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Outcome of Allogeneic Peripheral Blood Stem Cell Transplantation by Donor Graft CD3$^+$/Tregs Ratio: A Single-Center Experience

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The therapeutic efficacy of allogeneic peripheral blood stem cell transplantation (PBSCT) for hematological malignancies relies largely on the graft-versus-leukemia (GVL) effects exerted by the donor CD3 cells, but there is a risk of onset of uncontrolled graft-versus-host disease (GVHD). Regulatory T cells (Tregs) (CD4+CD25^{high} Foxp3+) are believed to maintain tolerance and to inhibit acute GVHD (aGVHD) after allogeneic PBSCT. Nevertheless, when looking at post-allotransplantation patient outcomes, although the impact of aGVHD on survival is amply documented, so far there is no evidence that the donor graft CD3/Tregs ratio may affect overall survival (OS), nonrelapse mortality (NRM), disease-free survival (DFS), and relapse rates. Our aim was to study the possible impact of the gCD3/Tregs ratio on survival after myeloablative allogeneic PBSCT. We analyzed 74 consecutive patients diagnosed with acute myeloid leukemia (n = 62), acute lymphoblastic leukemia (n = 10), and chronic myeloid leukemia (n = 2) who underwent transplantation with unmanipulated PBSCs from a human leukocyte antigen–identical related donor (n = 48) or a human leukocyte antigen–identical unrelated donor (n = 26). Patients were subdivided into a high gCD3/Tregs ratio (≥36) group (HR group, n = 30) and a low gCD3/Tregs ratio (<36) group (LR group, n = 44). The OS, DFS, NRM, and relapse rates at 3 years were 53%, 51%, 29%, and 34%, respectively. Comparing the LR and HR groups, a statistically significant difference was demonstrated for the 3-year OS, DFS, and NRM rates (65% vs 31%, $P = .0001$; 67 versus 26%, $P = .0001$; 5% versus 71%, $P < .0001$, respectively) but not for relapse (30% vs 25%, $P =$ ns). By multivariate analysis, LR significantly predicted better OS ($P = .019$), DFS ($P = .003$), and NRM ($P = .05$), whereas there was no statistically significant association between LR and relapse ($P = .155$). Overall, our data may suggest that LR preserves GVL effects but is also protective against aGVHD in allotransplantation patients.

INTRODUCTION

The contribution of regulatory T cells (Tregs) to post-transplantation immunological reconstitution has been clearly established, given their impact on T cell immunity [1] and on modulating graft-versus-host disease (GVHD) while preserving graft-versus-leukemia (GVL) effects in mouse models [2]. The pathophysiological link between GVHD and immune reconstitution is well defined [3,4]. Therefore, given that acute GVHD (aGVHD) is triggered by alloreactive mature donor CD3 T cells [5,6] and antagonized by Tregs [7,8], the immunity of patients undergoing allotransplantation might depend on the ratio between these two cellular populations, as we have already suggested in humans [9], in line with murine experimental models of aGVHD [10,11].

Moreover, it is well known that, apart from inducing prolonged immunosuppression [12] and a graft failure risk [13], T cell depletion of the donor graft results in a higher leukemia relapse rate [14]. Nevertheless, when looking at the outcomes of patients undergoing allotransplantation, although the impact of human leukocyte antigen (HLA) incompatibility (and of the consequent aGVHD) on patient

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Table 1
Patient Characteristics

	n	%
Patients	74	
Sex (male/female)	34/40	46/54
Median age, y	33 (18-61)	
Diagnosis		
Acute myeloid leukemia	62	84
Acute lymphoblastic leukemia	10	13
Chronic myeloid leukemia	2	3
Conditioning regimen		
Busulfan + cyclophosphamide	74	100
Donor		
Sibling-matched donor	48	65
Unrelated matched donor	26	35
HCT-CI		
0	40	54
1-2	23	31
≥3	11	15
Graft CD3/Tregs ratio		
LR <36	44	60
HR ≥36	30	40
rATG usage		
Yes	26	35
No	48	65
Source of stem cells		
PBSC	74	100
GVHD prophylaxis		
CsA + MTX	74	100

HCT-CI indicates hematopoietic cell transplantation-specific comorbidity index; rATG, rabbit antithymocyte globulin; CsA, cyclosporin; MTX, methotrexate.

survival is amply documented [15,16], there is no evidence that donor CD3 T cells, if analyzed together with Tregs (gCD3/Tregs ratio), may have an impact in terms of post-transplantation outcomes (ie, overall survival [OS], non-relapse mortality [NRM], disease-free survival [DFS], and relapse rates). In this study, we expanded the previous evaluation [9] of the contribution of the graft CD3/Tregs ratio on aGVHD and immune recovery to focus on the possible impact of a low gCD3/Tregs ratio (LR) or high gCD3/Tregs ratio (HR) on OS, DFS, NRM, and relapse rates.

METHODS

Patients

Seventy-four patients who underwent allogeneic peripheral blood stem cell (PBSC) transplantation between January 2006 and December 2011 were included in this retrospective study; written informed consent was obtained from all patients. Patient characteristics are shown in Table 1.

All patients received PBSC grafts from an HLA-identical sibling donor (n = 48) or an unrelated HLA-identical donor (n = 26). DNA-based HLA typing of donor and recipient was done using high resolution (four digits) for HLA-A, -B, -C, -DRB1, and -DQB1; in unrelated transplantation, a complete match (10 of 10) was documented in 19 donor–patient pairs (73%) and a partial match (9 of 10) in 7 donor–patient mismatched pairs (27%). As conditioning regimen, all patients underwent a conventional myeloablative regimen that included 16 mg/kg oral busulfan (12.8 mg/kg intravenously in the last 9 patients) and 120 mg/kg cyclophosphamide. Antithymocyte globulin 7.5 mg/kg was administered in unrelated transplantations.

All patients were administered prophylactic antibiotics (levofloxacin 500 mg/d) until neutrophil engraftment; we used fluconazole (400 mg/d to day 100) as antifungal prophylaxis and trimethoprim-sulfamethoxazole for *Pneumocystis jirovecii* prophylaxis. All patients received GVHD prophylaxis, using cyclosporine A (starting on day −1) and methotrexate (on days 1, 3, 6, and 11).

Graft Content

The numbers of total nucleated, CD34, CD3, CD4, CD8, natural killer, and Treg cells in the donor graft were assessed before PBSC infusion. Tregs were phenotypically analyzed by flow cytometry using a panel of directly conjugated antibodies to CD3, CD4, CD25 (M-A251), and CD45RO. Fluorescein isothiocyanate, Phycoerytherin, peridinin-chlorophyll protein, and allophycocyanin (Beckman Coulter, Miami, FL and BD Biosciences, Franklin Lakes, NJ) were used as fluorophores. Intracellular analysis of FoxP3 (eBioscience, San Diego, CA) was performed after fixation and permeabilization according to the manufacturer's recommendations. Flow cytometry was performed with a FACSCalibur flow cytometer (BD Biosciences), and data were acquired and analyzed using Cellquest software (Becton Dickinson, San Jose, CA). NK lymphocytes, identified as CD3 and CD16 and/or CD56 cells, were analyzed using a multicolor flow cytometric panel with B, T, and NK cell markers (BD Multitest 6-color TBNK reagent, BD Biosciences). Absolute counts of viable total nucleated cells and CD34 cells were performed using FlowCount beads solution on a Cytomics FC500 flow cytometer (Beckman Coulter) according to the International Society of Hematotherapy and Graft Engineering protocol.

Table 2
Comparison between Patients with High and Low Graft CD3/Tregs Ratio

	HR Group (n = 30)	LR Group (n = 44)	
Median age, y	37	35	ns
Median graft CD3/Tregs ratio value	83	15	<.001
Grades II-IV aGVHD, n (%)			<.001
Yes	26 (87)	8 (18)	
No	4 (13)	36 (82)	
Sex mismatched, n (%)	17 (57)	25 (57)	ns
HCT-CI, n (%)			ns
<3	28 (93)	35 (79)	
≥3	3 (7)	8 (21)	
HLA MUD disparity, n (%)			
10/10	8 (27)	11 (25)	ns
9/10	3 (10)	4 (9)	
Donor, n (%)			
Sibling	19 (63)	29 (66)	ns
MUD	11 (37)	15 (34)	
rATG usage, n (%)			ns
Yes	11 (37)	15 (34)	
No	19 (63)	29 (66)	
GVHD prophylaxis, n (%)			ns
CsA + MTX	30 (100)	44 (100)	

HCT-CI indicates hematopoietic cell transplantation-specific comorbidity index; MUD, matched unrelated donor; rATG, rabbit antithymocyte globulin; CsA, cyclosporin; MTX, methotrexate.

Evaluation of gCD3/Tregs Ratio, Grades II to IV aGVHD, and Group Subdivision

The gCD3/Tregs ratio was calculated for all patients at the time of PBSC infusion. The gCD3/Tregs ratio value associated with the appearance of aGVHD grade II or greater was ≥36 [9]; therefore, the cohort was subdivided into two groups: an LR group (<36) and an HR group (≥36) (Table 2). aGVHD was diagnosed based on clinical symptoms and/or analysis of biopsy specimens from skin, oral mucosa, liver, and gut and classified according to the standard Seattle criteria [17]. All patients with aGVHD grade II or greater were treated with methylprednisolone at an initial dose of 1 to 2 mg/kg body weight and then adjusting the dose according to the clinical response. All patients with aGVHD lower than grade II were classified as aGVHD− (Table 2).

Outcomes

OS was defined as the time from transplantation to death for any cause. DFS was defined as the time from transplantation to relapse or death for any cause. NRM was defined as death not related to disease recurrence or progression. Relapse was defined as disease recurrence.

Statistical Analysis

Patient characteristics were compared using the chi-square or Fisher exact test (as appropriate) in the case of discrete variables and the *t*-test or Mann-Whitney test in the case of continuous variables. The endpoints of the study were the cumulative incidences of relapse and NRM, comparing the HR and LR groups. Cumulative incidence curves were used in a competing-risk setting, with relapse and death due to causes independent of relapse each treated as a competing event to calculate the probability of NRM and relapse, respectively. The groups were compared with Gray's k-sample test. OS was estimated by the Kaplan-Meier method. The OS differences between groups were calculated by log-rank test. Multivariate analyses of NRM, OS, DFS, and relapse were carried out with Cox regression models, treating NRM and disease relapse as competing events. Critical level of significance was set at .05.

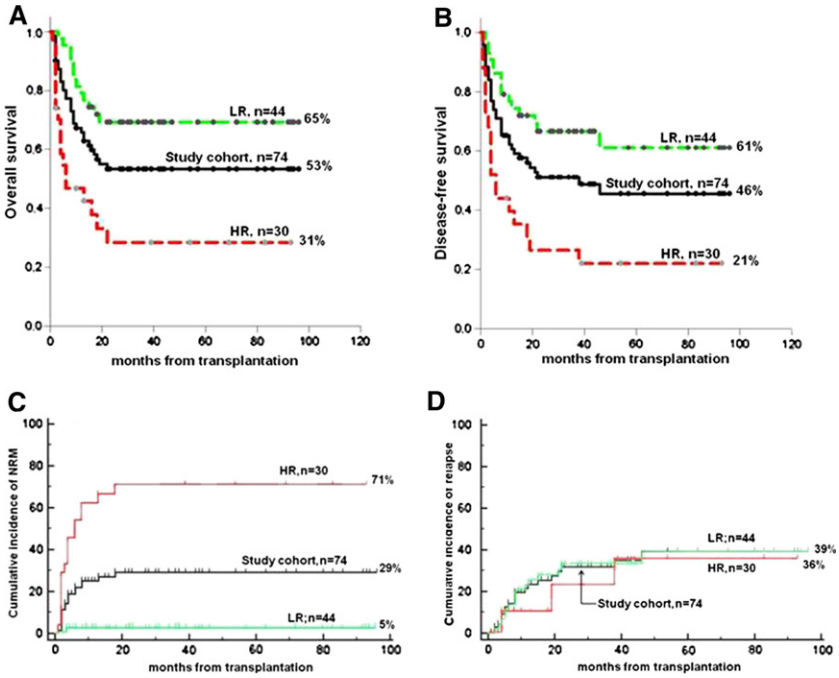

Figure 1. Study cohort and LR and HR group OS (A), DFS (B), NRM (C), and relapse rates (D). Three-year LR versus HR OS (65% vs 31%; $P = .0001$), DFS (67% vs 26%; $P = .0001$), NRM (3% vs 71%; $P < .0001$), and relapse (30% vs 25%; $P =$ ns).

RESULTS

LR Versus HR Group

The differences in terms of HLA disparity, sex mismatch, donor type, rabbit antithymocyte globulin (rATG) usage, grade II or greater aGVHD incidence, and hematopoietic cell transplantation-specific comorbidity index [18] between the HR (n = 30 patients) and LR groups (n = 40 patients) are summarized in Table 2.

When considering rATG usage, we did not find any correlation either with the HR or LR group (11 of 30 [37%] vs 15 of 44 [34%], $P =$ ns; Table 2) or with grades II to IV aGvHD incidence (11 of 34 [32%] vs 15 of 40 [37%]; $P =$ ns). There was no difference in terms of cGVHD incidence between the two groups (13 of 30 [43%] vs 16 of 44 [36%]; $P =$ ns).

OS, NRM, DFS, and Relapse Rates

Study cohort and LR and HR group univariate OS, DFS, NRM, and relapse rates are reported in Figure 1. OS, DFS, NRM, and relapse rates at 3 years for the study cohort were 53%, 51%, 29%, and 34%, respectively. OS, DFS, NRM, and relapse rates at 3 years for the LR group were 65%, 67%, 5%, and 30%, respectively, and for the HR group were 31%, 26%, 71%, and 25%, respectively.

Multivariate Analysis of Factors Affecting OS, NRM, DFS, and Relapse Rates

Variables included in multivariate analysis were gCD3/Tregs ratio (LR vs HR), type of disease (acute myelogenous leukemia vs no acute myelogenous leukemia), status at

Table 3
Cox Multivariate Analysis

	OS		DFS		Relapse		NRM	
	P Value	Hazard Ratio (95% CI)	*P* Value	Hazard Ratio (95% CI)	*P* Value	Hazard Ratio (95% CI)	*P* Value	Hazard Ratio (95% CI)
Ratio (LR vs HR)	**.019**	.319 (.122-.829)	**.003**	.231 (.087-.603)	.155	3.507 (.622-19.783)	**.05**	.300 (.120-1.120)
Disease (AML vs not AML)	.519	.769 (.360-1.675)	.846	.928 (.436-1.975)	.199	.474 (.151-1.481)	.750	.808 (.218-2.997)
Pretransplantation status (1° CR vs not 1° CR)	.563	.791 (.357-1.751)	.392	.712 (.327-1.550)	**.045**	.280 (.081-.971)	.074	.308 (.085-1.119)
Age, y (<55 vs ≥55)	.085	2.489 (.881-7.034)	.532	1.404 (.484-4.073)	.604	1.958 (.154-24.906)	**.006**	10.570 (1.950-57.295)
HCT-CI (<3 vs ≥3)	.848	1.129 (.326-3.907)	.238	2.147 (.604-7.638)	.270	3.816 (.353-41.201)	.169	.239 (.031-1.836)
CMV serostatus (high risk vs not high risk)	.625	1.330 (.424-4.169)	.529	1.433 (.468-4.391)	.119	.415 (.137-1.254)	.198	3.111 (.552-17.533)
HLA mismatched (yes vs no)	.244	.381 (.0751-1.934)	.817	.770 (.084-7.030)	.693	1.907 (.077-47.157)	**.009**	.021 (.001-.382)
Sex mismatched (RMDF vs other)	.139	.309 (.065-1.466)	.089	.163 (.020-1.316)	**.023**	4.358 (1.225-15.502)	.246	.330 (.501-2.150)
Donor (MUD vs sibling)	.734	1.109 (.436-3.244)	.865	.915 (.329-2.547)	**<.001**	.054 (.014-.213)	.690	1.339 (.319-5.626)
aGVHD grades II-IV (yes vs no)	.066	.356 (.118-1.072)	.722	.829 (.296-2.326)	.429	.585 (.155-2.207)	.996	.000 (.000-+inf)

AML indicates acute myeloid leukemia; CR, complete remission; HCT-CI, hematopoietic cell transplantation comorbidity index; CMV, cytomegalovirus; RMFD, recipient male, donor female; MUD, matched unrelated donor.
Significant values are shown in bold.

Table 4
Causes of Death

Cause	HR Group	LR Group
aGVHD grades II-IV	6 (30)	0
cGVHD	4 (20)	1 (7)
CMV infection	3 (15)	1 (7)
Multiple organ failure/sepsis	2 (10)	4 (29)
Primary disease	5 (25)	5 (36)
Graft failure	0	2 (14)
Toxicity	0	1 (7)
Total	20	14

CMV indicates cytomegalovirus.
Values are number of incidences with percentages in parentheses.

transplantation (one complete response vs no complete response), age (<55 and ≥55 years), cytomegalovirus (CMV) risk (high vs not high), HLA mismatched, gender mismatched, type of donor (sibling vs matched unrelated donor), and grades II to IV aGVHD. Data are summarized in Table 3.

Causes of NRM

Table 4 shows the causes of death for the LR and HR groups.

DISCUSSION

We already demonstrated a correlation between graft Tregs and immunological recovery [19] and then between the gCD3/Tregs ratio and aGVHD as well as immune reconstitution [9] in a cohort of patients undergoing allogeneic PBSC transplantation. We also defined a cut-off value of gCD3/Tregs ratio (≥36) associated with the appearance of aGVHD and CMV infection/disease, thus permitting us to distinguish between low and high gCD3/Tregs ratio allotransplantation patients at the time of the allograft reinfusion, in terms of aGVHD and CMV infection/disease risk during the post reinfusion time: LR predicts the absence of aGVHD and less risk of CMV infection [9]. However, although the risk factors for survival after allotransplantation are well defined [15,20], little is known about the possible contribution of gCD3/Tregs ratio to transplantation outcomes (ie, OS, NRM, DFS, and relapse rates) in the human setting of allotransplantation. We analyzed 74 allotransplantation patients: 65 from our earlier study [9] and 9 new ones.

In a large recent study including only patients affected by acute myeloid leukemia [21], the univariate 3-year estimated probabilities of survival were around 39%, 37%, and 34% for matched related donor, matched unrelated donor, and mismatched unrelated donor, respectively. Although our study included 82% of acute myeloid leukemia patients, the univariate 3-year probability of survival in the whole cohort was generally better (53%). This may be because in our study cohort, the hematopoietic cell transplantation-specific comorbidity index [18] was predominantly lower than 3 (85% of patients), which yields an OS rate at 2 years of 56% and 55% for a 0 and 1 to 2 hematopoietic cell transplantation-specific comorbidity index score, respectively [22]. Nevertheless, at univariate (Figure 1A, B) and multivariate analysis (Table 3), a low gCD3/Tregs ratio was the only factor correlated with better OS and DFS rates. On this theoretical basis, gCD3/Tregs ratio may be considered a useful tool for stratifying patients in terms of expected OS.

With regard to the 3-year NRM, our data (Figure 1C) are similar to those reported in larger studies [20,21]. Nevertheless, given there was no difference in terms of the factors having an impact on NRM [15,20-23] between the LR and HR groups (Table 2), the NRM disparity between the two groups may confirm that the gCD3/Tregs ratio is closely linked with the onset of aGVHD and related deaths (Table 4). In fact, it is well known that unless aGVHD patients benefit from steroid therapy, mortality is high, and standardized treatment algorithms are lacking [24-27]. Thus, our data confirm the clinical importance of improving the outcome of patients suffering from aGVHD, while maintaining the GVL effect to avoid a potential rise in relapse and relapse-related mortality. In fact, as already widely reported [16,28,29], we too observed a higher cumulative incidence of relapse in aGVHD patients (53%), although, surprisingly, analyzing the relapse incidence between the LR and HR groups in univariate (Figure 1D) and multivariate models (Table 3), we did not find any statistically significant difference.

Of note, gCD3/Tregs ratio does not show a similar impact on relapse and NRM. In fact, gCD3/Tregs ratio has no impact on relapse, while maintaining its contribution to NRM (Table 3). gCD3/Tregs ratio seems to be a better tool to predict patients' risk of NRM without affecting the antileukemic effects. In other words, LR allotransplantation patients have less risk of NRM and a similar risk of relapse, as compared with HR patients.

Actually, our data seem to confirm what was described in mice [2]: Tregs determine a suppressive effect on aGVHD while preserving the GVL action. The LR might mean that Tregs are able to act in suppressing or controlling aGVHD [30], which still remains the leading cause of death after allotransplantation. On the contrary, as already reported by Wolf et al. [31] regarding graft Tregs content, in our study cohort, the gCD3/Tregs ratio (regardless of LR and/or HR) did not penalize patients in terms of relapse (Figure 1D, Table 3), confirming a differential action by Tregs on controlling aGVHD and preventing relapse.

In conclusion, apart from an inhibition of aGVHD and related advantage in terms of OS due to a reduction in the NRM rate, the relapse rate does not seem to be affected by gCD3/Tregs ratio. By either Treg depletion or adoptive transfer of purified Treg cells, two studies [10,11] in mice had already demonstrated that the relative ratio of donor-type T reg cells and donor-type CD4+/CD25+ T cells (and not Tregs by themselves) would determine the outcome of experimental aGVHD in vitro. Thus, the best strategies targeting predominantly GVHD without affecting GVL effect might include a graft balance composition in favor of Tregs, although there is recent evidence [32] that high levels of graft Tregs might antagonize successful engraftment. Therefore, future clinical perspectives might be focused on both an optimal graft composition (by means of graft engineering) and a gCD3/Tregs ratio–based risk-stratified management (ie, differential aGVHD prophylaxis according to HR or LR status) after allotransplantation.

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