# Regulatory T-Cells in the Graft and the Risk of Acute Graft-Versus-Host Disease After Allogeneic Stem Cell Transplantation

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**Background.** FOXP3$^+$ regulatory T-cells (Treg) are important regulators of allo-reactivity and may therefore represent an important predictor for the risk of graft versus-host disease (GVHD) after allogeneic stem cell transplantation.
**Methods.** To determine the clinical significance of Treg-content in stem cell grafts, we analyzed 58 human leukocyte antigen (HLA)-identical sibling donors (34 patients received myeloablative and 24 patients reduced intense conditioning regimens) and correlated the Treg frequency with clinical outcome after stem cell transplantation (SCT).
**Results.** A mean value of $9.1\times10^6$ CD4$^+$FOXP3$^+$ Treg per kg body weight (bw) of the recipient was transplanted (ranging from 0.7 to $33.7\times10^6$ Treg/kg bw). Graft content of Treg correlated with mononuclear cells and CD3$^+$ T-cells. Patients receiving low numbers of Treg (Treg$^{low}$) after myeloablative conditioning for SCT had a significantly increased cumulative incidence of 76% for acute GVHD when compared with 23% for individuals receiving high numbers of Treg (Treg$^{high}$). This observation, however, was not made in patients after reduced intense conditioning-SCT. Notably, relapse rate was not significantly different between Treg$^{low}$ and Treg$^{high}$ patients in either patient group and overall survival was even increased in Treg$^{high}$ patients after myeloablative SCT. Finally, low Treg graft levels represent an independent prognostic factor in multivariate analysis for the appearance of acute GHVD.
**Conclusion.** Donor-derived Treg might be of particular significance for the development of acute GVHD after myeloablative SCT using HLA-identical sibling donors.

**Keywords:** Graft composition, Regulatory T-cell, Stem cell transplantation, Graft-versus-host disease.

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Acute graft-versus-host disease (aGVHD) is a serious complication after both standard conditioning (*1*) and reduced intense conditioning (RIC) (*2*) allogeneic stem cell transplantation (SCT). Notably, GVHD often is associated with the eradication of residual disease after SCT (*3*). If this graft-anti-tumor (GVT) effect is not present, relapse may occur, which is a major cause of treatment failure. Thus, strategies targeting predominantly GVHD without affecting GVT are warranted.

FOXP3-expressing CD4$^+$CD25$^+$ regulatory T cells (Treg) tip the balance between auto- and tumor-immunity (*4*, *5*). Increased levels of Treg have been described in a variety of tumors (*6–8*), whereas in autoimmune disorders, Treg seem to be numerically and/or functionally deficient (*9*, *10*). Treg also have been conferred to function as critical regulators of allo-reactivity in vitro and in vivo (*11–14*). Edinger and co-workers demonstrated the potent suppressor activity of isolated Treg in an experimental model of fully mismatched allogeneic SCT by preventing development of GVHD without affecting the GVT effect (*15*). To date, only little is known about the prognostic significance of Treg content in SC-grafts for Treg reconstitution and incidence of GVHD after hematopoietic SCT. Thus, it was the aim of our current project to determine the effect of Treg graft content on the outcome after allogeneic SCT.

## MATERIALS AND METHODS

### Patients

We evaluated 58 patients with hematological malignancies receiving consecutive allogeneic peripheral blood stem cells (PBSC) transplants from human leukocyte antigen (HLA)-identical siblings. SCT was performed between March 2000 and December 2005 after written informed consent was obtained. Thirty-four transplants were performed following standard intensity, myeloablative conditioning (ie, intravenous [IV] busulfan 12.8 mg/kg, n=14, or 12 Gy fractionated total body irradiation [TBI, 2 Gy×6 through 3 days; n=16], plus high-dose cyclophosphamide [120 mg/kg]). The CBV regimen (cyclophosphamide 6000 mg/m$^2$, carmustine 450 mg/m$^2$, etoposide 1600 mg/m$^2$) was applied for lymphoma patients (n=4). Twenty-four patients received a RIC regimen consisting of busulfan (12.8 mg/kg for patients ≤60 years; 6.4 mg/kg for patients >60 years or with previous hematopoietic stem cell transplantation) together with fludarabine (90 mg/m$^2$; n=23). Patients who were not in remission at the time of transplant additionally received thiotepa (250 mg/m$^2$). One patient received high-dose melphalan (200 mg/m$^2$). Standard risk disease at the time of transplant was defined as acute leukemia in first remission or chronic myelogenous leukemia

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**TABLE 1.** Patient characteristics

| | N |
|---|---|
| All pts. | 58 |
| Mean patient age, years (range) | 45 (17–69) |
| Mean donor age, years (range) | 44 (19–71) |
| Diagnosis | |
| AL (%) | 33 (57) |
| CML (%) | 8 (14) |
| MDS (%) | 6 (10) |
| Lymphoma (%) | 8 (14) |
| Others (%) | 3 (5) |
| Risk | |
| Standard risk (%) | 22 (37) |
| High-risk (%) | 36 (63) |
| Mean TREG ($\times 10^6$/kg; range) | 9.1 (0.7–33.7) |
| Mean CD34 ($\times 10^6$/kg; range) | 7.77 (1.11–17.36) |
| Mean CD3 ($\times 10^8$/kg; range) | 3.58 (1.31–7.89) |
| Mean MNC ($\times 10^8$/kg; range) | 12.1 (5.23–22.16) |
| Mean NK ($\times 10^8$/kg; range) | 0.4 (0.11–1.48) |
| Take (median days, range) | 13 (8–32) |
| Conditioning | |
| Myeloablative | |
| CY/fTBI (%) | 16 (27) |
| BUCY (%) | 14 (24) |
| CBV (%) | 4 (7) |
| Reduced intensity | |
| BUFLU (%) | 23 (40) |
| HD-MEL (%) | 1 (2) |
| Sex match | |
| M/F (R/D; %) | 10 (17) |
| Others (%) | 48 (83) |
| CMV | |
| R and/or D +ve (%) | 45 (78) |
| R and D −ve (%) | 13 (22) |

in first chronic phase. All other indications were classified as high-risk disease. Detailed patient and treatment characteristics are listed in Table 1.

### Prophylaxis, Diagnosis, and Treatment of GVHD

Following standard intensity myeloablative conditioning, GVHD prophylaxis consisted of cyclosporine A (CsA) monitored by serum levels plus short-course methotrexate (*16*). After RIC transplants, CsA and mycophenolate mofetil (MMF) were administered according to the Seattle protocol for nonmyeloablative transplants (*17*). Acute and chronic GVHD were diagnosed from clinical symptoms and/or biopsies from skin, oral mucosa, liver, and gut and classified according to the previously published standard Seattle criteria (*18*). Acute GVHD≥grade 2 was treated with steroids (methylprednisolon, 2 mg/kg/d).

### Cytomegalovirus Screening, Definition, and Treatment of CMV Infection

Surveillance for cytomegalovirus (CMV)-reactivation (pp65 antigenemia) was performed on a weekly basis until day +100. CMV infection was defined according to recently published standard criteria (*19*). Preemptive IV ganciclovir (5–10 mg/kg/d) or valganciclovir (900 mg PO BID) was immediately started at the time of first detection of CMV antigenemia (pp65) for at least 3 weeks followed by maintenance therapy three times weekly for additional two weeks.

### Supportive Care

No prophylactic antibiotics were administered during neutropenia. If leukocytes dropped <1.0 g/L, patients received granulocyte colony-stimulating factor (5 $\mu$g/kg/d) to accelerate hematopoietic recovery. All patients received *Pneumocystis jiroveci* pneumonia prophylaxis with trimethoprim-sulfamethoxazole (TMP/SMZ) for up to 6 months after SCT or until $CD4^+$ T-cell counts exceeded 200/$\mu$L. Valacyclovir 500 mg BID orally or low-dose acyclovir 250 mg/m$^2$ IV three times daily from the beginning of conditioning until the end of the first year was administered for Herpes simplex and Varicella-zoster virus prophylaxis. Antifungal prophylaxis consisted of fluconazole 400 mg/d from the beginning of conditioning until day +73. Irradiated (25 Gy) leukocyte-depleted random platelet units from single donors and red cells were given when platelet counts dropped below 20 G/L and hemoglobin levels decreased to <8.0 g/dL.

### Quantification of Treg

The content of regulatory T cells was determined after collection of stem cell grafts. Treg number was determined by FACS staining of CD4 together with FOXP3 (using the FoxP3 Staining Set [clone PCH101] from eBioscience) strictly according to the manufacturer's instructions. In brief, $1\times10^6$ cells were double stained with cell surface markers (FITC-conjugated anti-human CD4 together with PE-conjugated anti-human CD25 mAb) for 30 min at 4°C. After two washes, cells were fixed and permeabilized using Fix/Perm buffer for 45 min at 4°C followed by two washing steps. Finally, cells were stained with titrated amounts of antigen-presenting cell-conjugated anti-human FOXP3 mAb for 45 min at 4°C. Two milliliters of Perm buffer was added to each sample, and T cells were pelleted, resuspended in 200 $\mu$L of buffer, and analyzed on a flow cytometer (Becton Dickinson, FACSCalibur). Data acquisition and analysis were performed using CellQuest software. The number of Treg per kg body weight (bw) of the recipient was determined as follows: % $CD4^+$ $FOXP3^+$ Treg of viable mononuclear cell (MNC)×the total number of transplanted MNC/kg bw of the recipient.

### Statistics

Data were analyzed as of February 2006. Statistical significance was defined as $P<0.05$. SPSS (Version 14.0; SPSS Institute, Chicago, IL) and (to calculate cumulative incidences including the existance of competing risks) the NCSS statistical software package (Kaysville UT) were used for analyses. Correlations between individual graft cell subsets were calculated applying the Spearman rank correlation coefficient.

As cutoff-point, the mean value of Treg content in the graft was chosen. For univariate analyses of overall survival (OS), the Kaplan-Meier survival analysis was applied. OS was calculated from the date of SCT to the date of death or last follow-up, or to the date of documented disease relapse, respectively. Cumulative incidence estimates were calculated

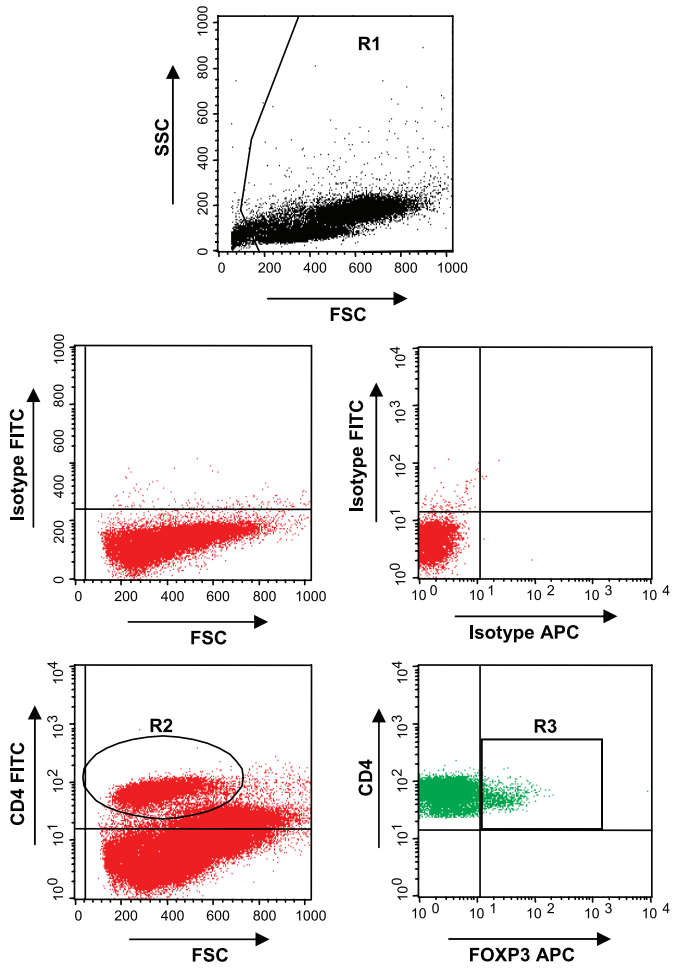


**FIGURE 1.** Gating strategy for the quantification of Treg in stem cell grafts. The gating strategy for determination for Treg is shown. The total number of Treg per kg bw of the recipient was determined as follows: % $CD4^+$ $FOXP3^+$ Treg of viable MNC× total number of transplanted MNC/kg bw of the recipient. A representative example of the gating strategy of $CD4^+FOXP3^+$ T-cells is given.

No. of transplanted $CD4^+FOXP3^+$ Treg was calculated as follows:

No. of living MNC (=R1) x % $CD4^+$ TC in R1 (=R2) x % $CD4^+FOXP3^+$ in R2 (=R3) / bw recipient [kg]

for relapse, CMV infection, acute and chronic GVHD, taking into account the existence of competing risks (*20*).

Multivariate analyses, applying the Cox (proportional hazards) regression, were calculated for the OS, relapse, aGVHD II-IV, and cGVHD and included the variables, Treg content ($Treg^{low}$ vs. $Treg^{high}$), type of conditioning (standard vs. RIC), sex mismatch (other vs. female donor/male recipient), risk by the underlying disease (standard risk vs. high risk), donor and recipient CMV serostatus, and donor and recipient age.

## RESULTS

### Treg Content in Stem Cell Grafts Correlates With Total MNC and $CD3^+$ T-Cell Numbers

The mean number of Treg, identified by their typical staining pattern of CD4 together with intracellular expression of FOXP3 (Fig. 1) transplanted with 58 HLA-identical stem cell grafts was $9.1\times10^6$ per kg bw of the recipient (ranging from 0.7 to $33.7\times10^6$ Treg/kg bw). Treg in stem cell grafts correlated with total MNC and $CD3^+$ T-cell numbers (correlation coefficient of $\rho=0.4$ [$P=0.002$] and $\rho=0.5$ [$P=0.0001$], respectively; Table 2) but not with $CD56^+$ NK cells or $CD34^+$ stem cell numbers (Table 2).

**TABLE 2.** Correlation of graft components

| | | MNC | CD3 | CD34 | NK |
|---|---|---|---|---|---|
| Treg | $\rho$ | 0.4 | 0.5 | −0.04 | 0.17 |
| | P | 0.002[a] | 0.0001[a] | 0.8 | 0.24 |
| | N | 58 | 53 | 58 | 52 |

[a] Significant.

### Graft Content of Treg Is Correlated With the Incidence of Acute GVHD

Low numbers of Treg in stem cell grafts were associated with a significantly increased cumulative incidence of aGVHD grade II-IV (76% in the $Treg^{low}$ and 31% in the $Treg^{high}$ group, $P=0.001$; Fig.2A). In contrast, relapse rate was not significantly affected by the number of transplanted Treg (36% in the $Treg^{low}$ and 23% in the $Treg^{high}$ group, $P=0.27$; Figure 2A). Other important clinical endpoints, such as cGVHD (59% in the $Treg^{low}$ and 43% in the $Treg^{high}$ group, $P=0.19$) or CMV-reactivation (31% in the $Treg^{low}$ and 38% in the $Treg^{high}$ group, $P=0.83$) were also not influenced by the number of transplanted Treg.

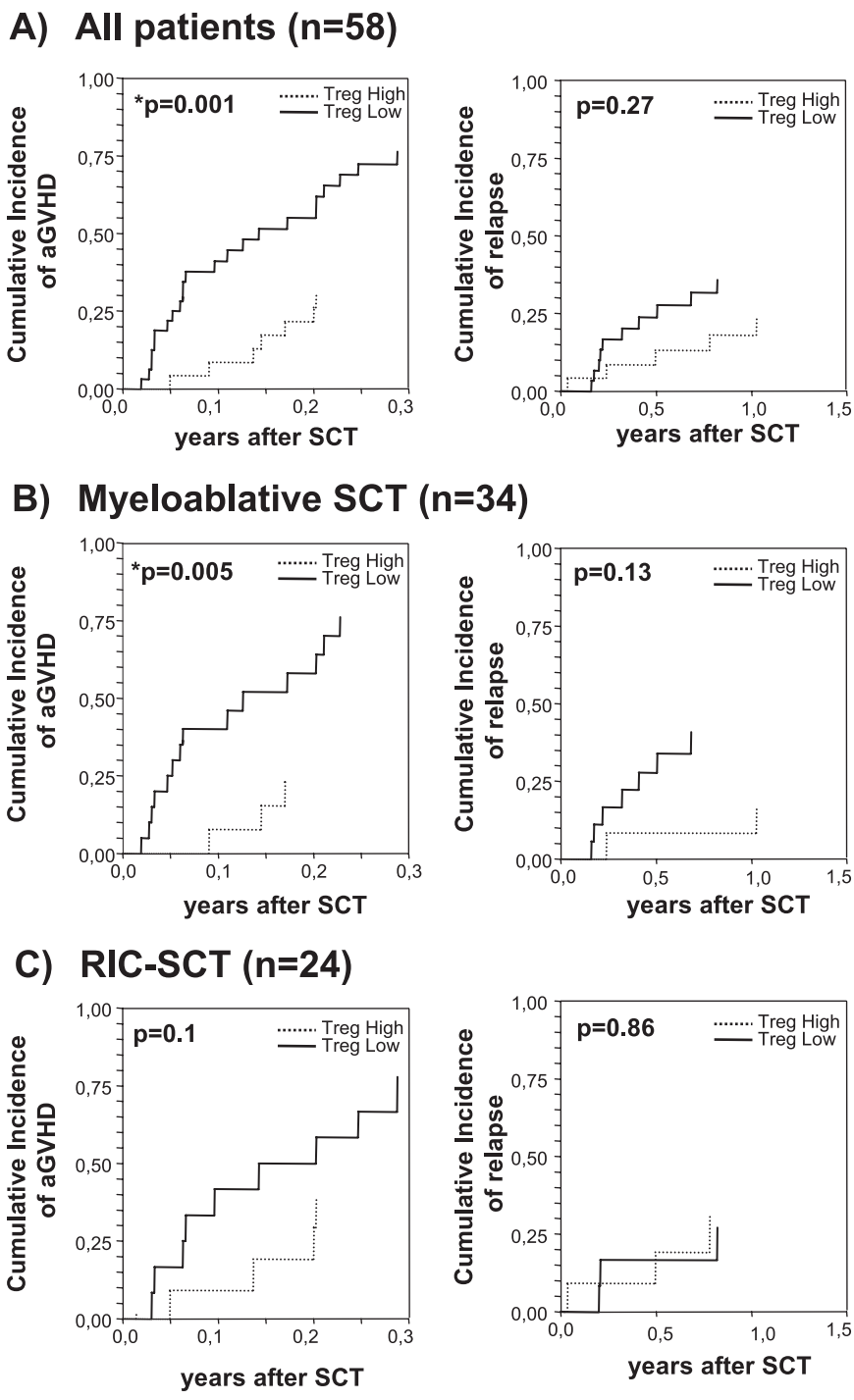


**FIGURE 2.** aGVHD and relapse-rate depending on Treg graft content. The cumulative incidence for $Treg^{low}$ and $Treg^{high}$ patients is shown for A) all 58 patients (including myeloablative and RIC-SCT), B) patients after myeloablative conditioning (n=34), and C) after RIC-SCT (n=24). *P* value was determined by log-rank test.

### Treg in SC Grafts Only Predict aGVHD in Myeloablative but Not in RIC Transplants

The patient cohort was next divided into individuals receiving SCT after either myeloablative or RIC regimens. Of note, a significant impact of Treg graft-content on aGVHD incidence was only detectable in patients undergoing myeloablative SCT (76% in the $Treg^{low}$ and 23% in the $Treg^{high}$ group, $P=0.005$; Fig. 2B), whereas in RIC-SCT only a trend toward a higher aGVHD incidence was determined in patients receiving low Treg numbers (78% in the $Treg^{low}$ and 39% in the $Treg^{high}$ group, $P=0.1$; Fig. 2C). Again, in none of the groups a significant impact of Treg graft content on relapse rate (Fig. 2B and C), cGVHD or CMV-infection (data not shown) was detected.

### High Treg Numbers in the Graft Are Associated With Improved Survival in Myeloablative SCT

Figure 3 depicts that high graft content of Treg is associated with a significantly increased overall survival (OS) rate ($P=0.01$), whereas this difference was neither observed in RIC-SCT patients ($P=0.9$) nor in the whole patient group ($P=0.1$).

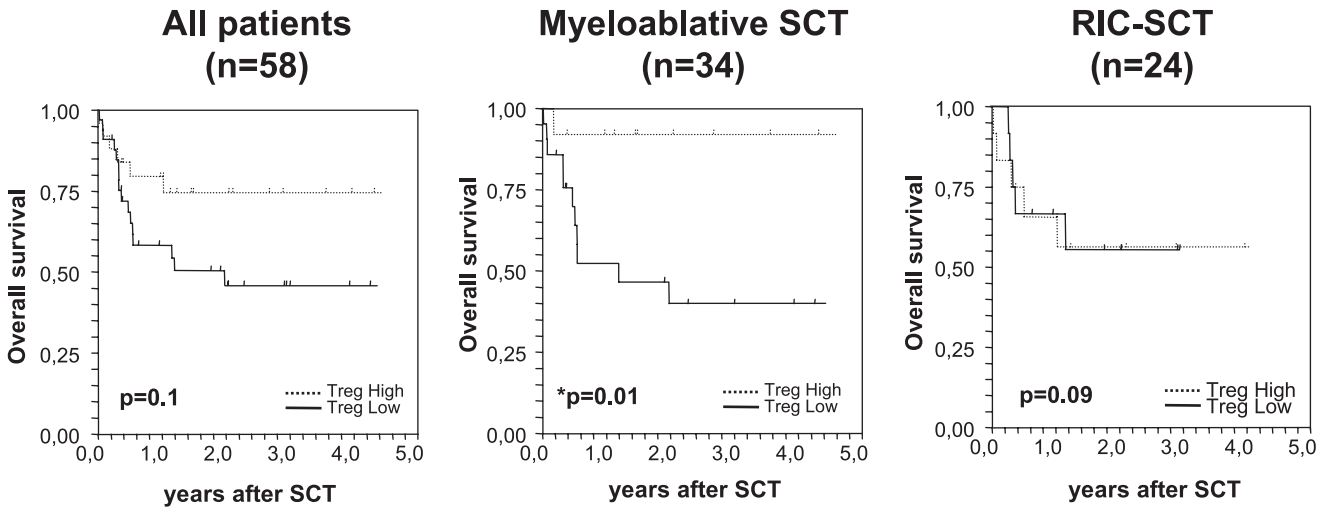


**FIGURE 3.** High levels of Treg in the graft are linked to a better overall survival in myeloablative SCT, but not in RIC-SCT. Kaplan-Maier curves for all patients (n=58) and the respective subgroups (ie, myeloablative [n=34] and RIC-SCT [n=24]) are shown. *P* value was determined by log-rank test.

### Treg Graft Content Is an Independent Predictor for aGVHD

Multivariate analysis identified Treg graft content as an independent predictor for appearance of aGVHD in the whole patient group as well as in SCT recipients after myeloablative conditioning (Table 3). In contrast, Treg graft content did not predict other postSCT events, such as cGVHD, OS, or relapse rate. Other significant variables for aGVHD and relapse are shown in Table 3. We could not detect significant variables associated with cGVHD, OS or CMV-infection in our patient group (data not shown).

## DISCUSSION

Our current report identifies $CD4^+FOXP3^+$ Treg as an additional graft component predicting the risk of aGVHD after HLA-identical sibling SCT. Patients undergoing SCT with unmanipulated SC-grafts receiving high numbers of Treg have a 45% lower cumulative incidence of aGVHD. This observation is in line with a very recent report from 32 HLA-identical sibling transplants showing that in patients receiving T-cell–depleted stem cell grafts after myeloablative conditioning and subsequent T-cell add-back, Treg content in PB of the sibling donors may predict for risk of GVHD (*21*). In contrast, a positive correlation between $CD4^+$ and $CD8^+$ T cells co-expressing CD25 with the incidence of GVHD was described by Stanzani and co-workers (*22*). Notably, the significant influence of Treg on aGVHD-incidence in our patient group was restricted to patients receiving myeloablative conditioning regimens (with a 53% lower cumulative incidence of aGVHD in $Treg^{high}$ patients). The role of Treg content in SC grafts of patients undergoing myeloablative SCT is further underscored by the observation that low Treg content is associated with an inferior overall survival rate and represents an independent risk factor for the appearance of aGVHD.

Apart from the limited patient number after RIC-SCT included in our study, immunological differences might account for the lack of a significant effect of Treg graft content with respect to aGVHD incidence in RIC-SCT. The latter is characterized by a longer time of donor/host cell chimerism, as well as aGVHD appears in general at later time points after SCT (reviewed in Mineishi and Schuening; *23*). Thus, rapid activation of aGVHD-inducing T-cell clones after myeloablative conditioning might be suppressed more efficiently in the presence of higher numbers of initially transplanted Treg. It is already known from in vitro experiments that the relative proportion of Treg in relation to the number of effector T-cells determines their suppressive activity (*24*, *25*). It is noteworthy, that in contrast to animal studies (*26*) we have currently no data on the role of Treg with respect to their repopulation of lymphoid organs after SCT in humans, the compartment where the induction of GVHD-inducing T-cell clones is supposed to take place (*27*). Thus, at least in the myeloablative setting, greater numbers of transplanted Treg

**TABLE 3.** Cox regression analysis for aGVHD and relapse

| Endpoint | Variable | RR [95% CI] | *P* value |
|---|---|---|---|
| All patients | | | |
| aGVHD | Treg (high *vs.* low) | 0.2 [0.06–0.52] | 0.002 |
| | Age D | 1.1 [1.04–1.2] | 0.004 |
| | Age R | 0.9 [0.87–0.99] | 0.02 |
| Relapse | Risk (high *vs.* standard) | 6.8 [1.2–39.0] | 0.02 |
| | Age D | 0.9 [0.8–0.99] | 0.04 |
| | Age R | 1.1 [1.02–1.25] | 0.03 |
| Myeloablative SCT | | | |
| aGVHD | Treg (high *vs.* low) | 0.1 [0.03–0.63] | 0.01 |
| | Age D | 1.2 [1.0–1.2] | 0.045 |
| | Age R | 0.9 [0.85–0.98] | 0.01 |
| Relapse | Risk (high *vs.* standard) | 9.4 [1.0–78.5] | 0.04 |

Only significant variables are listed.
D, donor; R, recipient.

may repopulate the host's secondary lymphoid organs more efficiently, thereby locally tipping the balance towards a more tolerogeneic milieu. Second, after RIC-SCT, MMF is used in combination with CsA, whereas most of our myeloablative conditioned patients received methotrexate in combination with CsA.

The exact role of MMF in combination with CsA and its in vivo role for the suppressive function of Treg remains to be determined yet. Recent data demonstrated that calcineurin-dependent interleukin-2 production is critically required for Treg function in vivo, as the combination of Treg with CsA, but not with rapamycin or MMF, led to suppressed Treg function with increased T-cell proliferation, GVHD severity and reduced survival in a BM-transplantation model in rodents (*28*). Thus, the establishment of preclinical SCT models including more appropriate models for RIC-SCT might help to define the role of Treg as inhibitors of GVHD more exactly with respect to the distinct transplant settings. In addition, the observation that Treg graft content is not correlated to Treg levels in the peripheral blood after SCT (Wolf et al., unpublished data) in combination with the recently published conflicting reports on the role of postSCT levels of Treg and/or the Treg-specific transcription factor FOXP3 for GVHD risk (*29–33*) strongly argues for further studies in larger patient cohorts addressing the question whether Treg graft content might represent a novel and clinically relevant predictor for aGVHD risk in SCT.

It is noteworthy, however, that apart from inhibition of aGVHD in myeloablative SCT, relapse rate was not affected by the number of transplanted Treg in our patient cohort. This observation is of particular interest, as it nicely fits to the concept proposed by Edinger and co-workers showing in a preclinical model that Treg are potent inhibitors of GVHD without affecting GVT-effects (*15*). Our clinical observation is in line with these findings and further extends them by showing that Treg numbers had also no impact on infectious complications such as CMV reactivation.

In summary, our report demonstrates for the first time that graft content of Treg is of particular significance for aGVHD risk in myeloablative SCT. This observation suggests that manipulation of the graft, ie, by adding donor-derived and in vitro expanded Treg might be a rational approach to reduce aGVHD incidence after myeloablative SCT without affecting GVT.

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