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The Impact of Graft CD3 Cell/Regulatory T Cell Ratio on Acute Graft-versus-Host Disease and Post-Transplantation Outcome: A Prospective Multicenter Study of Patients with Acute Leukemia Undergoing Allogeneic Peripheral Blood Stem Cell Transplantation

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Although it is well known that tumor site- or bone marrow-infiltrating regulatory T cells (Tregs) might be correlated with worse outcomes in solid tumors and acute leukemias by promoting immune surveillance escape, their contribution to the immediate post-allogeneic transplantation phase by peripheral blood (PB) allografts remains unclear. Moreover, the Treg content in stem cells harvested from PB has been suggested to be correlated with acute graft versus-host-disease (aGVHD) and immunologic recovery after allogeneic PB stem cell transplantation (allo-PBSCT). This study aimed to investigate the impact of the graft content of Tregs, as graft $CD3^+$/Tregs ratio (gCD3/TregsR), on acute GVHD and post-allo-PBSCT outcomes. We prospectively enrolled 94 consecutive patients at 9 Italian centers of the Gruppo Italiano Trapianto di Midollo Osseo (GITMO) with acute myelogenous (n = 71; 75%) or lymphoblastic (n = 23; 25%) leukemia in complete remission who underwent matched related donor (n = 35; 37%) or unrelated donor (n = 59; 63%) allo-PBSCT. The median graft $CD3^+$ cell, Treg, and gCD3/TregsR values were 196 × 10^6/kg body weight (range, 17 to 666 × 10^6/kg), 3 × 10^6/kg (range, 0.1 to 35 × 10^6/kg), and 71 (range, 1 to 1883), respectively. The discriminatory power of the gCD3/TregsR value to predict grade ≥II aGVHD was assessed by estimating the area under the receiver operating characteristic (ROC) curve (AUC). Any grade and grade ≥II aGVHD occurred in 24 (26%) and 17 (18%) allo-PBSCT recipients, respectively. By ROC analysis, AUC (0.74; 95% confidence interval [CI], 0.608 to 0.866; P = .002) identified 70 as the optimal gCD3/TregsR cutoff value predicting the appearance of grade ≥II aGVHD with 76% sensitivity and 71% specificity. Patients were subdivided into a high (ROC curve value ≥70) gCD3/TregsR group (HR; n = 48) and a low (ROC curve value <70) gCD3/TregsR group (LR; n = 46). The incidence of grade II-IV aGVHD was lower in the LR group compared with the HR group (9% [4 of 46] versus 27% [13 of 48]) in both univariate analysis (odds ratio [OR], 4.8; 95% CI, 1.44 to 16.17; P = .015) and multivariate analysis (OR, 5.0; 95% CI, 1.34 to 18.93; P = .017), whereas no differences were documented taking into account aGVHD of any grade. The overall survival, disease-free survival, nonrelapse mortality, and relapse

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rates at 2 and 3 years were 61% and 54%, 62% and 55%, 15% and 23%, and 27% and 30%, respectively. Of note, gCD3/TregsR did not significantly correlate with relapse ($P = .135$). Taken together, our data from this prospective multicenter study confirm the value of Tregs in preventing aGVHD while maintaining the graft-versus-leukemia effect.

INTRODUCTION

Regulatory T cells (Tregs) are the major regulators of immune responses in the periphery and maintain a state of self-tolerance free from autoimmune diseases [1]. Because of their inherent suppressive function [2-3], Tregs are being explored for their therapeutic potential in preventing autoimmunity [4] and improving survival of allografts [5]. In this regard, as already reviewed in the human setting [6], allogeneic hematopoietic stem cell transplantation (allo-HSCT) might serve as a model for studying how the allogeneic peripheral blood (PB) graft content of Tregs potentially impacts both immunologic reconstitution promoting tolerance against nonself (ie, protection from graft-versus-host disease [GVHD]) and, consequently, post-allo-HSCT survival outcomes.

In fact, given their impact on T cell immunity [7], Tregs are able to modulate GVHD while preserving the graft-versus-leukemia (GVL) effect in mouse models [8,9]. In particular, acute GVHD (aGVHD) is triggered by alloreactive mature donor CD3 T cells [10,11] and antagonized by Tregs [12,13]. As a result, different murine experimental models [14,15] have shown significant inhibition of rapidly lethal GVHD after infusion of grafts with an enriched Treg content. Moreover, T cell depletion of the graft is associated with prolonged immunosuppression [16], major risk of graft failure [17], and a higher rate of leukemia relapse [18]. Conversely, unmanipulated allografts, although causing aGVHD, are often associated with the eradication of residual disease and, consequently, a low frequency of relapse due to the GVL effect [18]. Despite this evidence, however, the impact of graft Tregs, as in the ratio of $CD3^+$ cells to Tregs (gCD3/TregsR), on aGVHD incidence and post-allo-HSCT outcomes (ie, overall survival [OS], nonrelapse mortality [NRM], disease-free survival [DFS], and relapse) remains incompletely understood.

In this study, we expanded our previous single-center evaluation of the contribution of the gCD3/TregsR on both aGVHD [19] and survival [20] with the aim of confirming our published conclusions in a prospective multicenter study of patients with acute leukemia in complete remission (CR) undergoing allogeneic PB stem cell transplantation (PBSCT), with a myeloablative conditioning regimen.

METHODS

Patients

From 9 Italian centers of the Gruppo Italiano Trapianto Midollo Osseo (GITMO), we prospectively enrolled patients (age 18 to 65 years) with acute myelogenous leukemia or acute lymphoblastic leukemia in CR who underwent matched related donor or matched unrelated donor (MUD) PBSCT. We calculated the Treg ($CD4^+/CD45RA^-/CD127^{low}/CD25^{high}$) content in the PB harvest together with the CD3, CD4, CD8, and NK populations. All patients provided written informed consent for the collection of personal data in accordance with the Declaration of Helsinki and Italian law. The study enrollment period extended from May 2015 to December 2018, with 18 months of follow-up from the last enrolled patient.

DNA-based HLA typing of donor and recipient was done using high-resolution (4 digits) for HLA-A, -B, -C, -DRB1, and -DQB1. All patients received a myeloablative conditioning regimen according to the current guidelines [21], with busulfan (Bu) + cyclophosphamide (Cy), Bu + fludarabine (Flu), thiotepa + Bu + Flu, or total body irradiation + Cy. Antithymocyte globulin (ATG) use and dosage and GVHD prophylaxis varied according to the local center policy.

Graft Content Evaluation by Flow Cytometry

The numbers of total nucleated, CD3, CD4, CD8, natural killer (NK), Treg, and CD34 cells in the donor grafts were assessed at each center (samples were not centralized) before PBSCT.

CD3, CD4, CD8, and NK Cells

To determine the percentages and absolute counts of CD3 and CD4 T cell subsets, 50 μL of the whole PB stem cell harvest were stained with CD45 PerCP-Cy5.5, CD3 FITC, CD4 PE-Cy7, CD8 APC-Cy7, CD16 PE, and CD56 PE in a calibrated number of fluorescent beads (Trucount; BD Biosciences, San Diego, CA). The absolute number of positive cells (cells/μL) was calculated by comparing cellular events to bead events using BD FACSCanto clinical software version 3 (BD Biosciences).

Tregs

There is no generally accepted method for defining $CD4^+CD25^{high}$ Tregs using flow cytometry, and FoxP3 is considered one of the most specific markers of Tregs [22]. Nevertheless, Tregs consistently express lower levels of IL-7R (CD127) than the majority of other $CD4^+$ T cells, and CD127 expression is inversely correlated with FoxP3 levels in Tregs [23-24]. Thus, CD127 might be a suitable alternative to FoxP3 in identifying Tregs. As a result, our Treg study population has been defined as $CD4^+/CD45RA^-/CD25^{high}/CD127^{low}$. Consequently, for Treg identification, 100 μL of the whole PBSC harvest were incubated with a lyophilized pellet of CD45RA FITC, CD25 PE, CD127 PerCP-Cy5.5, HLA-DR PE-CY7, CD39 APC, or CD4 APC-H7 monoclonal antibody (BD Biosciences). Samples were processed according to the manufacturer's guidelines, and data were acquired on a FACS Canto II flow cytometer (BD Biosciences). The absolute number (cells/μL) of positive cells was calculated as described above.

CD34

Absolute counts of viable total nucleated cells and CD34 cells were performed using FlowCount bead solution on a Cytomics FC500 flow cytometer (Beckman Coulter, Brea, CA) according to the International Society of Hematotherapy and Graft Engineering protocol.

aGVHD and gCD3/TregsR

aGVHD was graded using standard criteria [25]. Receiver operating characteristic (ROC) curve analysis was performed to define the gCD3/TregsR value associated with the appearance of aGVHD $\geq$II. The cohort was also divided into two groups based on gCD3/TregsR value: low gCD3/TregR (LR) and high gCD3/TregsR (HR) group. 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 at adjusted doses according to the clinical response.

Statistical Methods

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. OS was defined as the time from transplant to death from any cause, and surviving patients were censored at last follow-up. DFS from transplantation was calculated using death and disease progression and/or relapse as events. NRM was defined as death from any cause other than disease progression or relapse. The discriminatory power of the gCD3/TregsR value to predict grade $\geq$II aGVHD was assessed by estimating the area under the ROC curve (AUC) using the trapezoidal method. The optimal cutoff was determined by maximizing both sensitivity and specificity, computed at the optimal cutoff, as reported along with 95% confidence interval (CI).

Cumulative incidence curves were used in a competing-risk setting, with relapse, death due to causes independent of disease, and death due to causes independent of disease and/or aGVHD each treated as a competing event to calculate the probability of NRM, relapse, and aGVHD, respectively. The groups were compared using Gray's *k*-sample test. OS was estimated by the Kaplan-Meier method. The differences in OS and DFS between groups were calculated using the log-rank test. Multivariate analyses of variables with an impact on aGVHD and on OS, DFS, and NRM were carried out with binary logistic and Cox regression models, respectively. The critical level of significance was set at .05.

RESULTS

Pretransplantation Patient Characteristics

Patient characteristics are summarized in Table 1. All patients received a PBSC graft from an HLA-identical sibling donor (n = 35; 37%) or an unrelated HLA-identical donor (n = 59; 63%). DNA-based HLA typing of donor and recipient was done using high- resolution (4 digits) for HLA-A, -B, -C, -DRB1, and -DQB1; in unrelated transplantations (n = 59; 63%), an antigenic (9 out of 10) mismatch was documented in 8 (16%) donor-patient pairs and an allelic mismatch was documented in 4 (7%) donor-patient pairs. Sixty-five percent of the patients underwent transplantation in first CR (CR1), and 35% did so in second or greater CR (CR≥2). Myeloablative conditioning was BuCy-based in 15 patients (16%), BuFlu-based in 31 (33%), thiotepa + BuFlu-based in 31 (33%), and total body irradiation-based in 10 (10%). The donor/recipient cytomegalovirus (CMV) serostatus was high-risk in 54 (57%) pairs, and donor/recipient sex match was female/male in 11 (12%) pairs. The median graft $CD3^+$, Treg, and CD3/TregsR values were 196 × 10^6/kg of body weight (range, 17 to 666 × 10^6/kg), 3 × 10^6/kg (range, 0.1 to 35 × 10^6/kg), and 71 (range, 1 to 1883), respectively. The GVHD prophylaxis strategy was mostly ATG-based (80%), and Cy + methotrexate was used in 95% of the study population.

Table 1
Patient Characteristics

	Numbers (n) of patients
Number of patients	94
Age, yr, median (range)	49 (18-68)
Sex, male/female, n (%)	41 (44)/53 (56)
Karnofsky Performance Status <80, n (%)	11 (12)
Time from diagnosis to allo-PBSCT, d, median (range)	226 (86-8134)
Disease status at allo-PBSCT, n (%)	
CR1	61 (65)
CR2	21 (22)
CR>2	12 (13)
Disease, n (%)	
AML	71 (75)
ALL	23 (25)
Myeloablative conditioning regimen, n (%)	
BuCy	15 (16)
BuFlu	31 (33)
TBF	31 (33)
TBI-based	10 (10)
Other	7 (8)
CMV risk, n (%)	
Low	27 (29)
High	54 (57)
Very high	13 (14)
Sex match, n (%)	
Female donor/male recipient	11 (12)
Other combinations	83 (88)
Donor type, n (%)	
MRD	35 (37)
MUD	59 (63)
GVHD prophylaxis strategy, n (%)	
ATG-based	75 (80)
Not ATG-based	19 (20)
Associated immunosuppressive agents, n (%)	
Cy alone	0 (0)
Cy + methotrexate	89 (95)
Cy + mycophenolate mofetil	5 (5)
HLA disparity: antigenic mismatch, n (%)	
10/10	85 (90)
Not 10/10	9 (10)
gCD3/TregsR, median (range)	71 (1-1883)

AML indicates acute myelogenous leukemia; ALL, acute lymphoblastic leukemia; TBI, total body irradiation; TBF, thiotepa + busulfan + fludarabine; MRD, matched related donor.

ROC Analysis

By ROC analysis, the AUC (0.74; 95% CI, 0.608 to 0.866; P = .002) identified 70 as the optimal gCD3/TregsR cutoff value predicting the appearance of grade ≥II-IV aGVHD, with 76% sensitivity and 71% specificity. The same value has been confirmed in the whole study population (n = 94) and after excluding patients who did not receive ATG (n = 75).

No optimal cutoff value predicting the appearance of aGVHD grade II-IV was obtained for absolute counts of either CD3 cells (AUC, 0.61; 95% CI, 0.480 to 0.741; P = .154) or Tregs (AUC, 0.32; 95% CI, 0.191 to 0.453; P = .07). No cases of grade II-IV aGVHD were reported for gCD3/TregsR ≤30.

LR Group versus HR Group

As specified above, patients were subdivided according to the gCD3/TregsR associated with the appearance of grade ≥II aGVHD. Therefore, the cohort was subdivided into 2 groups: LR (gCD3/TregsR <70; n = 46 patients, 49%) and HR (gCD3/TregsR ≥70; n = 48 patients, 51%).

The differences in terms of age (P = not significant [ns]), time from diagnosis to transplantation (P = ns), HLA disparity (P = ns), sex mismatch (P = ns), donor type (P = ns), ATG use (P = ns), CMV risk (P = ns), disease status at allo-PBSCT (P = ns), type of myeloablative regimen (P = ns), type of donor (P = .022), and incidence of grade ≥II aGVHD (P = .015) between the HR and LR groups are summarized in Table 2.

Cumulative Incidence of aGVHD at 100 Days

The overall grading and the target organ staging of the aGVHD case series are reported in Table 3. The cumulative incidence of any grade aGVHD (24 events) and grade ≥II aGVHD (17 events) was 26% and 18%, respectively (Figure 1A).

The cumulative incidence of aGVHD of any grade was 25% for the LR group and 26% for the HR group (P = ns) (Figure 1B). When considering grade ≥II aGVHD events, a statistically significant difference was found between the LR and HR groups (9% versus 25%; P = .028) (Figure 1C). This statistically significant difference between the LR and HR groups remains unaffected when eliminating the patients not receiving ATG (6% versus 26%; P = .014) (Supplementary Figure S1).

The cumulative incidence of grade III-IV aGVHD (8 events) was 8% for the entire cohort. No difference in cumulative incidence was documented between the LR and HR groups (9% versus 8%; P = ns) (Supplementary Figure S2A), whereas a statistically significant difference was seen between the HLA-matched and HLA-mismatched pairs (5% versus 31%; P = .001) (Supplementary Figure S2B).

Univariate and Multivariate Analyses of Factors Affecting aGVHD

HLA mismatch (antigenic and/or allelic) and HR group were correlated with the incidence of grade ≥II aGVHD in both univariate analysis (HLA mismatch: odds ratio [OR], 5.5; 95% CI, 1.54 to 19.27; P = .012; HR group: OR, 4.8; 95% CI, 1.44 to 16.17; P = .015) and multivariate analysis (HLA mismatch: OR, 7.4; 95% CI, 1.8% to 30.12%; P = .005; HR group: OR, 5; 95% CI,

Table 2
Characteristics of the LR and HR Groups

Characteristic	LR (gCD3/TregsR <70) (N = 46)	HR (gCD3/TregsR ≥70) (N = 48)	*P*
aGVHD (grade ≥II), n (%)	4 (9)	13 (27)	.015*
gCD3/TregsR, median, range	33 (1-70)	140 (71-1883)	<.002†
Age, yr, median, range	50 (22-68)	47 (18-65)	.38†
Time from diagnosis to allo-PBSCT, d, median (range)	199 (86-8134)	249 (128-4483)	.12†
Female donor/male recipient, n (%)	6 (13)	5 (10)	.94*
MUD, n (%)	23 (50)	36 (75)	.022*
HLA disparity, n (%)			
Antigenic, not 10/10	4 (9)	5 (10)	.99‡
Allelic mismatch	3 (6)	1 (2)	.36‡
Antigenic and/or allelic mismatch	7 (15)	6 (12)	.93*
CR1 at allo-PBSCT, n (%)	32 (69)	29 (60)	.47*
CMV risk, n (%)			.93*
Low	14 (31)	13 (27)	
High	26 (56)	28 (58)	
Very high	6 (13)	7 (15)	
ATG-based GVHD prophylaxis, n (%)	39 (85)	36 (75)	.36*
Associated immunosuppressive agents: Cy + methotrexate, n (%)	44 (96)	45 (94)	.99‡
Myeloablative conditioning regimen, n (%)			.23*
BuCy	6 (13)	9 (19)	
BuFlu	18 (39)	13 (27)	
TBF	17 (37)	14 (30)	
TBI-based	4 (9)	6 (12)	
Other	1 (2)	6 (12)	

* Chi-square test.
† Mann-Whitney *U* test.
‡ Fisher's exact test.

Table 3
Overall Grading and Target Organ Staging of aGVHD Case Series-Study

Case	aGVHD Grade	Stage		
		Skin	Gastrointestinal	Liver
1	I	1	0	0
2	I	1	0	0
3	I	2	0	0
4	I	2	0	0
5	I	2	0	0
6	I	1	0	0
7	I	1	0	0
8	II	3	0	0
9	II	3	0	0
10	II	3	0	0
11	II	3	0	0
12	II	3	0	0
13	II	2	1, upper tract	0
14	II	3	0	0
15	II	2	1, upper tract	0
16	II	3	0	0
17	III	1	3, lower tract	0
18	III	2	1, upper tract	3
19	III	2	2, upper tract	0
20	III	2	0	2
21	III	0	3, lower tract	0
22	III	3	2, lower tract	0
23	IV	4	0	0
24	IV	2	0	4

1.34 to 18.93; *P* = .017). No correlation was reported for the other factors: CR1, low CMV risk, female donor/male recipient, ATG use, type of donor, and recipient age (data summarized in Table 4).

HLA mismatch (antigenic and/or allelic) was the sole variable correlated with grade III-IV aGVHD. HLA mismatch was reported in 4 of 8 (50%) grade III-IV aGVHD events versus 9 of 86 (10%) no grade III-IV aGVHD events (OR, 8.56; 95% CI, 1.82 to 40.24; *P* = .012).

Chronic GVHD (cGVHD)

Grading of chronic GVHD (cGVHD) was mild in 1, moderate in 10, and severe in 6 of the 17 reported episodes. The cumulative incidence of cGVHD was 39% for the whole cohort, 34% for the LR group, and 33% for the HR group (*P* = not significant) (Supplementary Figure S3).

Clinical Outcomes

Response to Steroid Therapy

According to local policy, all 17 patients with grade ≥II-IV aGVHD were treated with ≥1 mg of methylprednisolone (9 with 1 mg/kg and 8 with 2 mg/kg). gCD3/TregsR did not correlate with response rate; response was documented for 4 of 13 treated patients in the HR group and in 1 of 4 treated patients in the LR group (31% versus 25%; *P* = ns). In addition, no correlation with cGVHD was demonstrated.

OS, DFS, NRM, and Relapse According to gCD3/TregsR Group

Overall OS was 61% at 2 years and 54% at 3 years (Figure 2A). OS was not significantly different between the HR

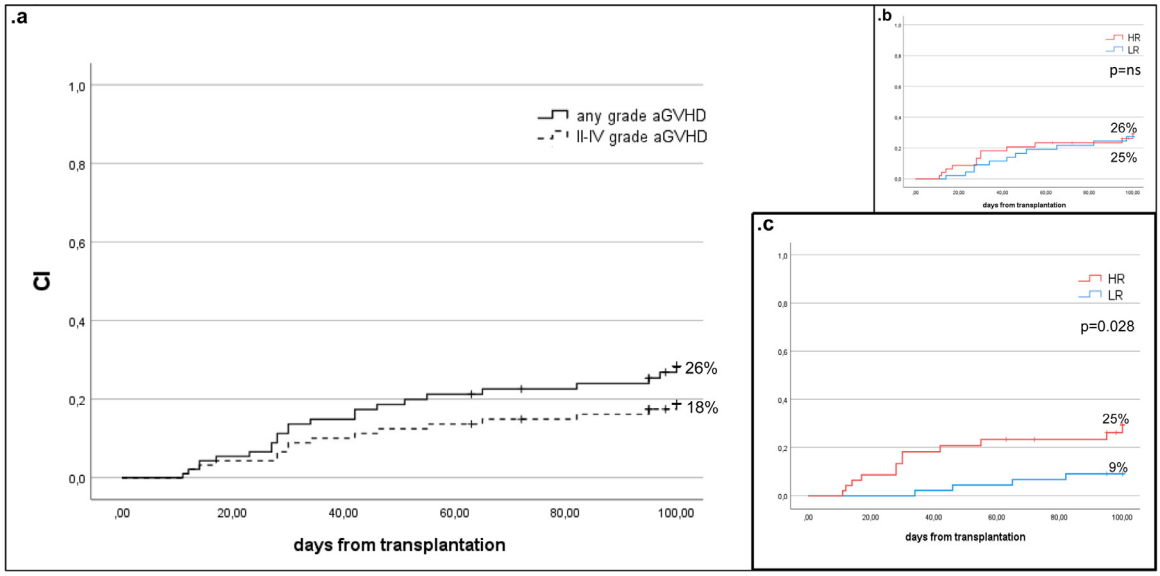

Figure 1. Cumulative incidence (CI) of aGVHD in the whole study group (A) and in the high gCD3/Tregs ratio (HR) and low gCD3/Tregs ratio (LR) groups for any grade aGVHD (B) and grade II-IV aGVHD (C).

Table 4
Variables with Impact on Grade II-IV aGVHD

Variable	With Grade II-IV aGVHD (N = 17)	Without Grade II-IV aGVHD (N = 77)	Univariate Analysis*			Multivariate Analysis†		
			Unadjusted OR	95% CI	*P*	Adjusted OR	95% CI	*P*
Female donor/male recipient	0 (0)	11 (14)	0	0-0	.20			
ATG use	12 (71)	63 (82)	0.5	0.16-1.76	.32			
gCD3/TregsR ≥70	**13 (76)**	**35 (45)**	**4.8**	**1.44-16.17**	**.015**	**5.0**	**1.34-18.93**	**.017**
Recipient age ≥50 yr	12 (71)	52 (67)	1.1	0.37-3.63	.97			
Low CMV risk	8 (47)	19 (25)	2.7	0.92-8.02	.08			
CR1	11 (65)	50 (65)	1	0.33-2.97	.79			
HLA mismatch‡	**6 (35)**	**7 (9)**	**5.5**	**1.54-19.27**	**.012**	**7.4**	**1.80-30.12**	**.005**
MUD	11 (65)	48 (62)	1.2	0.37-3.31	.92			
AML	12 (71)	59 (77)	0.7	0.23-2.36	.76			

Significant values are in bold type.
* Chi-square or Fisher exact test as appropriate.
† Binary logistic regression.
‡ Antigenic and/or allelic.

and LR groups (*P* = .35) (Figure 2B). DFS was 62% at 2 years and 55% at 3 years (Figure 2C) and was not significantly different between the HR and LR groups (*P* = .17) (Figure 2D). The cumulative incidence of NRM was 15% at 2 years and 23% at 3 years (Figure 3A) and was not significantly different between the HR and LR groups (*P* = .77) (Figure 3B). The cumulative incidence of relapse was 27% at 2 years and 30% at 3 years (Figure 3C) and was not significantly different between the 2 groups (*P* = .135) (Figure 3D).

OS, DFS, and NRM Risk Factors

The results of univariate and multivariate analyses of risk factors (ATG use, disease status at transplantation, type of donor, HLA mismatch, recipient age, sex match, CMV risk, disease, gCD3/TregsR) associated with OS, DFS, and NRM are presented in Table 5. Recipient age was confirmed as a factor impacting OS (HR, 0.27; 95% CI, 0.12 to 0.67; *P* = .004), DFS (HR, 0.50; 95% CI, 0.24 to 0.99; *P* = .05), and NRM (HR, 0.17; 95% CI, 0.04 to 0.65; *P* = .01).

DISCUSSION

It is widely accepted that Tregs in murine models of allo-HSCT may promote immune reconstitution [7] and prevent GVHD, while maintaining a graft-versus-tumor (GVT) response [8,9]. In particular, early recognition of disparate host antigens by donor T cells is critical for GVHD pathogenesis [26]. Consequently, it is crucial to study Tregs when they act proximal to the transplantation, as we did in allogeneic grafts at time of infusion. Moreover, Tregs actively traffic to the inflammation site and suppress activation, an action that is mainly cell-to-cell dependent [27]. Therefore, Tregs actively search for inflamed tissue and suppress only when inflammation occurs, and their activity is limited to the inflammation site. As a result, in the context of myeloablative conditioning, which is characterized by the most inflamed postconditioning phase, Tregs are expected to be useful against inflammation and consequently protective against aGVHD [28,29]. In our study cohort, homogenously composed of patients undergoing myeloablative conditioning and receiving PBSC grafts, the cumulative incidence of aGVHD grade ≥II was statistically correlated with HLA mismatch (Table 4) and HR group (Figure 1C, Table 4).

Moreover, it has been demonstrated that decreases in both PB Tregs [30-33] and Tregs infiltrating the sites of GVHD (eg, skin, intestinal mucosa) [33,34] were correlated with the onset

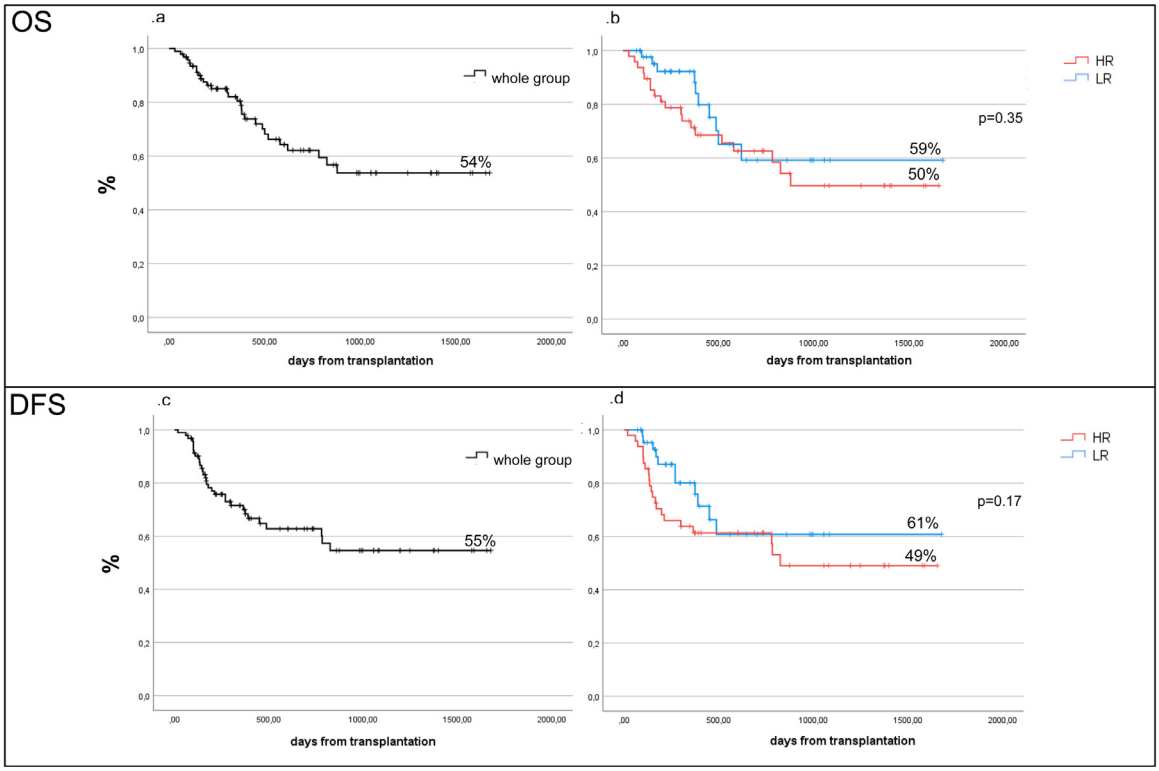

Figure 2. OS and DFS in the whole cohort (A and C) and the HR and LR groups (B and D).

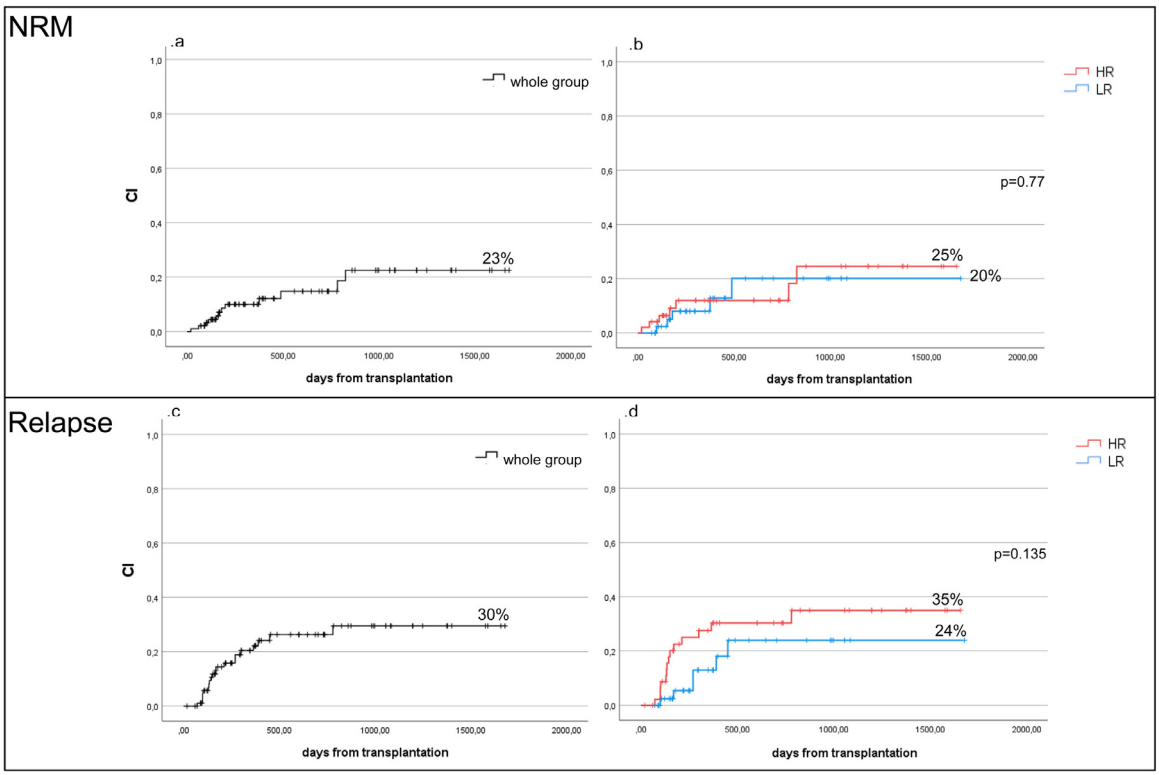

Figure 3. Cumulative incidence (CI) of NRM and relapse in the whole cohort (A and C) and the HR and LR groups (B and D).

Table 5
Univariate and Multivariate Analyses of Risk Factors for OS, DFS, and NRM

Risk Factor	OS					DFS					NRM				
	Univariate Analysis			Multivariate Analysis*		Univariate Analysis			Multivariate Analysis*		Univariate Analysis			Multivariate Analysis*	
	P	HR	(95% CI)	*P*	HR	(95% CI)	*P*	HR	(95% CI)	*P*	HR	(95% CI)	*P*	HR	(95% CI)
ATG usage (no vs yes)	**.015**	**2.67**	**(1.21-5.88)**	.11	2.27	(0.84-6.10)	**.019**	**2.52**	**(1.16-5.49)**	**.022**	**2.48**	**(1.14-5.39)**	.31	1.97	(0.53-7.37)			
CR (CR1 vs not CR1)	.15	0.56	(0.25-1.24)	**.03**	**0.39**	**(0.17-0.90)**	.32	0.68	(0.31-1.45)				.14	0.45	(0.14-1.41)	**.034**	**0.27**	**(0.08-0.90)**
Donor type (MRD vs MUD)	.28	0.65	(0.30-1.41)				.42	0.74	(0.35-1.53)				.51	0.67	(0.20-2.23)			
HLA mismatch (yes vs no)†	.48	1.38	(0.56-3.38)				.78	1.13	(0.47-2.76)				.40	1.74	(0.47-6.46)			
Recipient age (<50 yr vs ≥50 yr)	**.013**	**0.38**	**(0.18-0.81)**	**.004**	**0.27**	**(0.12-0.67)**	**.049**	**0.49**	**(0.24-0.99)**	**.05**	**0.50**	**(0.24-0.99)**	**.048**	**0.29**	**(0.09-0.99)**	**.010**	**0.17**	**(0.04-0.65)**
Donor/recipient sex match (M/M, F/F, M/F, vs F/M)	.96	1.03	(0.31-3.46)				.7	1.26	(0.38-4.20)				.68	1.53	(0.19-11.99)			
CMV risk (low vs high)	.57	0.78	(0.33-1.83)				.36	0.68	(0.27-1.55)				.47	0.61	(0.16-2.32)			
Type of disease (ALL vs AML)	.69	0.84	(0.36-1.97)				.44	1.34	(0.63-2.83)				.99	0.99	(0.26-3.66)			
gCD3/TregsR (LR vs HR)	.30	0.67	(0.31-1.45)				.15	0.60	(0.29-1.26)				.77	0.84	(0.26-2.67)			

Significant values are in bold type.
* Stepwise regression model.
† Antigenic and/or allelic.

and severity of aGVHD. Consequently, in our study cohort, the contribution of Tregs to protection from graft-related inflammation was not observed when considering aGVHD of all grades (Figure 1B), despite confirmation in grade ≥II cases (Figure 1C).

One-half of all grade III-IV aGVHD cases are HLA-mismatched, thus justifying the lack of association with gCD3/TregsR (Supplementary Figure S2A). In fact, although Tregs are crucial for recognition of host antigens as self, imposing anergy in fully HLA-matched donor-recipient pairs [35,36], and might be theoretically advantageous when some mismatches are present [37], the power of HLA mismatch in determining aGVHD remains to be proven (Table 4, Supplementary Figure S2B). Moreover, the lack of association between HLA disparity and grade ≥II aGVHD was demonstrated in the haploidentical setting [38], whereas HLA mismatch remains a risk factor for aGVHD in the myeloablative conditioning setting [39]. In addition, as expected, male recipients with female donors had no increased risk of aGVHD. In fact, this correlation has not been observed in the myeloablative conditioning setting, although it appears to be present in reduced-intensity conditioning allo-HSCT [40].

How the addition of in vivo T cell depletion (ie, ATG use) has led to a reduced incidence of cGVHD has been clearly established, particularly in patients receiving PBSCs as the stem cell source in both sibling donor [41] and MUD [42] grafts. Consequently, no impact on aGVHD due to ATG use was expected in our study cohort (Table 4). Of note, although there is in vitro evidence that the addition of ATG also induces the generation of Tregs [43], the correlation between Tregs and the incidence of aGVHD remained unaffected when excluding patients not receiving ATG (Supplementary Figure S1), thus confirming how graft Tregs might finally attenuate the risk of aGVHD, irrespective of ATG-based lymphodepletion or Treg promotion.

Tregs might be expected to increase the risk of disease recurrence by decreasing the GVT effect. Conversely, they preserve it while inhibiting GVHD, as demonstrated in murine models [8,9]. Of interest, in our human setting, Tregs did not seem to favor relapse (Figure 3D), thus confirming what has been observed and reviewed previously [6].

Given that aGVHD remains the leading cause of mortality in allo-HSCT [39], the correlation between gCD3/TregsR and aGVHD and the lack of correlation between Tregs and NRM demonstrated in our study (Figure 3B, Table 5) might appear contradictory. However, lower gastrointestinal tract aGVHD is the major driver of NRM [44], and in our cohort, lower gastrointestinal grade ≥II aGVHD occurred in only 3 of 17 cases (18%) (Table 3). Moreover, our study population was free of many factors known to impact NRM; all the enrolled patients were in CR and most had a good Karnofsky Performance Status (88%), and it is known that the type of donor (MUD; 59% of our study cohort) does not clearly impact NRM in myeloablative conditioning [45]. Furthermore, it has been reported that CMV risk preferentially correlates with survival outcome in T cell-depleted allo-HSCT [46]. Accordingly, patient age was the sole factor impacting OS, DFS, and NRM (Table 5).

Our study has several limitations of note. First were the heterogeneity of myeloablative conditioning and lack of longitudinal follow-up of Tregs, which could have supplied original data on their PB pharmacokinetics. In particular, the lack of association between the LR group and cGVHD (Supplementary Figure S3) might reflect these limitations, although the supposed protection from cGVHD by Tregs has been called into question by some reports [47,48]. Furthermore, T cell depletion with ATG has an inevitable impact on gCD3/TregsR that is difficult to decipher. Finally, the Treg phenotyping was neither centralized nor FoxP3-based.

Despite these complicating factors, the LR group seems to be protective against grade ≥II aGVHD without any impact on relapse and NRM. In the post-transplantation phase, these simultaneous effects might be further enhanced with additional drugs, such as rapamycin [49] and/or azacitidine [50,51], which are believed to induce Tregs, especially in those patients for whom a very low gCD3/TregsR might theoretically exclude severe aGVHD episodes. Moreover, in future clinical trials, it remains to be determined at what time after transplantation these agents might be used without penalizing the immune reconstitution that, conversely, Tregs are known to promote.

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SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.jtct.2021.08.008.

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