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Recovery of CMV-Specific CD8$^+$ T Cells and Tregs after Allogeneic Peripheral Blood Stem Cell Transplantation

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Recovery of cytomegalovirus (CMV)-specific CD8$^+$ T cells after allogeneic stem cell transplantation (SCT) is critical for protection against CMV infection and disease. Moreover, Foxp3$^+$CD4$^+$CD25^{high} regulatory T cells (Tregs) are a major regulator of adaptive immunity, preventing graft-versus-host disease (GVHD) and so promoting timely and complete immune recovery. The aim of our study was to evaluate the recovery of circulating tetramer-based CMV-specific CD8$^+$ T cells and T regs in 46 patients after allogeneic peripheral blood SCT (PBSCT). CMV infection and/or disease was observed in 7% and 94% of patients with or without CMV-specific CD8$^+$ T cells recovery ($P < .001$), and in 77% and 4% of patients with or without acute GVHD (aGVHD) ($P < .001$), respectively. T regs values were higher in patients without than with CMV infection and/or disease at 2 ($P < .001$) and 3 months ($P < .001$) after allogeneic PBSCT, respectively. Moreover, we observed a positive correlation between T regs and the recovery of CMV-specific CD8$^+$ T cells at 2 ($r = .61$, $P < .0001$) and 3 ($r = .72$, $P < .00001$) months, respectively. Tregs were higher in patients without than with aGVHD at 1, 2 ($P < .001$) and 3 months ($P < .0001$), respectively. At multivariate logistic regression, aGVHD (odds ratio [OR]: 2.60, 95% confidence interval [CI] [1.3-5.0], $P = .0006$) and CMV-specific CD8$^+$ T cells recovery (OR:2.25, 95% CI [1.2-4.8], $P = .05$) were correlated with CMV infection and/or disease, whereas no correlation was found for Tregs, absolute neutrophil count, patients' and donors' age, disease status pretransplantation, type of disease, and CMV serology. Taken together, our data may suggest the existence of a correlation between Tregs and the recovery of CMV-specific CD8$^+$ T cells; Tregs may preserve an optimal microenvironment for the reconstitution of functional immunity and mediate protective effects against aGVHD.

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KEY WORDS: Tregs, CMV-specific CD8$^+$ T cells, Allogeneic SCT

INTRODUCTION

Despite the recent development of potent antiviral medication, reactivation of cytomegalovirus (CMV) is one of the major complications in patients undergoing allogeneic stem cell transplantation (SCT), and is significantly related to morbidity and mortality rates [1-3]. The decision to administer antiviral therapy is currently based on the clinical risk [4,5] and the detection of CMV by antigenemia assay or polymerase chain reaction (PCR) for CMV-DNA [6,7]. However, prolonged treatment with antiviral drugs, such as gancyclovir and foscarnet, increases the risk of other infectious complications because of myelotoxicity [8], and impairs the reconstitution of CMV-specific CD8$^+$ T cell responses, thereby increasing the risk for late CMV disease [9]. Recovery of CMV-specific CD8$^+$ T cells plays an important role in the control of CMV reactivation [10,11], and monitoring their number [12-14] may be crucial to optimize the antiviral therapy with minimal drug exposure.

Moreover, Foxp3$^+$CD4$^+$CD25^{high} regulatory T cells (Tregs) are major regulators of adaptive immunity [15] and, given the pathophysiologic link between graft-versus-host disease (GVHD) and immune reconstitution [16,17], protection against GVHD by Tregs could potentially promote timely and complete immune recovery [18]. Depletion of Tregs enhances microbial and tumor immunity [19], whereas their adoptive transfer protects animals from autoimmune

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disease [15], induces tolerance following organ transplantation and prevents early GVHD in SCT models [20-22]. GVHD damages the stroma of the thymus and secondary lymphoid organs, thereby decreasing thymic output, impairing the peripheral expansion of T cells, and consequently leading to a prolonged immunodeficiency state [16,17]. However, the impact of Tregs on host immunity after allogeneic SCT is still poorly understood. In this study, we investigated the recovery both of Tregs and of tetramer-based CMV-specific $CD8^+$ T cells after allogeneic peripheral blood SCT (PBSCT).

PATIENTS, MATERIALS, AND METHODS

Forty-six patients who underwent allogeneic PBSCT between February 2005 and September 2008 were included in this study (Table 1); written informed consent was obtained from all patients. Patients' characteristics are shown in Table 1. As conditioning regimen, all patients received a conventional myeloablative regimen that included 16 mg/kg of oral busulfan and 120 mg/kg of cyclophosphamide. All patients were administered prophylactic antibiotics (levofloxacin 500 mg daily) until neutrophil engraftment; we used fluconazole (400 mg daily to day 100) as antifungal prophylaxis and trimethoprim-sulfamethoxazole for *Pneumocystis carinii* prophylaxis.

All patients received GVHD prophylaxis, using cyclosporine A (starting on day −1) and methotrexate (on days +1, +3, +6, +11). Heparinized blood samples were taken at 1, 2, 3, and 6 months after transplantation for the cytometric Tregs, CMV-specific $CD8^+$ T cells, and absolute neutrophil counts. Tregs evaluation was also performed in those patients with acute GVHD (aGVHD), at its precise onset, before starting any treatment for aGHVD, during the period from 0 to 3 months after allogeneic PBSCT. The HLA genotype was HLA-A01 in 6 patients, HLA-A02 in 23 patients, HLA-B01 in 1 patient, HLA-B02 in 2 patients, and HLA-B35 in 14 patients. Tregs evaluation was also performed in 24 of 46 (52%) donor grafts.

Table 1. Patient Characteristics

	N	%
Patients	46	
Sex (male/female)	25/21	54/46
Median age: years (range)	36 (13-61)	
Diagnosis		
Acute myelogenous leukemia	39	85
Acute lymphoblastic leukemia	4	9
Chronic myelogenous leukemia	2	4
Myelofibrosis	1	3
CMV serological status recipient/donor		
Positive/positive	40	87
Positive/negative	4	9
Negative/positive	2	4
Conditioning regimen		
Busulfan + cyclophosphamide	46	100
Donor		
HLA matched sibling	46	100
Median age, years (range)	40 (23-56)	
Disease status pretransplant		
CR	36	78
Not CR	10	22
Source of stem cells		
PBSC	46	100
GVHD prophylaxis		
CsA + MTX	46	100

CMV indicates cytomegalovirus; PBSC, peripheral blood stem cells; GVHD, graft-versus-host disease; CsA, cyclosporin; MTX, methotrexate; CR, complete remission.

$Foxp3^+CD4^+CD25^{high}$ Regulatory T Cells (Tregs) Count

Cells were phenotypically analyzed by flow cytometry using a panel of directly conjugated antibodies to CD3, CD4, CD25 (M-A251), and CD45RO. Fluorescein isothiocyanate (FITC), phycoerythrin (PE), PercP, and allophycocyanin (APC) were used as fluorophores (Beckman Coulter, Miami, FL, USA; and Becton Dickinson, Franklin Lakes, NJ, USA). Intracellular analysis of FoxP3 (eBioscience, San Diego, CA, USA) was performed after fixation and permeabilization according to the manufacturer's recommendations. Flow cytometry was performed by a flow cytometer (FACS Calibur, Becton Dickinson) and data were acquired and analyzed using Cellquest software.

Tetramer Staining

We used fluorochrome-conjugated tetrameric complexes of $CMVpp50_{245-253}$ VTEHDTLLY-HLA-A0101, $CMVpp65_{495-503}$ NLVPMVATV-HLA-A 0201, $CMVpp65_{417-426}$ TPRVTGGGAM-HLA-B 0702, $IE\text{-}1_{199-207}$ ELRRKMMYM-HLA-B0801, and $CMVpp65_{123-131}$ IPSINVHHY-HLA B3501 to monitor recovery of CMV-specific $CD8^+$ T cells (according to the patients' HLA); 5 μL CD8-FITC, CD3-P5, CD4-P5, CD19-P5, and CD13-P5 and 2 μL fluorochrome-conjugated tetrameric HLA (according to the patients' HLA) purchased from Beckman Coulter Inc., were added to 100 μL heparinized blood and incubated for 30 min. After the RBC were lysed and washed twice, the cells were fixed and acquired on a flow cytometer (FACS Calibur, Becton Dickinson). More than 20000 cells in the lymphocyte gate were acquired and analyzed using Cellquest software. The $CD4^-$, $CD19^-$, $CD13^-$ and $CD3^+$, $CD8^+$ CMV-tetramer positive fraction of the lymphocyte gate was defined as CMV-specific $CD8^+$ T cells.

Preventing CMV Infection and Disease

CMV seropositivity was assessed by the detection of IgG antibodies to CMV late antigen. CMV recipient/donor serologic status is shown in Table 1; no negative/negative serologic status was found in our case study. All patients received prophylactic high-dose

acyclovir 500 mg/m^2 intravenously 3 times daily, followed by 800 mg orally (when feasible) 4 times daily, throughout the period from engraftment to 100 days after allogeneic PBSCT [6]. During this time, patients underwent viral monitoring by CMV pp65 antigen (Ag) in peripheral blood leukocytes (PBLs) (CMV Brite Immunofluorescence kit Biotest Diagnostics, Denville, NJ, USA) once a week and received preemptive antiviral therapy if evidence of CMV replication was found [6]. Following this period, patients who had experienced CMV reactivation or had severe GVHD continued to be monitored weekly, whereas the other patients were monitored every 2 weeks. CMV disease was defined according to Ljungman et al [23].

Preemptive Antiviral Therapy

On detection of CMV pp65 Ag in PBLs, preemptive therapy was started [6]. The initial therapy was ganciclovir 5 mg/kg twice per day for a minimum of 7-14 days, which was then adjusted according to the follow-up CMV antigenemia value [6].

CMV-Specific Immune Response Recovery

Reconstitution of CMV-specific CD8$^+$ T cells, as evaluated by quantitative tetramer to levels >10/μL, was considered adequate for protection against CMV infection [11,12,14,24,25]. Receiver operating characteristic (ROC) curve analysis was performed to define the threshold number of CMV-specific CD8$^+$ T cells in association with a CMV pp65 Ag-positive test.

Acute aGVHD Treatment

All patients were treated with methylprednisolone at doses of 2 mg/kg body weight, which were then adjusted according to clinical improvement or not.

Table 2. Correlation between CMV Infection/Disease and CMV-Specific Immune Recovery, acute GVHD, or Absolute Neutrophil Count (ANC)

CMV-Specific IR$^+$(%)	CMV-Specific IR$^-$(%)	P*
2 (7)	16 (94)	<.001
aGVHD$^+$ (%)	**aGVHD$^-$ (%)**	
17 (77)	1 (4)	<.001
ANC < 500/μL (%)	**ANC ≥ 500/μL (%)**	
9 (37)	9 (41)	Ns

aGVHD indicates acute graft-versus-host disease; CMV, cytomegalovirus; ANC, absolute neutrophil count.
Patients with CMV-specific immune recovery (IR$^+$); without CMV-specific IR (IR$^-$); with acute GVHD (aGVHD$^+$); without aGVHD (aGVHD$^-$); No. of patients IR$^+$: 29; No. of patients aGVHD$^+$: 22; No. of patients with <500 ANC/μL: 24.
*Fisher's Exact test.

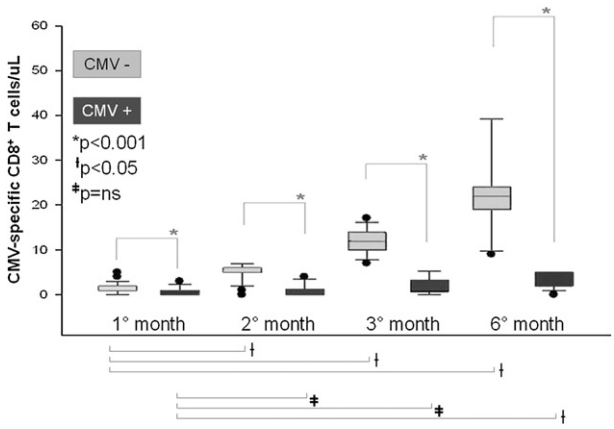

Figure 1. Box plots showing values of circulating CMV-specific CD8$^+$ T cells at 1, 2, 3, and 6 months after allogeneic peripheral blood stem cell transplantation in patients with (CMV+) and without (CMV−) CMV infection/disease. The top, bottom, and middle lines through the box correspond to the 75th, 25th, and 50th percentiles, respectively. The bars on the bottom and top extend from the bottom 10th percentile and top 90th percentile, respectively.

Statistical Analysis

The Mann-Whitney Rank Sum test was used for comparison of absolute cell counts between 2 groups, and Kruskal-Wallis One Way Analysis of Variance on Ranks test for multiple comparisons versus the control group (Dunn's Method). Fisher's exact test (2-sided) was performed to compare proportions, and the Pearson product for correlation. Logistic regression was performed for univariate and multivariate analysis.

RESULTS

CMV Infection

CMV antigenemia was positive in 18 of 46 (39%) patients, between day 28 and day 93 (median, day 47) after transplantation, 3 of 46 (6%) patients developed CMV disease (2 pneumonitis, 1 colitis) and died. CMV infection was controlled in 2 of 2 (100%) and in 5 of 16 (31%) patients with and without recovery of CMV-specific CD8$^+$ T cells, respectively.

Table 3. Median CMV-Specific CD8$^+$ T Cells and Tregs at Any Time Point

	Median CMV-Specific CD8$^+$ T Cells (r)		Median Tregs (r)	
	with CMV infection	w/o CMV infection	with aGVHD	w/o aGVHD
1 mo.	0 (0-3)	2 (0-5)	5 (0-7)	10 (0-14)
2 mo.	0 (0-4)	5 (0-9)	6 (0-9)	15 (4-18)
3 mo.	1 (0-8)	12 (5-23)	6 (2-9)	19 (8-38)
6 mo.	3 (0-5)	22 (5-53)	7 (3-10)	21 (9-41)

CMV indicates cytomegalovirus; aGVHD, acute graft-versus-host disease; mo, month; r, range; w/o, without.

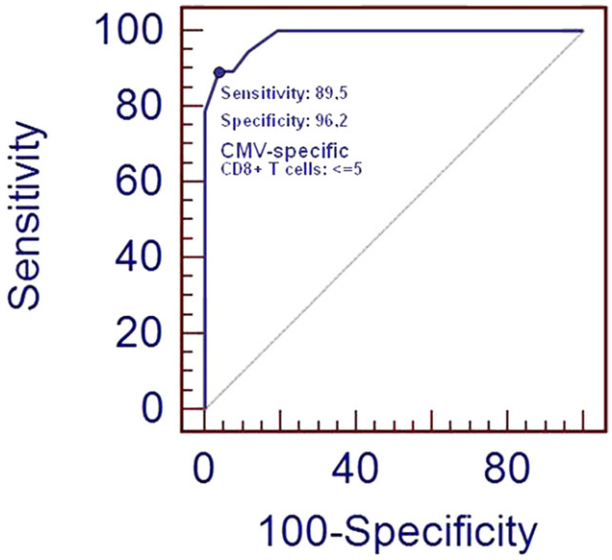

Figure 2. Receiver operating characteristic (ROC) curve of CMV-specific $CD8^+$ T cells for antigenemia CMV diagnostic test; area under curve: 0.985 (95% CI: 0.894-1.000). The performance is summarized in Table 3.

A second episode of CMV reactivation occurred in 2 of 18 (11%) patients. CMV infection/disease correlation with CMV-specific immune recovery, aGVHD, and absolute neutrophil count is shown in Table 2.

$CD4^+$, $CD8^+$, and CMV-Specific $CD8^+$ T Cell Counts

The median value of $CD4^+$ T cells was 110/μL (range: 30-380), 160 (range: 43-430), 209 (range: 40-420), and 346 (range: 80-450) at 1, 2, 3, and 6 months, respectively. The median value of $CD8^+$ T cells was 280/μL (range: 130-456), 454 (range: 159-1230), 573 (range: 174-1692), and 722 (range: 482-2242) at 1, 2, 3, and 6 months after allogeneic PBSCT, respectively. The median value of $CD4^+$ and $CD8^+$ showed no difference in patients with and without CMV infection/disease. Median CMV-specific $CD8^+$ T cells were significantly higher in patients without than with CMV infection/disease at 1 (2 cells/μL versus 0, $P < .001$), 2 (5 cells/μL versus 0, $P < .001$), 3 (12 cells/μL versus 1, $P < .001$), and 6 months (22 cells/μL versus 3, $P < .001$), respectively (Figure 1 and Table 3), with a progressive increase of CMV-specific $CD8^+$ T cells as from the first month in patients without CMV infection/disease (Figure 1). We did not find any correlation between $CD34^+$ stem cells and T cell graft content with T cells recovery.

By ROC analysis, a CMV-specific $CD8^+$ T cells value of ≤5/μL is associated with a pp65 Ag CMV-positive test (Figure 2 and Table 4).

Tregs Evaluation

The median Tregs values at any time point are summarized in Table 3; they were significantly higher in patients without than with CMV infection/disease at 2 (15/μL versus 3, $P < .001$, Figure 3A) and 3 months (22/μL versus 6, $P < .001$, Figure 3B) after allogeneic PBSCT. Moreover, we observed a good correlation between Tregs and recovery of CMV-specific $CD8^+$ T cells at 2 ($r = .61$, $P < .0001$) (Figure 4A) and 3 ($r = .72$, $P < .00001$) (Figure 4B) months, respectively.

aGVHD

aGVHD was observed in 22 of 46 patients (48%). Median time of aGVHD onset was 50 days (range: 33-77). Tregs were significantly higher in patients without than with aGVHD (Figure 5).

Tregs Graft Content (Evaluation Performed on 24 of 46 [52%] Donor Grafts)

Tregs graft content counts was higher in patients who did not develop aGVHD compared with those who did (12 versus 2 cells $\times 10^6$/kg, $P < .001$) (Figure 6).

Univariate and Multivariate Logistic Regression: Impact on CMV Infection and/or Disease

Data are summarized in Tables 5 and 6.

Relapse and Survival Rate

Relapse and overall survival (OS) rates did not differ between patients with or without CMV-specific immune recovery, whereas OS rate is significantly different in patients with or without aGVHD (Table 7).

Table 4. Diagnostic Performance of CMV-Specific $CD8^+$ T Cells Values

Criterion	Sensitivity	95% CI	Specificity	95% CI	+LR	−LR
<0	0.00	0.0-17.6	100.00	86.8-100.0		1.00
≤4	78.95	54.4-93.9	100.00	86.8-100.0		0.21
≤5*	89.47	66.9-98.7	96.15	80.4-99.9	23.26	0.11
≤7	89.47	66.9-98.7	92.31	74.9-99.1	11.63	0.11
≤8	94.74	74.0-99.9	88.46	69.8-97.6	8.21	0.059
≤9	100.00	82.4-100.0	80.77	60.6-93.4	5.20	0.00
≤23	100.00	82.4-100.0	0.00	0.0-13.2	1.00	

CI indicates confidence interval; CMV, cytomegalovirus; +LR: positive likelihood ratio; −LR: negative likelihood ratio. *, optimal diagnostic point. Criterion: CMV-specific $CD8^+$ T cells.

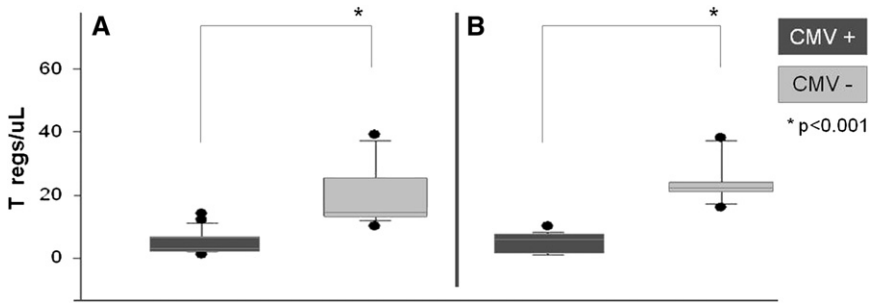

Figure 3. Box plots showing values of circulating T regs in patients with (CMV+) and without (CMV−) CMV infection/disease at 2 (A) and 3 months (B).

DISCUSSION

Despite major advances in the diagnosis and treatment of infectious complications in transplantation patients, CMV infection/disease remains a major cause of treatment-related morbidity and mortality after allogeneic SCT [1-3]. Protective immunity toward CMV is maintained by CMV-specific $CD8^+$ T cells [10,11]. Additionally, naturally arising $CD4^+CD25^+$ regulatory T cells are major regulators of adaptive immunity [15] and, in a murine GVHD model, adoptive transfer of Tregs leads to improved immunity to CMV, with a significant reduction in viral load and increased survival [18]. In our experience, in a homogeneously treated population (busulfan and cyclophosphamide, and HLA-matched sibling donor transplantation in 46 of 46 patients [100%]), we observed a correlation between increased Tregs and protection against CMV at 2 and 3 months after allogeneic PBSCT (Figures 3 and 4).

So far, little is known about the contribution of Tregs to protective immunity against CMV and, consequently, to the number of circulating CMV-specific $CD8^+$ T cells. Particularly, immunity against CMV may be altered by GVHD status [16,17] and related functional defects [26,27]. Thus, evaluation of the CMV-specific immune response, regardless of GVHD status, might appear to have a limited value for estimating its real contribution to lower CMV infection risks. Nevertheless, in accordance with other authors [12-14], we believe that monitoring CMV-specific $CD8^+$ T cells may help to identify the risk of CMV infection/disease after allogeneic SCT. Functional depression of the lymphocytes because of GVHD, corticosteroids, or other immunosuppressive therapy for GVHD seems to be the major cause of CMV reactivation after allogeneic SCT [4,26,27], although the

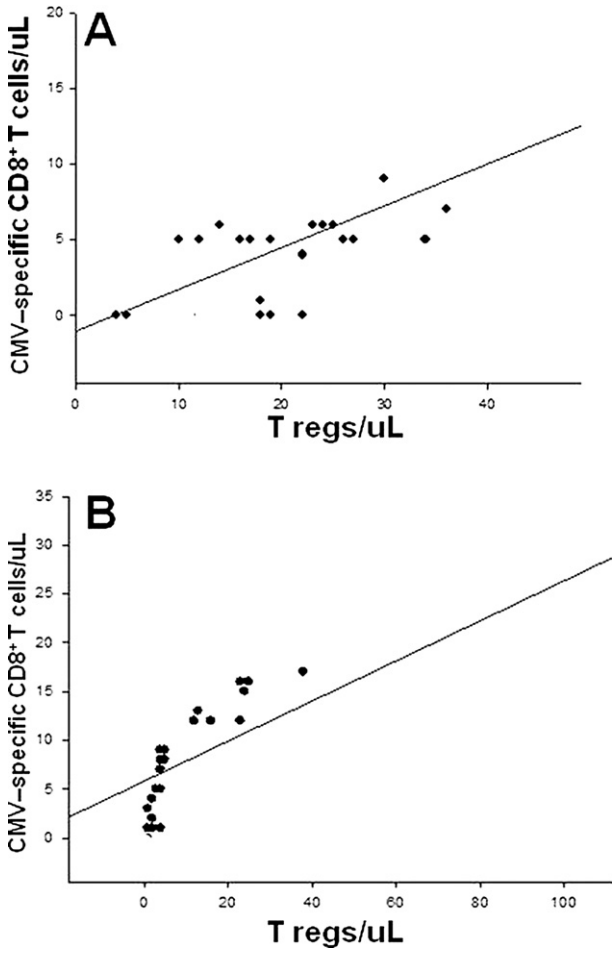

Figure 4. Correlation between T regs and CMV-specific $CD8^+$ T cells. Dots correspond to samples from patients undergoing allogeneic peripheral blood stem cell transplantation after 2 (A) and 3 (B) months. A significant positive correlation was seen between Tregs and CMV-specific $CD8^+$ T cells, as shown by the trend line (A, r = .61; $P < .0001$; B, r = .72; $P < .00001$).

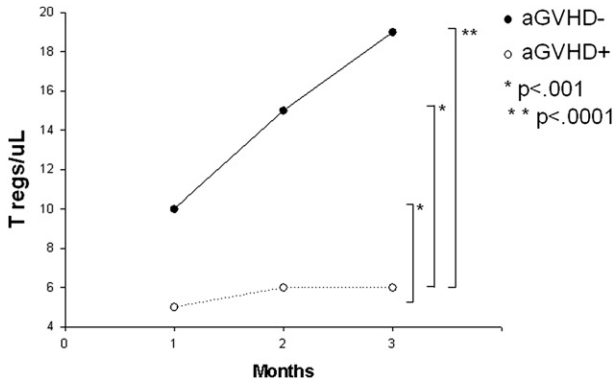

Figure 5. Median value of circulating T regs in patients with aGVHD (aGVHD+) compared with those without aGVHD (aGVHD−) at 1, 2, and 3 months.

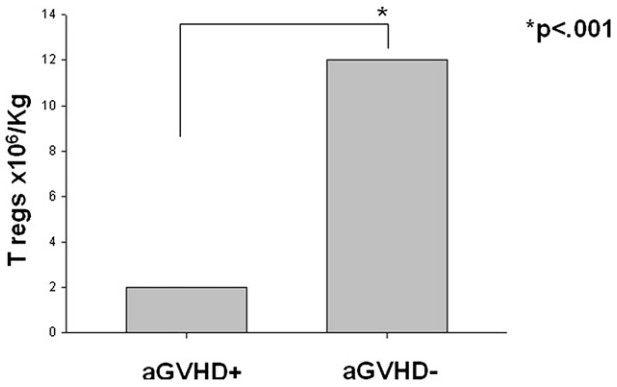

Figure 6. Median value of graft T regs content in patients with aGVHD (aGVHD+) and without aGVHD (aGVHD−).

influence of corticosteroids on reconstitution of $CD8^+$ T cells is controversial. Some studies [11,14] have reported a significant reduction of CMV-specific $CD8^+$ T cells with corticosteroid therapy. Evidence from another study [26] has indicated that the frequency and the absolute number of these cells were similar in patients receiving corticosteroids and those who did not, whereas CMV-specific $CD8^+$ T cells showed a decreased cytokine production, suggesting a qualitative rather than a quantitative alteration [26]. In any case, our data confirm the correlation of CMV infection/disease with both graft status and CMV specific immune response (Tables 2, 5, and 6).

Some authors [24,25,27] have reported that it is difficult to predict CMV infection by the evaluation of CMV-specific T cell reconstitution using HLA tetramer assays as these do not correlate with the incidence and severity of CMV infection and/or disease. Conversely, we and other authors [12-14] have shown that the numeric reconstitution of CMV-specific immune response after allogeneic SCT may be protective against the development of CMV infection/disease (Figure 1). Moreover, the best sensitivity and specifity threshold number of CMV-specific $CD8^+$ T cell for CMV Ag positive test (Figure 2 and Table 4) is below the one considered protective against CMV replication [11,12,14,24,25]. Therefore, close monitoring of CMV-specific $CD8^+$ T cells, in a cohort of patients early after transplantation, could constitute a rapid and sensitive tool to identify allogeneic SCT recipients at risk for developing CMV infection and/or disease. It should be noted that 46 of 46 (100%) of our patients must be considered at risk and 4 of 46 (9%) at high risk for CMV reactivation in view of the donor/recipient CMV serologies [4-6]. Moreover, it has been demonstrated [28] that short courses of antiviral treatment allow a rapid reconstitution of CMV-specific $CD8^+$ T cells in most patients, at least in those given a transplant from a CMV-seropositive donor (42/46 of our cases). Therefore, although few studies [11,12,14,24,25] have reported thresholds of T cell immunity that can be considered protective against CMV and the only pilot study [29] using T cell responses as the guide for withholding antiviral therapy was for patients more than 100 days after SCT, we consider that close monitoring of CMV specific $CD8^+$ T cells may enable us to distinguish those patients who might benefit from short courses of preemptive antiviral therapy from the ones who might

Table 5. Univariate Logistic Regression: Impact on CMV Infection/Disease

Variables	Odd Ratio (95% CI)	*P*
aGVHD (Grade II-IV)		
Yes/No	2.65 (1.40, 4.75)	.001
CMV-specific CD8+ T cells recovery		
No/yes	2.60 (1.35, 4.80)	.001
Tregs	2.35 (1.06, 5.00)	.03
ANC		
<500 μL/≥500 μL	1.30 (0.70, 3.10)	.30
Patient age	1.00 (0.95,1.05)	.75
Donor age	1.01 (0.96, 1.04)	.55
Disease status pretransplantation		
Remission/not remission	2.05 (0.84, 4.70)	.10
Diagnosis		
Myeloyd/not myeloid malignancy	1.10 (0.70, 2.10)	.84
CMVserology		
High risk/not high risk	1.45 (0.65, 3.30)	.30

CMV indicates cytomegalovirus; CI, confidence interval; ANC, absolute neutrophil count.
Donor type: 100% sibling.
Conditioning regimen intensity: 100% full ablative.
Transplant source: 100% PBSC.

Table 6. Multivariate Logistic Regression: Impact on CMV Infection/Disease

Variables	Odd Ratio (95% CI)	*P*
aGVHD (grade II-IV)		
Yes/no	2.60 (1.30, 5.00)	.006
CMV-specific $CD8^+$ T cells recovery		
No/yes	2.25 (1.20, 4.30)	.05
Tregs	2.35 (0.90, 5.50)	.06

aGVHD indicates acute graft-versus-host disease; CI, confidence interval; CMV, cytomegalovirus.

Table 7. Correlation between Relapse and Overall Survival with CMV-Specific Immune Recovery or aGVHD

	CMV-Specific IR^+		CMV-Specific IR^-
Relapse incidence @	34	versus	35*
Overall survival rate @	72	versus	65*
	$aGVHD^+$		$aGVHD^-$
Relapse incidence @	27	versus	42*
Overall survival rate @	82	versus	58†

CMV indicates cytomegalovirus; aGVHD, acute graft-versus-host disease.
@: % at 2 years; Log Rank test.
Patients with CMV-specific immune recovery (IR^+); without CMV-specific IR (IR^-); with aGVHD ($aGVHD^+$); without aGVHD ($aGVHD^-$).
*P = ns.
†P = .04.

avoid treatment while monitoring viral replication, if CMV-specific $CD8^+$ T cells are detectable. Moreover, although a preemptive treatment strategy is highly effective at managing CMV infection [7], late presentation of CMV disease can occur [9,30], and this has a high mortality rate [3].

Several studies [20-22,31] have demonstrated that Tregs suppress GVHD in animal models. This is consistent with our experience, in which higher levels of Tregs are associated with the absence of aGVHD (Figure 5). Moreover, it has been demonstrated [32] that increased numbers of Tregs are associated with chronic GVHD (cGVHD), indicating that cGVHD does not occur as a result of a deficiency of these cells. Patients with a profound immune dysregulation occurring more than 100 days after allogeneic SCT do not seem to benefit by Tregs [32] as much as early after transplantation [33]. Particularly in this early phase, as demonstrated in murine models, Tregs contribute to protect from GVHD lethality [20,22], preserve thymic and peripheral lymph node architecture, and promote an accelerated donor lymphoid reconstitution, which can be more effective against CMV [18]. In a clinical setting, soon after allogeneic SCT, Tregs may protect from aGVHD in humans [33] and, not only as a consequence of less immunosuppressive therapies, from CMV reactivation. This is consistent with the correlation we found between Tregs and protection against CMV in the early phase after transplantation (Figures 3 and 4). Of note, the median time of CMV reactivation and aGVHD was 47 and 50 days, respectively. This quite contemporary onset could suggest that Tregs, although they act through different mechanisms, drive both CMV-specific $CD8^+$ recovery and protection against GVHD. Moreover, the Tregs graft content is significantly lower in patients with aGVHD than in those without aGVHD (Figure 6), confirming the thymic preservation by Tregs.

In conclusion, we suggest that there is a good correlation between the recovery of Tregs and of CMV-specific $CD8^+$ T cells; Tregs mediate the protective effects against aGVHD and the maintenance of an optimal microenvironment for the reconstitution of functional immunity. This supports further consideration of Tregs immunotherapy for clinical allogeneic SCT. Particularly, adoptive transfer of Tregs may be useful, with the aim of abrogating ongoing aGVHD and promoting a more rapid immune reconstitution in patients at risk of CMV infection and/or disease.

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