and by qRT-PCR (Figure 3C). The SD product contained CD4+ FOXP3+ cells despite complete CD25 depletion (Figure 3A-C). Figure 3A shows that 2.9% of the CD4+ cells in the SD product were CD25– and FOXP3+, and Figure 3C shows that FOXP3 mRNA-containing CD4+ cells persisted after CD25 depletion. CD25– FOXP3+ CD4+ cells could be also found in the donor (3.8%) and in the patient before (2.9%) and 30 (1.9%), 60 (2.6%), and 90 (2.4%) days after transplantation (Figure 3A). In this patient, the fraction of CD25– FOXP3+ Teffs declined from 4.9% at day 30 to 1.7% at day 90. After transplantation, the majority of CD4+ T cells expressed CD27. In CD4+ cells, expression of CD27 in CD25– and CD25+ Tregs was relatively increased compared with FOXP3– T cells (data not shown).

Treg recovery was confirmed in the entire study population (displayed in Figure 4). Median FOXP3 mRNA levels increased after transplantation and exceeded the levels of both the donors and the patients before transplantation (Figure 4A). The increase in FOXP3 mRNA levels was associated with a relative increase of FOXP3– and CD25– cells within CD4+ cells as depicted in Figure 4B-C. In contrast, CD25– FOXP3– Teffs levels declined after transplantation with the lowest median values observed 90 days after transplantation (Figure 4D), whereas median levels of CD25– FOXP3+ Tregs increased after transplantation reaching their highest values 90 days after transplantation (Figure 4E). All SD products contained FOXP3 mRNA and FOXP3 protein but were completely negative for CD25 (Figure 4A-C). The FOXP3 content in the SD product was attributed to a CD25– FOXP3+ fraction of Tregs that persisted after CD25 depletion. These CD25– Tregs were also present in patients before and after transplantation and at lower levels in donors (Figure 4F).

Absolute numbers of Tregs were calculated for peripheral blood samples of patients before and after transplantation as well as of their respective donors for whom a complete set of ALCs and flow cytometric profiles were available (Figure 5). Both the medians of absolute FOXP3 copy numbers per cell per volume and the absolute number of CD4+ FOXP3+ cells per volume increased during the first 90 days after transplantation coinciding with the results described for relative Treg recovery (Figure 5A-B). Thirty days after transplantation, patients had a median of 10 CD4+ FOXP3+ cells/μL, increasing to 14 at day 60 and to 19 at day 90. Patients before transplantation had a median of 18 and donors of 16 CD4+ FOXP3+ cells/μL (Figure 5A-B). Median absolute numbers of all CD4+ FOXP3+ cells (Figure 5C) and CD25– CD4+ FOXP3+ Tregs (Figure 5E) followed this trend but not median absolute numbers for CD25+ CD4+ FOXP3+ Teffs (Figure 5D) and CD25– CD4+ FOXP3+ Tregs (Figure 5F).

Association between Treg recovery and transplantation outcome

To explore the association of Treg with the onset of aGvHD, we compared the levels of FOXP3+ CD4+ T cells between patients with no or grade I aGvHD and patients with aGvHD (≥ grade II). We investigated the effects of Treg in the patient before and 30 days after transplantation, the donors, and the SD products at the onset of aGvHD (≥ grade II) (Figure 6). Levels of FOXP3+ cells in the CD4+ content in patients before and early after transplantation and in the SD product were not associated with the onset of aGvHD (Figure 6A-C). However, aGvHD (≥ grade II) was restricted to 5 patients whose donors had significantly (P = .019) fewer Treg compared with those with no or grade I aGvHD (Figure 6D). This effect of the donor Treg was also seen in association with the absolute number of CD25– CD4+ FOXP3+ Tregs (P = .045) but not with CD25– CD4+ FOXP3+...


T<sub>reg</sub> (P = .22) (Figure 6E,F). There was no association of T<sub>reg</sub> with cGvHD or relapse (data not shown).

**Discussion**

The stimulation of donor T cells with recipient antigen-presenting cells (APCs) and the concurrent removal of host-reacting T cells during the selective depletion process promises transplantation outcomes with a reduced incidence of aGvHD, while providing a fully functional donor immune system retaining its antileukemic potential uninhibited by immunosuppression. We previously showed that removal of host-reactive donor T cells from allografts by anti-CD25 IT was clinically feasible and reduced the frequency of severe aGvHD in a high-risk group of elderly patients undergoing matched-sibling transplantation who received only low-dose...
cyclosporine for immunosuppression. However, it was a concern that acute and chronic GvHD in our patients could have been associated with the concurrent removal of Tregs, with the alloactivated CD25+ T cells. In the present study, we found efficient Treg reconstitution in all patients. Using FOXP3 as a marker to distinguish between effector and regulator populations in CD4+ cells, we found a relative decline of T effs in contrast to a relative increase of Tregs over different time points after transplantation diminishing the effector-to-regulator ratio in favor of a more suppressive T-cell environment.

To further characterize Tregs, we also investigated the expression of CD27, a TNFR-family member that was found to be consistently expressed on Tregs. CD27 is absent on effector T cells and is rapidly lost on CD27+ naive and memory T cells after activation. In our series, the majority of reconstituted CD4+ T cells were CD27+. Nevertheless, CD27 expression in both CD25+ and CD25− Tregs was relatively increased compared with FOXP3− CD4+ T cells.

Regarding potential sources for Treg reconstitution, we considered the possibility that the reduced-intensity conditioning regimen could have conserved some patient-derived T cells leading to mixed T-cell chimerism after transplantation. However, by chimerism analysis the T-cell repertoire of our patients was found to be predominantly of donor origin. Furthermore, 5 patients who had full donor T-cell chimerism also reconstituted considerable numbers of Tregs as early as 30 days after transplantation. Therefore, it is unlikely that the Treg reconstituting in our patients were derived from recipient T cells surviving the conditioning procedure. In view of the advanced age of our patient population and the fact that we studied early (fewer than 100 days after transplantation) Treg reconstitution, the potential thymic contribution in the de novo generation of Tregs from CD34+ stem cells can be disregarded. Thus, the donor T cells appear to be the most likely source of Treg reconstitution. Donor Tregs could have been derived from 2 sources, a predominant population of CD25-depleted T cells in the SD product (1 × 10^6/kg) and a 4-log smaller amount of residual T cells (0.25 × 10^6/kg) contained in the stem-cell product. Although we confirmed that SD products did not contain CD25+ Tregs, we identified a population of CD25− (FOXP3+) Tregs persisting after CD25 depletion. FOXP3, the recently identified key player in Tregs, is predominantly but not exclusively expressed in CD4+ CD25+ T cells.33-35 FOXP3 expression in CD25− CD4+ cells also exerts suppressive and regulatory function as recently shown in mice41 and humans.42 We also found these CD25− Tregs in our patients before and after transplantation and in the donors, suggesting that the residual unselected T cells in the stem-cell product provide a small number of CD25− and CD25+ Tregs. Thus, it seems likely that Treg recovery following SD is derived mainly from CD25− Tregs which escape the depletion process with a further contribution of CD25− and CD25+ Tregs delivered in the stem-cell product. Regarding the developmental pathways of Tregs, it has already been suggested that CD25− Tregs may up-regulate their CD25 receptor following antigen stimulation43 and thereby contribute to the pool of CD25+ Tregs. This conversion would explain why we observed increasing levels of CD25+ Tregs after transplantation, while levels of CD25− Tregs remained rather stable. Finally, all these CD25+ donor-derived Treg fractions could have undergone lymphopenia-driven expansion44 after transplantation, thereby contributing to GvHD control and compensating for thymic failure of de novo Treg production.

Reconstituting donor postthymic T cells are responsible for establishing early immune function after SCT. While early studies investigating the relationship between Treg recovery and occurrence of aGvHD in humans were limited by the difficulty in distinguishing Treg from T eff populations, FOXP3 analysis has provided a more consistent picture where high FOXP3 levels in blood or tissue were...
associated with less aGVHD. Here, we found an association between the donor Treg content and the occurrence of clinically significant aGVHD. In contrast, neither the reconstituted level of Treg in the patient nor the Treg content of the SD product was associated with GVHD suppression. These results concur with our findings in unselected, T-cell-depleted SCT and emphasize that donor-derived Treg contribute to GVHD suppression in the host at a very early stage.

In mice, CD25 depletion was associated with the development of significant autoimmune disease, and in humans a clinical trial attempting the in vivo CD25 depletion after allografting was terminated due to an increased rate of aGVHD, attributable to Treg removal. In this study, we showed efficient Treg reconstitution despite giving a CD25-depleted allograft. Thus, the occurrence of aGVHD in our series of SD stem-cell transplant recipients cannot simply be explained by the removal of Tregs, but rather to an insufficient removal of alloreactive T cells by the SD process. Indeed, we previously reported that in patients developing aGVHD involving the gastrointestinal tract and the liver, there was persistent donor-versus-recipient alloreactivity as measured by helper T lymphocyte precursor frequencies. Either the recipient T-cell APCs used to stimulate the donor did not present all the potential GVHD-related antigens or the subsequent depletion did not remove all the alloactivated donor cells. The latter scenario could be partly due to a down-regulation of CD25 antigen during the coculture period allowing some alloactivated cells to escape the depletion process. Thus, future clinical developments of SD may also need to focus on varying the type of recipient APCs (eg, Epstein-Barr virus–transformed lymphoblastoid cell lines or unmanipulated PBMCs) as well as explore novel targets of donor T-cell activation (eg, CD137 or different depletion techniques (eg, TH9402-based photodepletion). In this study, we showed for the first time that SD using CD25 as the depleting target may protect against aGVHD, both by allowing efficient Treg reconstitution as well as by allodepletion. It should be emphasized that our study population is small and these results will need validation in a larger patient cohort. The SD approach nevertheless seems worthwhile since the technique offers the possibility of removing GVHD reactivity while preserving other donor immune function. Unlike other techniques involving T-cell subset depletion, SD appears to least perturb the balance of immune reactivity between regulator and effector cells, and conservation of Treg recovery may provide further GVHD protection when alloredepletion is incomplete.

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Authorship

Contribution: S.M. conceived and designed the study, performed experiments, analyzed data, performed statistical analyses, and wrote the paper; K.R. contributed to the study, performed experiments, analyzed data, and commented on the paper; B.N.S. collected patient data and provided clinical care; R.N. performed experiments; A.S.M.Y. advised on experimental design and commented on the paper; J.S. was involved in development and chemical manufacture of the CD25-immunotoxin for selective depletion and commented on the paper; R.K. provided the chimerism data; V.G. was involved in development and chemical manufacture of the CD25-immunotoxin for selective depletion; E.J.R. supervised the clinical selective depletion process, provided data, and commented on the paper; S.R.S. was the principal investigator on the selective depletion trial, collected patient data, and provided clinical care; E.S.V. was involved in development and chemical manufacture of the CD25-immunotoxin for selective depletion and commented on the paper; A.J.B. supervised this study, supervised the selective depletion trial, provided clinical care, and wrote the paper. S.M. and K.R. contributed equally to this work.

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