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ORIGINAL ARTICLE

Correlation of the $CD4^+CD25^{high}$ T-regulatory cells in recipients and their corresponding donors to acute GVHD

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Summary

Graft-versus-host disease continues to be a major life-threatening complication after allogeneic hematopoietic stem cells transplantation (aHSCT). The relationship of acute GVHD (aGVHD) with the levels of peripheral $CD4^+CD25^{high}$ T cells in patients after aHSCT and in their corresponding donors is not fully investigated. We examined the levels of $CD4^+CD25^{high}$ T cells in patients after aHSCT and in their corresponding donors, and analyzed the relationship of $CD4^+CD25^{high}$ T cells to the incidence and prognosis of aGVHD. The recipients with normal or high $CD4^+CD25^{high}$ T cells (three of eight, 37.5%) had no or mild aGVHD (grade I), and all survived during the follow-up period. In striking contrast, the recipients with lower or no $CD4^+CD25^{high}$ T cells suffered from greater than grade II aGVHD (four of four, 100%), and all died within 1-year post-aHSCT. Moreover, the number of $CD4^+CD25^{high}$ T cells in recipients correlated significantly with that of their corresponding donors. The $CD4^+CD25^{high}$ T cells from the recipients and their corresponding donors expressed high levels of Foxp3, and effectively suppressed the proliferation of $CD4^+CD25^-$ responder T cells. This study suggests that human Treg cells may play an important role in aGVHD, as has been seen in murine models. The levels of peripheral $CD4^+CD25^{high}$ T cells in recipients and donors could be helpful for predicting of the onset and outcome of aGVHD.

Introduction

Acute and chronic graft-versus-host disease (GVHD) are major causes of morbidity and mortality after allogeneic hematopoietic stem cell transplantation (aHSCT). Despite close molecular matching, GVHD occurs in an unpredictable fashion in 30–50% of patients after matched, related transplantation and in a higher fraction of patients after transplantation performed using HLA-mismatched or matched-unrelated donors [1–3]. In the past 5 years, many studies have investigated the relationship of GVHD onset, severity, and response to therapy with the immune status of donor and recipient, including the cellular infiltrate of the graft and cytokines [4,5], but none of these factors is able to clearly predict occurrence and prognosis of GVHD in clinical practice.

Recently, the role of a particular subpopulation of $CD4^+$ T cells that constitutively express the α-chain of the receptor for interleukin 2 (IL-2; CD25) has been characterized in suppression of autoimmunity and induction of transplantation tolerance [6]. Several studies in murine GVHD models have shown that $CD4^+CD25^+$ T-regulatory cells (Tregs) play a critical role in this disease. These studies demonstrated that depletion of $CD25^+$ T cells accelerated the development of aGVHD. Additionally, the infusion of *ex vivo* activated and expanded $CD4^+CD25^+$

T cells ameliorated the development of aGVHD after aHSCT in a dose-dependent fashion [7–14]. Thus, it has been speculated that the same association between $CD4^+CD25^+$ T cells and GVHD would be seen in humans. Interestingly, the expression of CD25 in human T cells is not identical to that of the mouse. It has been reported that in humans $CD4^+CD25^{high}$ T-cell subset form the specific CD4 Treg population [15–18]. In this study, we examined the relationship between $CD4^+CD25^{high}$ T cell numbers in patients undergoing aHSCT and the subsequent occurrence of aGVHD.

Objective, materials and methods

Healthy adults

To establish the normal reference range for $CD4^+CD25^{high}$ T cells, 57 healthy volunteer adults (male 31, female 26) were examined. The median age was 29 (range: 16–50).

Recipients and donors

Twelve consecutive adult patients who received aHSCT between June 2004 and the end of 2005 and their HLA-matched stem cell donors were examined and analyzed to investigate the relationship between human CD4 Treg cells and the aGVHD. All transplantations were performed in the Department of Hematology (Anhui Provincial Hospital, China), for the treatment of a variety of hematological malignancies. Samples of patients were collected at days between 22 and 35 post-aHSCT when the white blood cell count (WBC) exceeded 2×10^9/l; samples from donors were collected premobilization. According to the normal reference range of $CD4^+CD25^{high}$ T cells at its 95% confidence interval, recipients were divided into two groups: Group I with normal or high $CD4^+CD25^{high}$ T-cell counts and Group II with fewer or even no $CD4^+CD25^{high}$ T cells (Tables 1 and 2). Acute GVHD was diagnosed and graded according to international standard criteria [19]. Recipients were followed up to 6- to 15-month post-aHSCT. Investigators were blinded with respect to clinical outcome while analyzing $CD4^+CD25^{high}$ T cells and other laboratory data. The study was performed according to research protocols approved by the Hospital Review Board.

Antibodies, regents, and flow cytometry

The following monoclonal antibodies (mAbs) specific for human surface antigens were purchased from Beckman Coulter-Immunotech (Marseille, France): phycoerythrin

Table 1. Patient characteristics.

	Group I ($CD4^+CD25^{high}$ T cells $\geqq$ 0.3%, $n = 8$)	Group II ($CD4^+CD25^+$ T cells <0.3%, $n = 4$)
Age (years)	16–45 (mean 28.5)	25–46 (mean 32.8)
Sex (M/F)	3/5	3/1
Diagnosis		
Chronic myelogenous leukemia	7	3
Acute leukemia	1	
Lymphoma		1
Donor†		
HLA-identical related donor	8	3
HLA-matched VUD		1
Source of HSC		
PB	2	2
BM	1	
PB + BM	5	2
Preparative* regimen	TBI/cyclophosphamide Busulphan/cyclophosphamide	
Analysis for implantation†	Assay of sex chromosome or genetic fingerprint	
GVHD prophylaxis	Cyclosporine/prednisone	
Follow up after HSCT (day)	180–420	
Incidence of aGVHD	37.5% (3/8)	100% (4/4)
Grade of aGVHD	3 cases with Grade I	1 case with Grade II and 3 with Grade IV
Mortality within 1 year	0%	100%

VUD, volunteer-unrelated donor; HSC, hematopoietic stem cells; BM, bone marrow; PB, peripheral blood; TBI, total body irradiation.

*GVHD prophylaxis was with cyclosporine/methotrexate.

†HLA typing was performed by molecular techniques for HLA class I A, B, and C and for class II HLA DRB1 and DQB1, in all donors and recipients.

Table 2. Frequency of $CD4^+CD25^{high}$ T cells in recipients and their corresponding donors.

	Recipients (%†)	Donors (%†)
Group I ($n = 8$)	2.23 ± 1.52	1.41 ± 0.76
Group II ($n = 4$)	0.13 ± 0.10	0.69 ± 0.48*

*$P < 0.01$ vs. Group I.
†Percentage of $CD4^+$ T cells.

(PE)-conjugated anti-CD4 antibodies (13B8.2 clone); fluorescein isothiocyanate (FITC)-conjugated anti-CD25 (B1.49.9 clone); three color reagent kit for T cell subtype containing of PE-cychrome5 (PE-Cy5) conjugated anti-CD3, FITC-conjugated anti-CD4, and PE-conjugated anti-CD8 (UCHT1, 13B8.2 and B911 clone); two color reagent kit for natural killer cells (NK) containing of FITC-conjugated anti-CD3, PE-conjugated anti-CD16 and CD56 (UCHT1, 3G8 + N901 and NKH-1 clone); and its isotype control antibody.

Human intracellular transcription factor forkhead box P3 (Foxp3) staining kit containing PE-conjugated anti-Foxp3 (PCH101 clone), FITC-conjugated anti-CD4 (RPA-T4 clone) and allophycocyanin (APC) conjugated anti-CD25 (BC96 clone), and its isotype control antibody, permeabilization buffer, Fix/Perm concentrate, Fix/Perm diluent, red blood cells lysis buffer (RBC Lysis Buffer) was purchased from eBioscience (San Diego, CA, USA).

Peripheral blood mononuclear cells (PBMC) were isolated from PB samples by density centrifugation over Ficoll-Hypaque gradient (Tianjin Hao Yang Biological Manufacture Co., Tianjin, China). Human T cells were cultured in complete medium consisting of RPMI 1640 supplemented with 10% heated inactivated fetal calf serum (FCS), penicillin (100 IU/ml)/streptomycin (100 mg/ml), and 2 mM L-glutamine, all purchased from Invitrogen (Carlsbad, CA, USA). Cell proliferation reagent (Cat No. 1644807) was purchased from Roche Diagnostics (Indianapolis, IN, USA).

Cells were analyzed and sorted using Coulter Epics XL flow cytometer (FCM) with SYSTEM II software and COULTER EPICS ALTRA HyPerSort™ System with EXPO 32 MULTICOMP Software (Beckman Coulter, Miami, FL, USA).

Examination of peripheral $CD4^+CD25^{high}$ cells

About 2–5 ml of PB was collected. All samples were anticoagulated with heparin and examined within 4 h. About 100 µl of anticoagulated blood was incubated at 25 °C for 15 min with the FITC-conjugated CD25-specific mAb and PE-conjugated CD4-specific mAb, and their appropriate isotype controls. After incubation, red blood cells were lysed and washed in PBS two times. Stained cells were quickly detected using the FCM and analyzed using SYSTEM II software. The frequency of $CD4^+CD25^{high}$ T cells was expressed as a percentage of $CD4^+$ T cells by sequential gating on lymphocytes and $CD4^+$ T cells (Fig. 1a–c).

Isolation of $CD4^+CD25^{high}$ and $CD4^+CD25^-$ cells

For functional assays, $CD4^+CD25^{high}$ and $CD4^+CD25^-$ cells were sorted using the gates (see Fig. 3a) on a Becton Counter flow-cytometric cell-sorter (ALTRA HyPerSort™ System), after cells were dyed with FITC-conjugated anti-CD4 and APC-conjugated anti-CD25. Consistent purity of >93% was obtained for both $CD4^+CD25^{high}$ T cell and $CD4^+CD25^-$ cell fractions.

Detection of intracellular Foxp3

According to the manufacturer's instructions, 100 µl of prepared cells (5×10^5) or whole blood were added to each tube. Cell surface staining was performed using APC-conjugated anti-CD4 and FITC-conjugated anti-CD25 for 20 min at room temperature in the dark followed by lysing RBC (for whole blood) or washing in PBS (for purified cells). Then cells were fixed and permeabilized with Fix/Perm reagent, incubated with

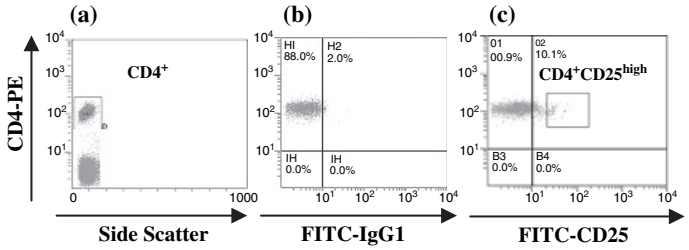

Figure 1 The analysis of $CD4^+CD25^{high}$ T cells from a healthy adult. Whole peripheral blood cells were stained with phycoerythrin (PE)-conjugated anti-CD4 and fluorescein isothiocyanate-conjugated anti-CD25. (a) The $CD4^+$ T cells were gated via CD4-PE fluorescence intensity and side-scatter properties. (b) The threshold for CD25 background fluorescence was scored based on its isotype control staining. (c) The $CD4^+CD25^+$ T cells population (upper right of the quadrant) exhibits a continuous and primarily low expression of CD25. In contrast, the $CD4^+CD25^{high}$ T-cells subset (in the box) is more homogeneous which mean fluorescence intensity about doubles that of $CD4^+CD25^+$ T cells.

PE-conjugated anti-Foxp3 and its isotype control antibody, washed with PBS and analyzed using FCM.

Functional assay of $CD4^+CD25^{high}$ T cells

Freshly purified $CD4^+CD25^{high}$ cells from the healthy adults, recipients in Group I (two with grade I and one without aGVHD) and their corresponding donors were assayed for suppressive activity in allogeneic mixed lymphocyte response (MLR). Irradiated (3000 rad) PBMCs from another healthy donor were used as allogeneic stimulator cells. $CD4^+CD25^-$ cells were used as responder cells. $CD4^+CD25^-$ cells (10^4 cells per well) were co-cultured with $CD4^+CD25^{high}$ cells (10^4 cells per well, 1:1 ratio) in the presence of irradiated PBMCs (2×10^4 cells per well) in a 96-well flat-bottom plate. Wells without $CD4^+CD25^{high}$ cells (responders and stimulators only) served as positive controls. Wells containing $CD4^+CD25^{high}$ cells and irradiated PBMCs (none responders) served as baseline controls. All incubations were run in triplicate with a final volume of 150 μl at 37 °C and 5% CO_2. After 48 h of incubation, 10 μl per well Cell Proliferation Reagent WST-1 was added. After incubating for more 4 h, the absorbance of the samples against a background control as blank was measured at 450 nm using 650 nm as a reference wavelength on Biocell HT1 ELISA microplate reader (Salzburg, Austria). Suppression was expressed as percentage of positive control.

WBC, T lymphocyte subtype, and NK cells in peripheral blood

White blood cell was determined visually using a hemacytometer (Beckman Coulter). The relative distributions of the T-cell subsets ($CD3^+$ T cells, $CD3^+CD4^+$ helper T cells, $CD3^+CD8^+$ cytotoxic T cells) and the NK cells in PB were stained with two- and three-color mAb, and then analyzed with multiparameter FCM.

Statistical analyses

Data were processed and analyzed using statistical software (SPSS 11.0, LEAD Technologies, Inc., Chicago, IL, USA). Statistical significance for the difference between different groups was assessed by two-sample *t*-test with equal variance. *P*-value of <0.05 was considered to be statistically significant.

Results

$CD4^+CD25^{high}$ T cells in healthy adults

Fifty-seven healthy adults were examined for CD25 expression on PB $CD4^+$ T cells. Using the gating strategy illustrated in Fig. 1a–c, the frequency of $CD4^+CD25^{high}$ T cells was 1.41 ± 0.52%. The 95% confidence interval of the normal reference range was from 0.30% to 2.43%.

Frequency of $CD4^+CD25^{high}$ T cells in recipients

Using the normal reference range for $CD4^+CD25^{high}$ T cells, we divided recipients into two groups: Group I with normal or higher numbers of $CD4^+CD25^{high}$ T cells and Group II with lower or undetectable levels of $CD4^+CD25^{high}$ T cells (Tables 1 and 2). The recipients in Group I had no or only grade I aGVHD (three of eight, 37.5%), and survived throughout the follow-up period. In striking contrast, all of the recipients in Group II developed severe aGVHD (above grade II) and died within 1-year post-aHSCT (four of four, 100%). The incidence of aGVHD in Group I recipients was significantly lower than in Group II (37.5% vs. 100%, $P < 0.05$, by Fisher's exact test). However, the levels of the other immunocytes including WBC, $CD3^+$, $CD3^+CD4^+$, $CD3^+CD8^+$ T cells, and NK cells were not significantly different between the two groups (Table 1–3).

$CD4^+CD25^{high}$ T cell numbers in donors

To determine whether the number of $CD4^+CD25^{high}$ T cells in recipients was related to that of their donors, we examined the relationship of $CD4^+CD25^{high}$ T cells between in donors and their corresponding recipients; there was a significant correlation ($R = 0.694$, $P < 0.05$, by Pearson's coefficient test). When donors were also divided into two groups according to $CD4^+CD25^{high}$ T cell numbers of their respective recipients, the numbers of $CD4^+CD25^{high}$ T cells in Group I donors was also significantly higher than that in Group II (Table 2 and Fig. 2).

Foxp3 expression and suppressive function of $CD4^+CD25^{high}$ T cells

In order to determine the function of $CD4^+CD25^{high}$ T cells from both recipients and donors, we further detected

Table 3. WBC, $CD3^+$, $CD3^+CD4^+$, $CD3^+CD8^+$ T cells, NK cells, and CD4 to CD8 ratio in recipients post-HSCT.

	WBC ($\times 10^9$/l)	$CD3^+$ (%*)	$CD3^+CD4^+$ (%*)	$CD3^+CD8^+$ (%*)	NK (%*)	CD4/CD8
Group I	5.16 ± 4.49	58.17 ± 22.77	20.42 ± 13.82	37.47 ± 20.83	30.73 ± 19.77	0.74 ± 0.25
Group II	4.34 ± 2.85	42.36 ± 25.42	13.06 ± 9.10	26.02 ± 15.82	19.40 ± 12.14	0.66 ± 0.22

*Percentage in lymphocytes of peripheral blood (PB).

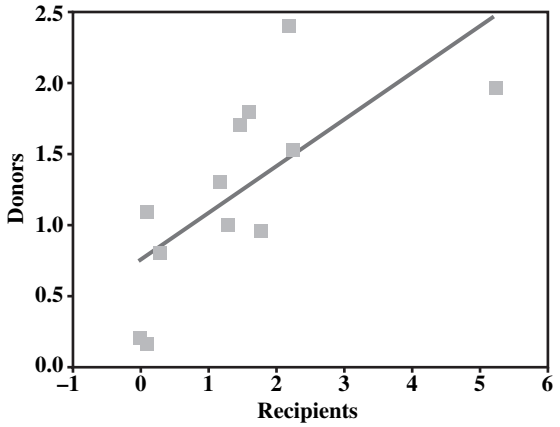

Figure 2 Correlation of $CD4^+CD25^{high}$ T cell numbers between recipients and donors. The $CD4^+CD25^{high}$ T cells as a percentage of $CD4^+$ lymphocytes in recipients and donors were plotted. Regression analysis is shown (solid line, $R = 0.694$).

the endogenous Foxp3 and suppressive function of $CD4^+CD25^{high}$ T cells from three individual recipients and their donors in Group I. $CD4^+CD25^{high}$ T cells separated from the whole blood of recipients and donors represented 88.3 ± 2.2% and 86.2 ± 3.5% of Foxp3-positive cells, respectively. Similarly, most of the $CD4^+CD25^{high}$ T cells in unseparated whole blood cells demonstrated Foxp3-positive staining (Fig. 3b,c).

The suppressive function of $CD4^+CD25^{high}$ T cells was demonstrated by their ability to inhibit the proliferation of $CD4^+CD25^-$ cells in functional assays of three healthy adults, recipients, and their corresponding donors from Group I. $CD4^+CD25^-$ cells showed a decreased proliferative rate, from 100% to 53.3 ± 1.5%, 52.9 ± 1.3% and 52.2 ± 3.8%, respectively, when they were co-cultured with $CD4^+CD25^{high}$ T cells from healthy adults, recipients and their donors (Fig. 3d). There are no significant differences between them (all $P > 0.1$).

Discussion

A number of studies in murine models have shown that $CD4^+CD25^+$ T cells play a very important role in the onset, control, and treatment of GVHD after aHSCT [13,14]. $CD4^+CD25^+$ T cells are able to reduce the incidence, and suppress the development of GVHD after major histocompatibility complex (MHC)-mismatched or

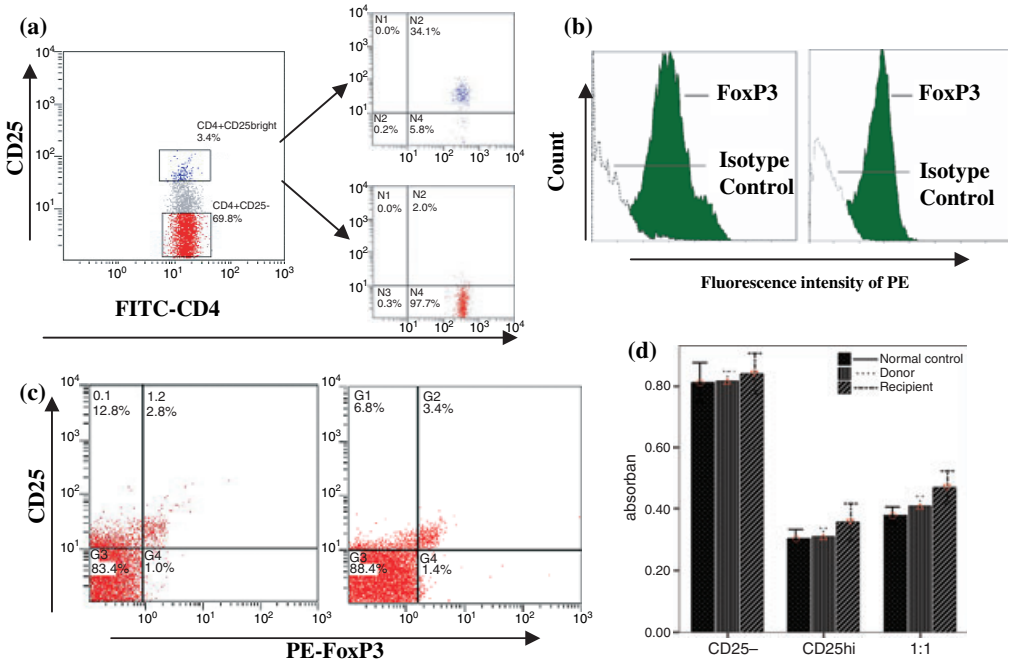

Figure 3 The functional assay of the $CD4^+CD25^{high}$ T cells from donors. (a) The dot plots show the $CD4^+CD25^{high}$ (upper box) and $CD4^+CD25^-$ (lower box) T cell-gating strategy pre-flow sorting and the subsequent purified $CD4^+CD25^+$ subsets. The purity of $CD4^+CD25^{high}$ and $CD4^+CD25^-$ T cells isolated were >93% for each fraction (from three separate experiments). (b) Over 85% of the freshly purified $CD4^+CD25^{high}$ T cells, respectively, from recipients (left) and the donors (right) express Foxp3. (c) Whole blood cells stain followed by RBC lysis shows that the $CD4^+CD25^{high}$ T cells from the recipients (left) and the donors (right) mostly expressed Foxp3. (d) Direct suppression by $CD4^+CD25^{high}$ T cells of proliferation of $CD4^+CD25^-$ T cells, performed using mixed lymphocyte response. Freshly isolated $CD4^+CD25^-$ T cells were cultured alone or co-cultured with $CD4^+CD25^{high}$ T cells at 1:1 ratio and stimulated with allogeneic-irradiated PBMCs. Proliferation was assessed by WST-1 reagent kit (Roche Diagnostics). The results represent the average of absorbance unit ($n = 3$, mean ± SD).

even haploidentical murine HSCT [7,8]. More recently it has been shown that $CD4^+CD25^+$ T cells infused into HSCT recipients after *ex vivo* activation and expansion could maintain their suppressor characteristics and reduce GVHD lethality [9,12]. The impairment of a competent peripheral regulatory system is one of important causative mechanisms underlying GVHD mediated by either alloreactive or autoreactive T cells [10,11,14].

The relationship of $CD4^+CD25^+$ T cells to GVHD in humans is unclear, and studies on regulatory T cells in clinical patients after aHSCT are limited. The current study showed that recipients with higher $CD4^+CD25^{high}$ T cells had no or only grade I aGVHD and survived throughout the follow-up period. In contrast, all of the recipients with lower levels of $CD4^+CD25^{high}$ T cells suffered severe (above grade II) aGVHD and died within 1-year post-aHSCT. Interestingly, the number of $CD4^+CD25^{high}$ T cells in recipients correlated significantly with that in their corresponding donors.

Because CD25 not only defines Tregs, but also activated lymphocytes, studies performed to effectively distinguish Treg cells have indicated that Foxp3 is a specific marker for regulatory T cells [17,20] and that $CD4^+CD25^{high}$ T cells have significant suppressive activity *in vitro* [15–18]. Our research showed that the majority of $CD4^+CD25^{high}$ T cells from recipients and their donors expressed Foxp3 and that $CD4^+CD25^{high}$ T cells effectively suppressed proliferation of $CD4^+CD25^-$ T cells. The data from this study prove that subset of $CD4^+CD25^{high}$ T cells reflects effectively the characteristics of human Tregs. A reduction in number of $CD4^+CD25^{high}$ Treg cells in recipients post-aHSCT may trigger lethal aGVHD. The cause of low levels of $CD4^+CD25^{high}$ T cells is still not clear, and could be related to lower levels of $CD4^+CD25^{high}$ T cells received from corresponding donors. GVHD is mediated by alloreactive lymphocytes in the stem cell graft. *Ex vivo* T-cell depletion of all T cells in the graft can prevent development of GVHD but can lead to an increase of infections and leukemic relapses. This has led to the approach of using anti-CD25 monoclonal antibody to eliminate autoreactive lymphocytes. However, based on this study, therapeutic intervention with anti-CD25 should be viewed with great caution as anti-CD25 may not only remove activated lymphocytes, but also Tregs and consequently cause lethal aGVHD. This may partly explain the phenomenon that Lee *et al.* [21] reported in a randomized study of corticosteroids with or without daclizumab (an anti-IL-2 receptor monoclonal antibody) for the initial treatment of aGVHD, which showed a significantly worse 100-day survival among patients who received corticosteroids plus daclizumab than in patients who received corticosteroids only (77% vs. 94%).

In addition, our treatment of a patient with CML using homogenic hematopoietic stem cells from his twin is instructive. The frequency of $CD4^+CD25^{high}$ T cells in the donor premobilization and in the recipient on day 28 post-transplantation was very high (15.9% and 6.1%) but there was no delay in immune reconstitution, infections, and leukemic relapses. The patient survived 18 months without any acute or chronic GVHD (data not shown in results).

Collectively, our results suggest that the role of Treg cells in aGVHD in human is similar to that seen in murine models. Monitoring of $CD4^+CD25^{high}$ Treg cells in donor and recipient should be valuable for predicting the onset, severity, and prognosis of aGVHD. Increasing the levels of $CD4^+CD25^{high}$ T cells in recipients by expansion *ex vivo* of Tregs from recipients or corresponding donors may be a potential therapeutic approach to the prevention and treatment of aGVHD.

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