

Regulatory T Cell Content in the Bone Marrow Graft Does Not Predict the Occurrence of Acute GVHD

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The subpopulation of regulatory T cells (Treg) was shown to play a key role in alloreactive responses. In allogeneic hematopoietic stem cell transplantation, several groups tested whether Treg content in transplants correlates with graft-versus-host disease (GVHD) with controversial results. In a retrospective study of 49 consecutive HLA-matched sibling transplantations, we studied the relationship between Treg content in bone marrow transplants and acute GVHD (aGVHD) occurrence. We observed a large variability in Treg in bone marrow grafts. However, contrary to previous observations in peripheral blood stem cells transplantation, we report that the Treg content of allogeneic bone marrow transplantation did not predict the occurrence of aGVHD.

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INTRODUCTION

CD4⁺CD25⁺FoxP3⁺ regulatory T cells (Treg) have an important role in the control of alloreactivity in allogeneic stem cells transplantation [1]. The first direct demonstration of Treg immunosuppressive effect on alloreactivity was made in experimental bone marrow (BM) transplantation (BMT) in mice, where complete Treg depletion before grafting significantly accelerated the mortality linked to graft-versus-host disease (GVHD) [2,3]. These observations led to the study of Treg content in transplants as a predictive factor for GVHD in humans. However, despite increasing evidences that Treg content could have

diagnostic and prognostic value for acute GVHD (aGVHD) when assessed in grafted patients [4], clinical studies were never performed in BM transplants and remain controversial in peripheral blood stem cell (PBSC) transplants (for review, see [5]).

Here, we studied the relationship between Treg content in nonmanipulated BM transplants and aGVHD occurrence in a retrospective study of 49 consecutive HLA-matched sibling transplantations.

MATERIALS AND METHODS

Patients

Forty-nine consecutive patients transplanted grafted at the Pitié Salpêtrière Hospital between February 2000 and July 2008, with BM from HLA-matched sibling donors, were included in this single center retrospective study. Written informed consent was obtained from all donors prior to sampling. The institutional review board "Ile-de-France IX" reviewed and approved the study.

Acute GVHD (grade II to IV) defined according to published criteria [6], has occurred in 65% (32 of 49) of the patients. All patients received a myeloablative conditioning regimen (total body irradiation [TBI] and high-dose cyclophosphamide or high-dose cyclophosphamide plus busulfan) and GVHD prophylaxis with a short course of methotrexate (15 mg/m² on day 1, 10 mg/m² on day 3, and 10 mg/m² on day 6) and cyclosporine. They all engrafted successfully. Grade II-IV

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Table 1. Candidate Variables in Cox's Model Analysis of Time to acute GVHD

Variables	Acute GVHD		Univariate Cox model analysis
	No (n = 17)	Yes (n = 32)	HR [95% CI]*
D/R gender: number of female/male (%)	3 (17.7)	8 (25.0)	1.35
CMV status donor/recipient -/+ (%)	5/17 (29.5)	8/31 (25.8)	1.08
Complete remission at HSCT (%)	7/14 (50.0)	15/31 (48.39)	0.97
Age of recipients (year): median [range]	41 [21-53]	40 [17-55]	1.005 [0.97-1.04]
Age of donors (year): median [range]	37 [12-60]	39 [12-62]	1.01 [0.99-1.04]
CD34 ⁺ /kg × 10 ⁶ : median [range]	2.56 [0.76-5.46]	1.80 [0.81-5.93]	0.96 [0.69-1.28]
CD3 ⁺ /kg × 10 ⁷ : median [range]	2.79 [1.52-5.30]	3.14 [0.18-8.40]	5.21 [0.54-44.2]
CD3 ⁺ CD4 ⁺ /kg × 10 ⁷ : median [range]	1.26 [0.62-2.66]	1.55 [0.11-2.88]	2.24 [0.42-11.4]
CD4 ⁺ CD25 ^{high} /kg × 10 ⁵ : median [range]	3.32 [0.79-14.77]	4.64 [0.42-9.46]	1.00 [0.89-1.10]
CD4 ⁺ Foxp3 ⁺ /kg × 10 ⁵ : median [range]	2.07 [0.50-14.54]	3.16 [0.40-10.53]	1.01 [0.90-1.11]
CD25 ^{high} /CD3 (%): median [range]	1.21 [0.45-3.98]	1.37 [0.48-2.73]	0.97 [0.70-1.30]
Foxp3/CD3 (%): median [range]	0.97 [0.17-3.72]	0.95 [0.21-2.83]	0.99 [0.72-1.31]

GVHD indicates graft-versus-host disease; CMV, cytomegalovirus; D/R, donor/recipient; HSCT, hematopoietic stem cell transplant; HR, hazard ratio; CI, confidence interval.

Forty-nine patients were grafted for acute leukemia (n = 18), lymphoma or chronic lymphocytic leukemia (n = 16), multiple myeloma (n = 3), or other diseases (n = 12). Twenty-two patients were in complete remission at time of transplantation. The patient population was broken down according to acute GVHD outcome (grade II to IV). For quantitative variables, groups (aGVHD yes versus no) were compared with the Mann-Whitney test, and for qualitative ones with the Fisher exact test. No comparisons achieved the statistical significance threshold ($P_{(\alpha\ 2\text{-sided})} < .05$).

*Univariate Cox (proportional hazards model) regressions of time to aGVHD (rightmost column) were performed for the candidate variables in the table. Data in cells are Hazard Ratio [95% confidence intervals].

aGVHD were treated with corticosteroids (n = 32) with anti-rIL2 (n = 5) or mycophenolate mofetil (n = 3) or both (n = 1).

BM Analysis

The concentration (cells/ μ L) of T lymphocytes was established from fresh bone marrow samples with a 3-color flow cytometry using BD Multitest (CD3/CD19/CD45) with Trucount tubes containing fluorescent beads as internal standard (BD Biosciences, Mountain View, CA). Treg analysis was performed on BM sample aliquots after thawing. No difference before and after cryopreservation/thawing was observed (Figure S1). Intranuclear forkhead box P3 (FoxP3) labeling was performed after CD3 (Clone UCHT1 Beckman Coulter, Villepinte, France), CD4 (Clone SFC12T4D11) and CD25 (Clone M-A251; BD Biosciences) membrane staining using antihuman Foxp3 kit (PCH101 clone, eBioscience, San Diego, CA), with rat IgG2a isotypic control. Cells acquisition and analysis was performed on a FACS Calibur cytometer using CellQuest software (BD Biosciences).

Statistical Analysis

A Mann-Whitney test was used to compare clinical and biologic characteristics in patients with aGVHD versus patients who did not experience aGVHD. Univariate and multivariate Cox model regression analysis of time to aGVHD was used to evaluate the influence of each candidate prognostic factor on the risk of aGVHD. Data were analyzed using JMP software (version 8.0; SAS Institute Inc., Cary, NC), and statistical threshold for significance set at $P_{(\alpha\ 2\text{-tailed})} < .05$. Sample size was calculated based on the 3 previous studies and powered to detect a similar difference [6-8]. We

calculated that with our cohort (aGVHD, n = 32; no-aGVHD, n = 17), the power was >80% (at $p_{(\alpha\ 1\text{-tailed})} < .05$) to detect a 60% increase in the bone marrow graft Treg content in the no-aGVHD group versus aGVHD 1 (by the *t*-test) [6], or to detect a 35% increase in aGVHD-free survival in high-Treg versus low-Treg group (by the log-rank test) [7,8].

RESULTS AND DISCUSSION

Following BMT, 32 (65%) patients developed an aGVHD (grade II, n = 24, grade III-IV, n = 8). Cox regression analysis showed a trend in increase in aGVHD risk with increasing CD3⁺ T cell graft content (HR [95% CI] = 0.21 [0.54-44.2]). None of the other candidate variables were found to have a statistical significance either in group comparisons or as prognostic factors in Cox model analysis (Table 1). Treg were identified by either CD25^{high} or FoxP3 expression on CD4⁺ T cells (Figure 1A) with a good correlation ($\rho = 0.85$) between these 2 markers (Figure S2). Analysis of Treg numbers revealed a large variability within the donor population (mean = $4.85 \times 10^5/\text{kg} \pm 3.08$ for CD25 and $3.72 \times 10^5/\text{kg} \pm 3.13$ for FoxP3) (Figure 1B). However, no correlation between occurrence of aGVHD and the proportion or absolute number of infused Treg was found (regardless of aGVHD grades cutoff point used; 0-I versus II-IV in Figure 1B, but also 0 versus I-IV or 0-II vs III-IV, data not shown). Overall aGVHD-free survival was not statistically different in patients who received above or below the median graft Tregs (median CD25⁺ count = $4.25 \times 10^5/\text{kg}$) (Figure S3). The analysis of the Treg ratio to CD3⁺ T cells yielded similar results. In the entire cohort, CD3 and Treg content were significantly correlated

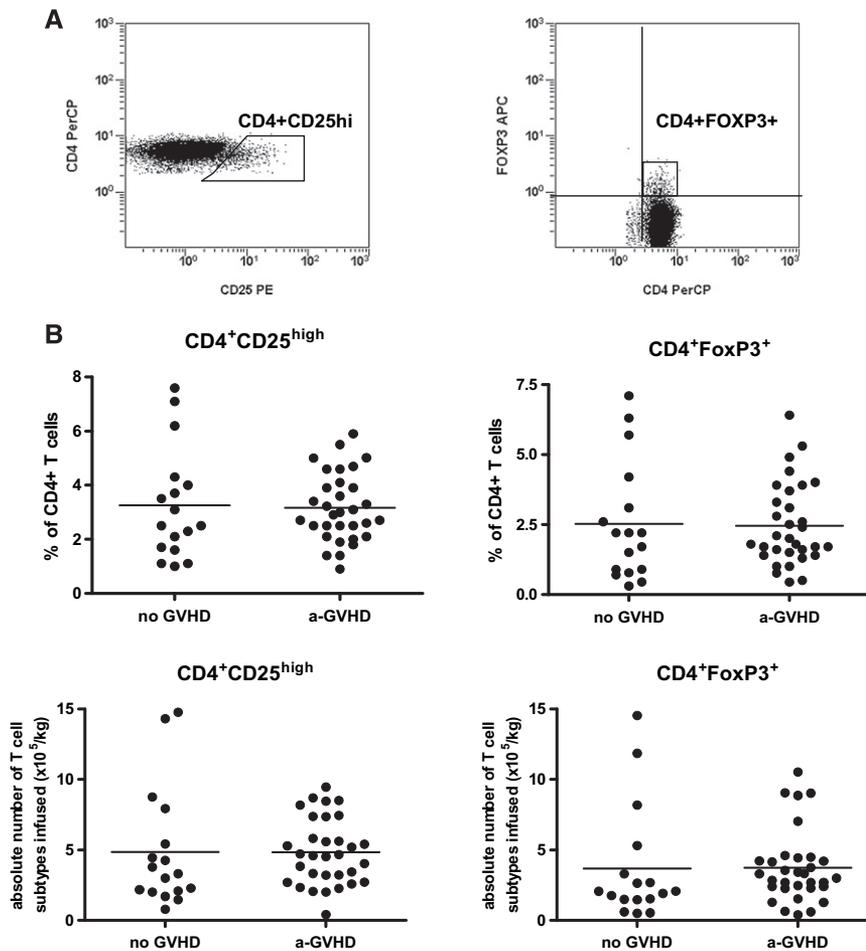


Figure 1. Regulatory T cell content of the grafts does not correlate with incidence of GVHD. (A) FACS plots showing the gating criteria used to quantify regulatory T cells upon CD4⁺CD25^{hi} and CD4⁺FoxP3⁺ phenotypes. (B) Scatter plot showing the percentage (up) or the absolute number (low) of infused Treg with respect to the grade of GVHD. Horizontal bars represent the mean. Mean % of CD25^{high} = 3.25 ± 0.49 for the no GVHD group and 3.16 ± 0.22 for the aGVHD group; mean % of CD4⁺FoxP3⁺ = 2.51 ± 0.51 for the no GVHD group and 2.45 ± 0.26 for the aGVHD group; mean number of CD25^{high} = 4.85 ± 1.02 × 10⁵/kg for the no GVHD group and 4.84 ± 0.41 × 10⁵/kg for the aGVHD group; mean number of CD4⁺FoxP3⁺ = 3.67 ± 0.98 × 10⁵/kg for the no GVHD group and 3.73 ± 0.45 × 10⁵/kg for the aGVHD group.

($r^2 = .22, P = .0007$, and $r^2 = .15, P = .006$, for CD25⁺ and Foxp3⁺, respectively). A multivariate Cox model analysis showed that Treg content was not an independent predictor (CD25, $P = .18$; CD3 $P = .01$) of aGVHD even following adjustment for CD3 content. We thus conclude that, in contrast to previous observations in PBSC transplantation, our results do not support the usefulness of assessing allogeneic BM Treg content to predict aGVHD. However, given the limited dimension of these cohorts we cannot rule out an effect of smaller magnitude. As a consequence, our conclusions probably need to be confirmed in a larger sample.

Three previous studies [7-9] showed that a high level of Treg in donor peripheral blood was associated with reduced cumulative incidence of aGVHD in cohorts of 22, 63, and 34 patients, respectively. In 1 of these studies Treg measurement was performed before granulocyte-colony stimulating factor (G-CSF) administration [7], whereas it was performed after for the 2 remaining. These results were observed both in related and unrelated PBSC myeloablative transplantations but

not with reduced-intensity conditioning (RIC). In addition, these studies focused on absolute numbers of Treg but not on the percentage of Treg among CD3⁺ or CD4⁺ T cells within graft, which is a determinant parameter regarding the suppressive effect of Treg [10].

The source of HSC (PBSC versus BM) may also impact the Treg content as recently observed by Blache et al. [11], and consequently, relationship between the Treg content of the transplant and the risk of aGVHD [12,13]. In our study involving BM only, patients received about 10-fold fewer CD3⁺ T cells compared with studies performed with PBSC [8,9]. Despite a similar incidence of aGVHD observed in these 2 settings, it remains possible though highly speculative, that Treg variation may have more relevance when increased numbers of T cells are injected. In addition, CD3 and Treg should have a dual and opposite effect on the risk of aGVHD, although both are linearly related as shown by the high correlation in our study. Hence, the modeling of the effect of the variation of these 2 parameters on the risk of aGVHD is likely to

be nonlinear. Indeed, in our study as in Pabst et al. [8,9], in multivariate analysis, Treg is not an independent predictor of the risk of aGVHD.

Finally, G-CSF treatment may have modified the Treg content and/or the rate of GVHD occurrence. Unfortunately, the impact of G-CSF on T cells is not entirely understood [11,14,15]. Our study has the advantage that neither donors nor recipients were treated with G-CSF, thereby avoiding a possible confounding factor.

The hypothesis that the Treg graft content may be related to GVHD occurrence originated from mouse experiments. Notably, accelerated GVHD was observed only when the transplant was fully Treg depleted [2,3], and protection against GVHD was only achieved when using Treg at the very high ratio of 1:1 Treg to conventional CD4⁺ T cell [2,3,16-19]. Despite the substantial variation in Treg concentration among donors (near 1 log in our study), the mean ratio of Treg to conventional CD4⁺ T cells (1:35 in BM samples) is far lower than the 1:1 ratio required for a therapeutic effect in murine models. Consequently, the impact of variations of Treg content that we observed in nonmanipulated human transplants may be of lesser significance. However, in the absence of correlation in this study and based on previous studies in mice, it may be worthwhile to manipulate Treg to a greater degree than that which occurs naturally. aGVHD may be attenuated or prevented by increasing the ratio of Treg in the transplant. Alternatively, Treg depletion may improve the graft-versus-tumor (GVT) effect of donor lymphocytes [20]. We have recently successfully tested this approach in a clinical trial which demonstrates that Treg depletion is a safe approach that induces GVHD and antitumoral effect in patients not responding to a classical donor lymphocyte infusion [21].

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AUTHORSHIP STATEMENT

M.R. and N.D. performed research and analyzed the data; S.M., G.B., D.A.L., and D.K. analyzed data and wrote the paper; F.N., H.T.N., and M.U. performed the research; J.P.V. designed the research; J.L.C. designed the research, analyzed the data, and wrote the paper.

SUPPLEMENTARY DATA

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbmt.2010.07.024.

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