Biol Blood Marrow Transplant 21 (2015) 2192–2202

Biology of Blood and Marrow Transplantation

journal homepage: www.bbmt.org

ASBMT American Society for Blood and Marrow Transplantation

Reconstitution of Human Cytomegalovirus–Specific CD4$^+$ T Cells is Critical for Control of Virus Reactivation in Hematopoietic Stem Cell Transplant Recipients but Does Not Prevent Organ Infection

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Article history:
Received 24 June 2015
Accepted 3 August 2015

Key Words:
Hematopoietic stem cell transplantation
Human cytomegalovirus
Specific T cell immunity
CD4$^+$ T cells
CD8$^+$ T cells
Gastrointestinal disease

A B S T R A C T

The relative contribution of human cytomegalovirus (HMCV)–specific CD4$^+$ and CD8$^+$ T cells to the control of HCMV infection in hematopoietic stem cell transplant (HSCT) recipients is still controversial. HCMV reactivation and HCMV-specific CD4$^+$ and CD8$^+$ T cell reconstitution were monitored for 1 year in 63 HCMV-seropositive patients receiving HSCT. HCMV reactivation was detected in all but 2 patients. In 20 of 63 (31.7%) patients (group 1) HCMV infection resolved spontaneously, whereas 32 of 63 (50.8%) patients (group 2) controlled the infection after a single short-course of pre-emptive therapy and the remaining 9 (14.3%) patients (group 3) suffered from relapsing episodes of HCMV infection, requiring multiple courses of antiviral therapy. The kinetics and magnitude of HCMV-specific CD8$^+$ T cell reconstitution were comparable among the 3 groups, but HCMV-specific CD4$^+$ T cells were lower in number in patients requiring antiviral treatment. HCMV-seronegative donors, as well as unrelated donors (receiving antithymocyte globulin) and acute graft-versus-host disease (GVHD) were associated with both delayed HCMV-specific CD4$^+$ T cell reconstitution and severity of infection. Conversely, these risk factors had no impact on HCMV-specific CD8$^+$ T cells. Eight patients with previous GVHD suffered from HCMV gastrointestinal disease, although in the presence of HCMV-specific CD4$^+$ and CD8$^+$ systemic immunity and undetectable HCMV DNA in blood. Reconstitution of systemic HCMV-specific CD4$^+$ T cell immunity is required for control of HCMV reactivation in adult HSCT recipients, but it may not be sufficient to prevent late-onset organ localization in patients with GVHD. HCMV-specific CD8$^+$ T cells contribute to control of HCMV infection, but only after HCMV-specific CD4$^+$ T cell reconstitution.

INTRODUCTION

Human cytomegalovirus (HCMV) infection remains a significant cause of morbidity and mortality after hematopoietic stem cell transplantation (HSCT). Although ganciclovir (GCV) prophylaxis and pre-emptive therapy effectively reduce the risk of HCMV disease, a minority of patients still develop recurrent episodes of HCMV reactivation and end-organ disease [1]. Since the 1980s, it has been known that the reconstitution of HCMV-specific CD4$^+$ and CD8$^+$ T cells is required to confer protection against HCMV reactivation [2,3]. Donor HCMV seronegativity, recipient HCMV seropositivity, T cell depletion, and the use of high-dose corticosteroids as treatment against graft-versus-host disease (GVHD) are among the factors influencing the recovery of HCMV-specific T cell immunity after HSCT [4,5].

To better define the role of both HCMV-specific CD4$^+$ and CD8$^+$ T cells in the control of HCMV infection after HSCT, we used HCMV-infected immature dendritic cells [6] for ex vivo stimulation of HCMV-specific T cells in a population of adult allogeneic HSCT recipients (HSCTR). The main objective of this study was to correlate the recovery of HCMV-specific immunity in this population with different clinical

Financial disclosure: See Acknowledgments on page 2201.

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http://dx.doi.org/10.1016/j.bbmt.2015.08.002
1083-8791/

presentations of HCMV infection: (1) immune control of HCMV infection, (2) initial lack of immune control of HCMV infection followed by short duration pre-emptive antiviral treatment, and (3) sustained lack of immune control of HCMV infection requiring multiple courses of pre-emptive antiviral therapy. In addition, we studied the impact of risk factors on the differential recovery of HCMV-specific $CD4^+$ and $CD8^+$ T cell responses and severity of HCMV infection. Finally, we analyzed the role of peripheral blood immunity in preventing HCMV gastrointestinal (GI) disease (GID). Results of the present study indicate that recovery of HCMV-specific $CD4^+$ T cells is mandatory for control of systemic HCMV infection, but it may not be sufficient to avoid late-onset GID.

METHODS

Study Population

From June 2011 through May 2014, 77 HCMV-seropositive patients receiving HSCT at Fondazione IRCCS Policlinico San Matteo, Pavia, Italy, were enrolled in the study. Fourteen patients were lost to follow-up because of early (within 3 months) death not related to HCMV infection (n = 7) or underlying disease relapse (n = 3), systemic HCMV infection at the time of transplantation (n = 1), or post-transplantation follow-up performed in other centers (n = 3). Thus, 63 patients, with a median follow-up of 358 days (range, 90 to 483) were analyzed. Their characteristics are shown in Table 1.

GVHD prophylaxis and treatment were previously reported [7]. Patients with steroid-resistant GVHD were treated with extracorporeal photochemotherapy (hepatic GVHD), mofetil mycophenolate, or infliximab (gut disease).

Thirty HCMV-seropositive adult healthy subjects were used as controls. The study was approved by the Fondazione IRCCS Policlinico San Matteo bioethics committee and institutional review board (Protocol n. 20100005053), and patients gave written informed consent before entering the study.

Management of HCMV Infection

HCMV reactivation (virus detection in blood or tissue biopsies) and disease (HCMV end-organ infection in association with clinical symptoms) were diagnosed by real-time PCR [8,9]. HCMV DNAemia was determined twice each week until discharge from the hospital, and then once each week for the first 3 months [8]. Subsequently, patients were monitored for HCMV at scheduled medical visits or in the presence of HCMV-related clinical symptoms. In patients receiving immunosuppressive therapy for acute GVHD and/or after detection of HCMV DNA in blood, monitoring of DNAemia was resumed twice each week.

No patient received anti-HCMV prophylaxis. Pre-emptive therapy was administered with a HCMV DNAemia cutoff of ≥30,000 DNA copies/mL whole blood, unless clinical conditions of patients suggested an earlier initiation of treatment (≥10,000 copies/mL) [10], and consisted of administration of intravenous GCV (5 mg/kg twice each day), replaced by foscarnet (90 mg/kg twice each day) in case of neutropenia. Antiviral treatment was stopped after 2 consecutive DNA-negative blood samples. Biopsy-proven HCMV GID was treated similarly until resolution of clinical symptoms and/or endoscopic intestinal lesions.

Immunologic Monitoring

Immunological monitoring was performed before the pre-transplantation myeloablative regimen, and at least at 30, 60, 90, 120, 180, and 360 days after transplantation. Additional controls were performed in patients with GVHD just before the onset of steroid treatment, a week after and, in patients showing a decrease in HCMV-specific T cells, every 2 weeks until recovery of HCMV-specific immunity. Subjects with protective virus-specific cellular immunity were considered those with at least 1 HCMV-specific $CD4^+$ and 3 HCMV-specific $CD8^+$ T cells/μL blood [7,11].

Total and HCMV-specific T cells were determined by flow cytometry analysis as reported [6,12] and are summarized in the Supplementary Methods.

Statistical Analysis

The number of total or HCMV-specific T cells between 2 groups were compared by using the Mann-Whitney *U*-test. The Kruskall-Wallis test was used to compare more than 2 groups, with Dunn's post test and correction for multiple comparisons. The association of different factors (age, GVHD, donor type, and serostatus) with the severity of HCMV infection was analyzed by the chi-square test. Time to HCMV-specific T cell reconstitution was calculated using Kaplan-Meier curves, which were compared by the log-rank test.

Table 1
Characteristics of the 63 HSCTR

Characteristic	n (%)
Gender	
Male	37 (59)
Female	26 (41)
Age at transplantation, median (range), yr	53 (26-68)
Underlying disease	
Acute myeloid leukemia	30 (48)
Myelodysplastic syndrome	9 (14)
Acute lymphocytic leukemia	7 (11)
Non-Hodgkin lymphoma	5 (8)
Chronic lymphocytic leukemia	4 (6)
Myelofibrosis	4 (6)
Multiple myeloma	3 (5)
Chronic myelomonocytic leukemia	1 (2)
Disease status at transplantation	
Complete remission	38 (60)
Partial remission	2 (3)
Active disease	22 (35)
Front line	1 (2)
Myeloablative (chemotherapy based) conditioning regimen	
Standard intensity	19 (30)
Reduced intensity	44 (70)
ATG administration	38 (60)
Stem cell source	
Peripheral blood	51 (81)
Bone marrow	12 (19)
Donor type	
Sibling	26 (42)
Unrelated	37 (58)
Donor/recipient HCMV serostatus	
D+/R+	46 (73)
D−/R+	17 (27)
GVHD	
No acute or chronic GVHD	30 (48)
Acute (grade I-II)	16 (25)
Acute (grade III-IV)	9 (14)
Chronic	12 (19)

D indicates donor; R, recipient.

RESULTS

HCMV Infection Reactivation and T Cell Immune Reconstitution

Three groups of patients were identified

The 63 patients were divided into 3 groups on the basis of the severity of systemic HCMV infection and the relevant requirement for pre-emptive therapy (Table 2). The development of late-onset GID, which occurred in some patients in complete dissociation from systemic infection and regardless of peripheral blood T cell recovery, is analyzed separately (see below).

Three representative cases are reported in Figure 1A-C.

Group 1 included 20 of 22 patients who developed self-resolving systemic HCMV infection (SI) without reaching the established cut-off of 30,000 HCMV DNA copies/mL blood. The median viral load peak was 2400 (range, 0 to 11,200) copies/mL blood and was reached at a median time of 53 (range, 26 to 270) days after transplantation (Table 2). However, 3 of these patients developed late-onset (between 6 and 12 months after HSCT) HCMV GID in the absence of detectable virus in blood (see below), whereas 2 patients had no detectable infection throughout the study period.

Group 2 included 32 patients who underwent a single course of antiviral therapy for SI either reaching (n = 23) or approaching (n = 9) the cut-off of 30,000 copies/mL. The median viral load peak was 37,100 (range, 10,300 to 100,000)

Table 2
Immunological and virological characteristics of HSCTR divided into three groups

Group No.	Type of Infection, n		Systemic HCMV Infection		Preemptive* Antiviral Treatment, median day (range)	Specific HCMV immunity			
			DNA Peak			T-cell count/μL blood (median, range)			
			Copies/L, median (range)	Median Day After Transplantation (range)		$CD4^+$		$CD8^+$	
	SI	SI + GID				Day 60	Day 360	Day 60	Day 360
Group 1 (self-resolving infection, n = 22)†	17	3	2400 (0-11,200)	+ 53 (26-270)	0	3.6 (.2-132.2)	4.5 (.5-80.0)	81.9 (2.5-326.3)	97.1 (8.4-461.1)
Group 2 (infection requiring treatment, n = 32)	29	3	37,100 (10,300-100,000)	+45 (25-124)	26 (9-49)	.2 (.1-34.9)	8.0 (.2-154.7)	23.2 (0.1-753.1)	214 (14.0-767.2)
Group 3 (recurrent infections, n = 9)	6	2	69,300‡ (16,200-203,900)	+47‡ (33-52)	66 (44-180)	.1 (.1-.4)	.9 (.1-232.8)	18.6 (1.1-55.9)	41.7 (25.0-1950)

* Patients with GID were subsequently treated for the disease as shown in Table 3.
† Two patients had no detectable infection.
‡ First peak of infection.

copies/mL at a median time of 45 (range, 25 to 124) days after transplantation (Table 2). Median duration of antiviral pre-emptive therapy was 26 (range, 9 to 49) days. GCV therapy controlled SI in all patients. However, 3 patients developed late-onset GID in the absence of virus in blood.

Group 3 included 9 patients, who underwent multiple courses of antiviral pre-emptive therapy because of relapsing systemic infection reaching or exceeding the cutoff. The median viral load peak was 69,300 (range, 16,200 to 203,900) copies/mL at a median time of 47 (range, 33 to 52) days after transplantation. Two patients underwent antiviral therapy before reaching this threshold, based on medical decision. As reported in Table 2, the total median duration of antiviral therapy was 66 (range, 44 to 180) days. Two patients also suffered from late-onset GID (with very low level or absent virus in blood).

Risk factors for HCMV infection severity were also investigated (see Supplementary Results).

Kinetics of Total and HCMV-Specific $CD4^+$ and $CD8^+$ T Cells in the 3 Groups of Patients

In group 1, median level of total $CD4^+$ T cells at day +60 (the time point nearest to the infection peak) was 310 (range, 51 to 1036) cells/μL and did not change significantly until 1 year after transplantation, never reaching levels of healthy controls (Supplemental Figure 1A). At day 60, total $CD8^+$ T cells (409 [range, 245 to 1717] cells/μL) were already similar to controls, and remained stable until 1 year after transplantation (611 [range, 88 to 2270] cells/μL), as illustrated in Supplemental Figure 1B.

In group 2, median level of total $CD4^+$ T cells at day +60 was 95 (range, 16 to 480) cells/μL, significantly lower than in group 1 patients, reaching comparable values 4 to 6 months after transplantation (Supplemental Figure 1A). At day +60, total $CD8^+$ T cells (513 [range, 32 to 986] cells/μL) were not different from group 1 patients. These cells progressively increased until 1281 (range, 206 to 4445) cells/μL 1 year after transplantation (Supplemental Figure 1B).

Almost all group 3 patients showed levels of total $CD4^+$ T cells under 100 cells/μL until 4 months after transplantation. Only 4 of 9 (55%) of these patients exceeded this level at 6 months after transplantation (Supplemental Figure 1A). Conversely, at day +60, median total $CD8^+$ T cells (371 [range, 74 to 808] cells/μL) were not significantly different from controls and from total $CD8^+$ T cells of group 1 patients, remaining stable throughout follow-up (594 [range, 181 to 7789] cells/μL at 12 months), as shown in Supplemental Figure 1B.

As for HCMV-specific immunity of group 1 patients (Table 2), HCMV-specific $CD4^+$ T cells were comparable to control levels, and thus were near or above the cut-off of 1 HCMV-specific T cell/μL, starting from day +60 through day +360 (Figure 2A). HCMV-specific $CD8^+$ T cells (Figure 2B) showed high levels, superior to those of healthy controls, from day +60 throughout the follow-up.

In group 2, HCMV-specific $CD4^+$ T cells were significantly lower than in group 1 patients (Table 2). Yet, 4 months after transplantation, 8 of 29 (27%) patients had specific-$CD4^+$ T cells below the cut-off of 1 cell/μL (Figure 2A). Almost all group 2 patients exceeded the cut-off 6 months after transplantation. HCMV-specific $CD8^+$ T cells (Figure 2B) were lower than those of healthy controls and group 1 patients until day +60; afterwards levels of HCMV-specific $CD8^+$ T cells were superior to controls and similar to group 1.

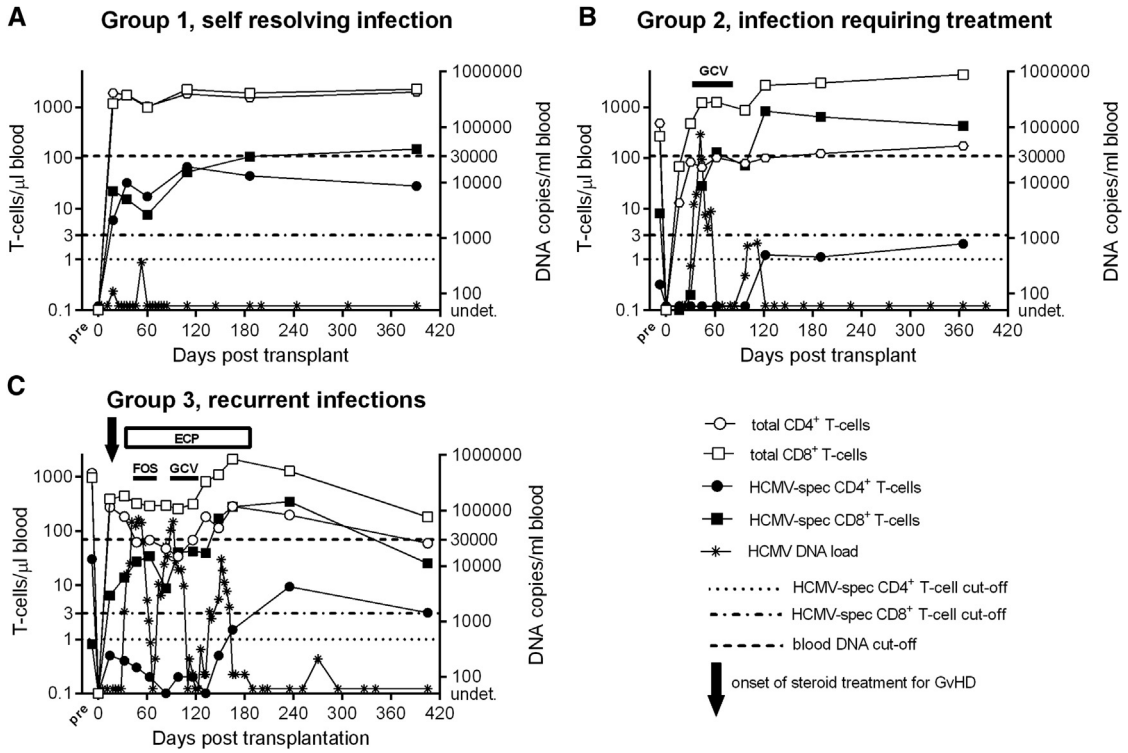

Figure 1. Kinetics of total and HCMV-specific CD4$^+$ and CD8$^+$ T cells in 3 representative HSCTR patients, 1 for each group. Panel A (group 1 patient): self-resolving infection in the presence of low viral load and HCMV-specific CD4$^+$ and CD8$^+$ T cells above the cut-off thresholds. Panel B (group 2 patient): HCMV infection GCV-treated for viral load above the cut-off, with undetectable HCMV-specific CD4$^+$ T cells until day 120, when they exceeded the cut-off level indicating control of HCMV infection. Panel C (group 3 patient): recurrent infections in a patient treated twice for systemic infection (viral load above the cut-off). This patient received steroids and extracorporeal photochemotherapy as anti-GVHD treatment. HCMV-specific CD4$^+$ T cells showed levels below the cut-off until 6 months after transplantation. GCV, ganciclovir, FOS, foscarnet, ECP, extracorporeal photochemotherapy.

In group 3 patients (Table 2), HCMV-specific CD4$^+$ T cells were almost absent at day 60, and 6 months after transplantation they were still below the cut-off of 1 cell/µL in nearly all patients (Figure 2A). Strikingly, HCMV-specific CD8$^+$ T cells (Figure 2B) were at high levels, comparable to those of healthy controls and group 1 patients throughout the follow-up.

Polyfunctional analysis of HCMV-specific CD4$^+$ and CD8$^+$ T cells is described in the Supplementary Results (Supplemental Figure 2), indicating that patients of group 2 developing HCMV infection requiring treatment in the presence of HCMV-specific CD4$^+$ T cells show a lower frequency of CD4$^+$ T cells producing IL-2 and TNFα together with IFN-γ than patients controlling the infection. On the contrary, the expression of perforin by HCMV-specific T cells was high in all patients examined (median, 87%; range, 63% to 100% and median, 95%; range, 52% to 100% of HCMV-specific CD4$^+$ and CD8$^+$ T cells, respectively) and did not discriminate between patients controlling or not the infection.

Factors Associated With Specific T Cell Immunity Reconstitution

The same factors associated with severity (Supplemental Table 1) of HCMV infection (unrelated donor/use of antithymocyte globulin [ATG], HCMV-seronegative donor, and acute GVHD) were also associated with delayed HCMV-specific CD4$^+$ T cell reconstitution (Figure 3). In particular, a strong correlation was found between donor HCMV serostatus and HCMV-specific CD4$^+$ T cell recovery (Figure 3B): ie, 1 year after transplantation, 5 of 13 (38%) donor negative/recipient positive patients were found to not have reconstituted HCMV-specific CD4$^+$ T cells above the protective cut-off, when the majority (32 of 40, 80%) of donor positive/recipient positive HSCTR already reconstituted HCMV-specific CD4$^+$ T cells 90 days after transplantation. In contrast, no parameter influenced the reconstitution of HCMV-specific CD8$^+$ T cells (data not shown).

To better determine the impact of the steroid treatment for acute GVHD on HCMV-specific T cell immunity, the 25 HSCTR who suffered from acute GVHD were divided into 2 subgroups, according to the development of acute GVHD before or after the reconstitution of detectable levels of HCMV-specific CD4$^+$ T cells (Figure 4).

The first subgroup (Figure 4A,B) included 15 patients starting steroid therapy at a median time of 17 days (range, 9 to 52) after transplantation, when HCMV-specific CD4$^+$ T cells were not yet detected. Median onset of antiviral therapy (administered in 13 of 15 patients) was at day 46 (range, 28 to 146). In this case, it was found that the time required to restore HCMV-specific CD4$^+$ T cells was significantly ($P = .04$) longer than in patients without GVHD (Figure 4A). No significant difference ($P = .35$) was found with respect to HCMV-specific CD8$^+$ T cell reconstitution between patients with and without GVHD (Figure 4B).

The second subgroup (Figure 4C,D) included 10 patients starting anti-GVHD therapy at a median time of 45 days (range, 25 to 114) after transplantation, when HCMV-specific CD4$^+$ T cell immunity was already detectable. Median onset of antiviral therapy for these patients was at day 52 (range,

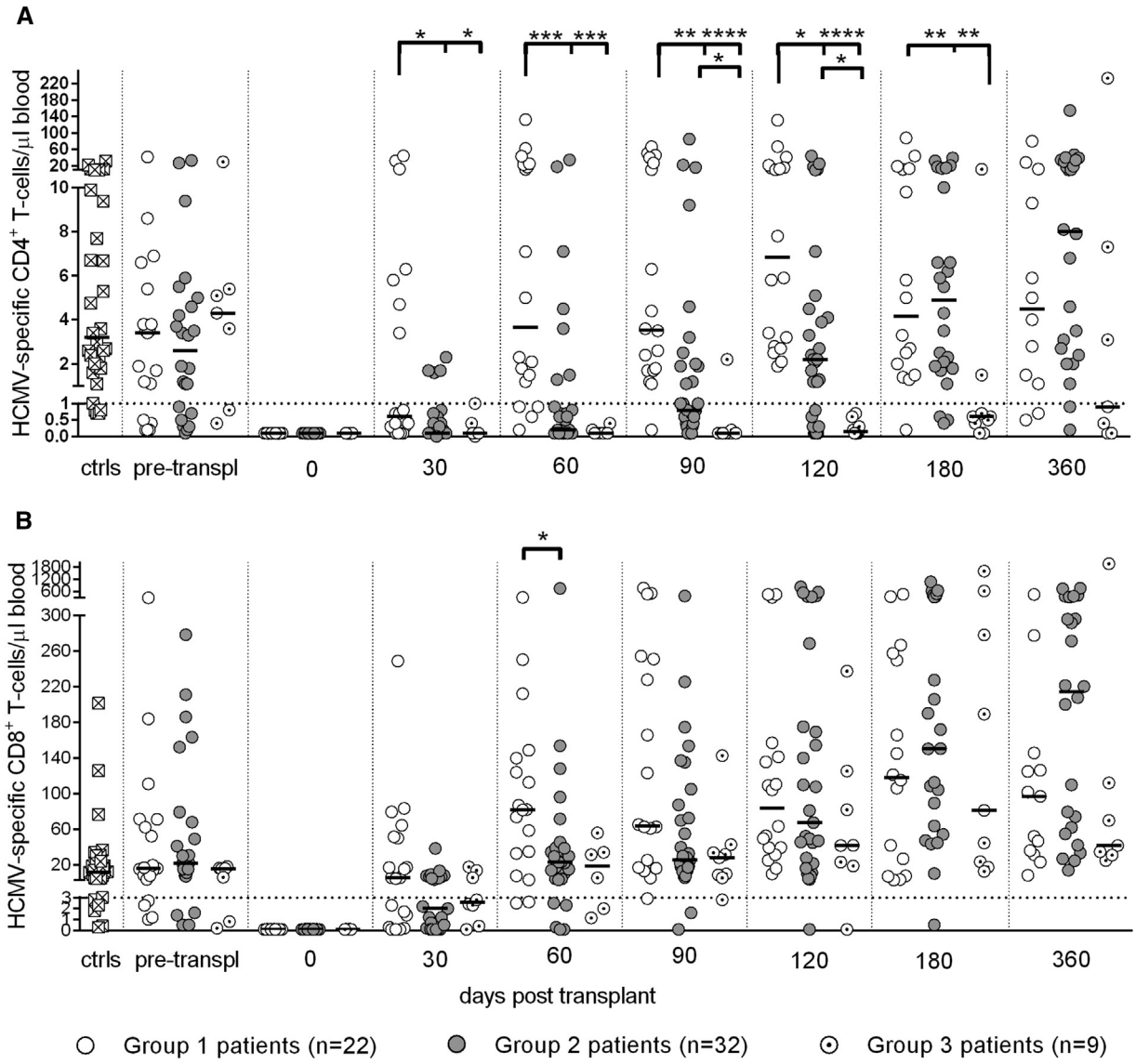

Figure 2. Kinetics of HCMV-specific (A) $CD4^+$ and (B) $CD8^+$ T cells in the 3 groups of HSCTR. Statistical significance of the differences between the 3 groups of patients is reported as follows: $^*P < .05$; $^{**}P < .01$; $^{***}P < .001$; $^{****}P < .0001$.

47 to 124). Steroid treatment determined a strong decrease (greater than 60% compared with presteroid immunity) in HCMV-specific $CD4^+$ T cells in patients belonging to groups 2 and 3 with HCMV SI requiring antiviral treatment. In patients who had recurrent infections (group 3), low levels of HCMV-specific $CD4^+$ T cells persisted for at least 3 months after onset of therapy. In 2 of 4 patients of group 1, steroid therapy caused a reduction in HCMV-specific $CD4^+$ T cells, which, however, remained above the protective cut-off level (Figure 4C). The other 2 group 1 patients, who received steroids for GVHD treatment at a very low dosage, maintained specific $CD4^+$ immunity at the presteroid level and surpassed the protective cut-off level within a few weeks. Steroid therapy for GVHD had no substantial impact on HCMV-specific $CD8^+$ T cell level (Figure 4D).

It was also investigated whether steroid treatment determined a reduction in some specific functional subset of HCMV-specific $CD4^+$ T cells. Results showed that multifunctional and monofunctional $CD4^+$ T cells decreased in parallel (data not shown).

Some patients received extracorporeal photochemotherapy (FEC), mofetil mycophenolate (MMF), or infliximab as second-line GVHD treatment. Among these, 3 patients received FEC before and 11 patients after immune reconstitution. Only in 3 patients we observed a reduction in HCMV-specific $CD4^+$ T cells (along with specific $CD8^+$ T cells in 2 cases) followed by HCMV reactivation. No significant change in HCMV-specific T cells was observed in the other cases, as well as in the 8 patients receiving MMF. Finally, in 1 patient receiving infliximab in association with FEC, a reduction in HCMV-specific $CD4^+$ T cells was observed without subsequent HCMV reactivation.

HCMV GID

All patients suffering from GID were endoscopically biopsied. HCMV organ localization was diagnosed in 8 of 63 patients (13%) on the basis of HCMV DNA quantification in organ biopsies (Table 3). For each patient, multiple biopsies of the upper and lower GI tract were taken: in Table 3 biopsies with the highest viral load are listed, along with

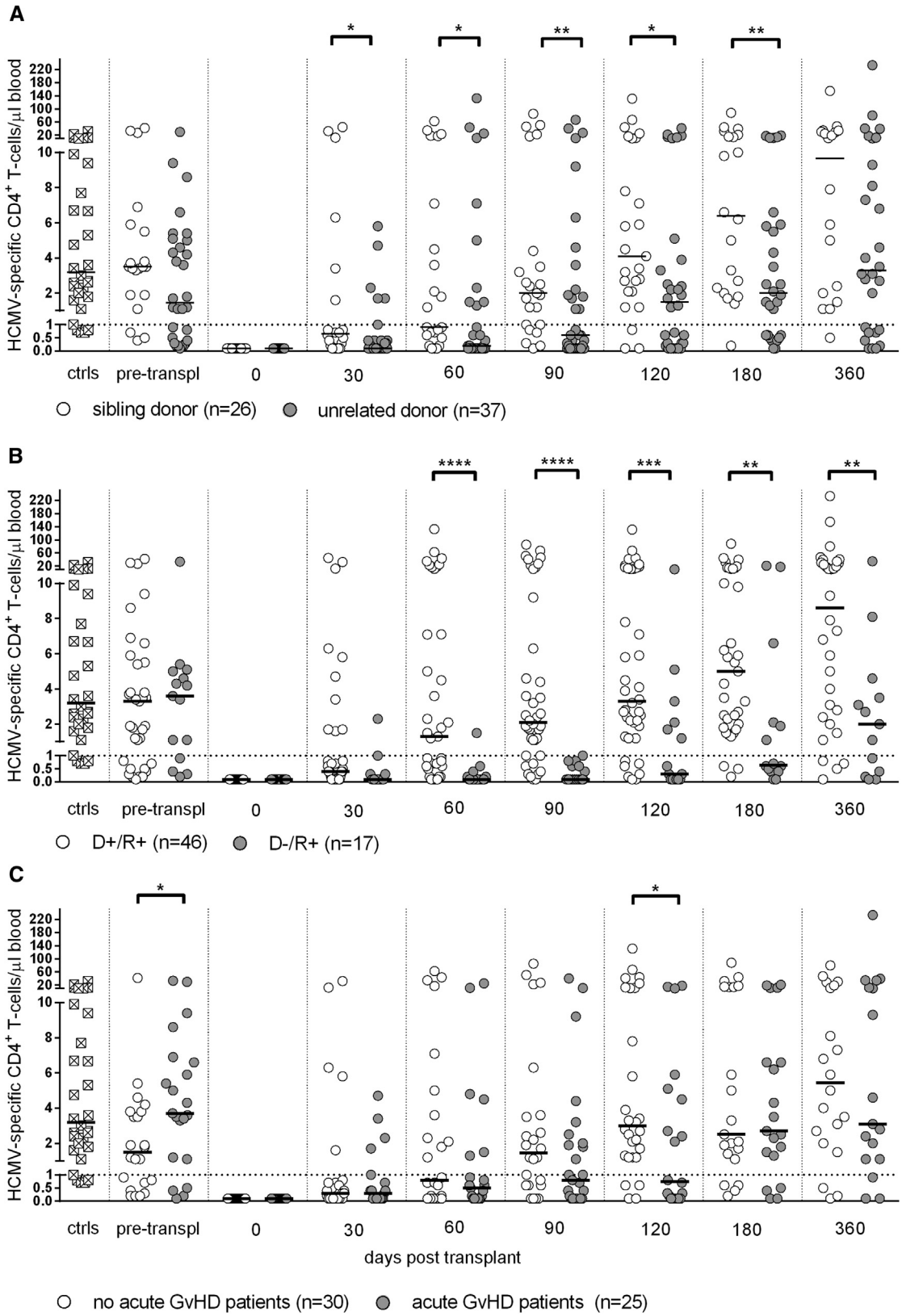

Figure 3. Kinetics of HCMV-specific CD4$^+$ T cells in HSCTR patients receiving stem cells (panel A) from sibling or unrelated donors, or (panel B) from HCMV-seropositive or seronegative donors and (panel C) with/without GVHD. Statistical significance of the differences between the 2 groups of patients reported in each panel is as follows: $^*P < .05$; $^{**}P < .01$; $^{***}P < .001$; $^{****}P < .0001$.

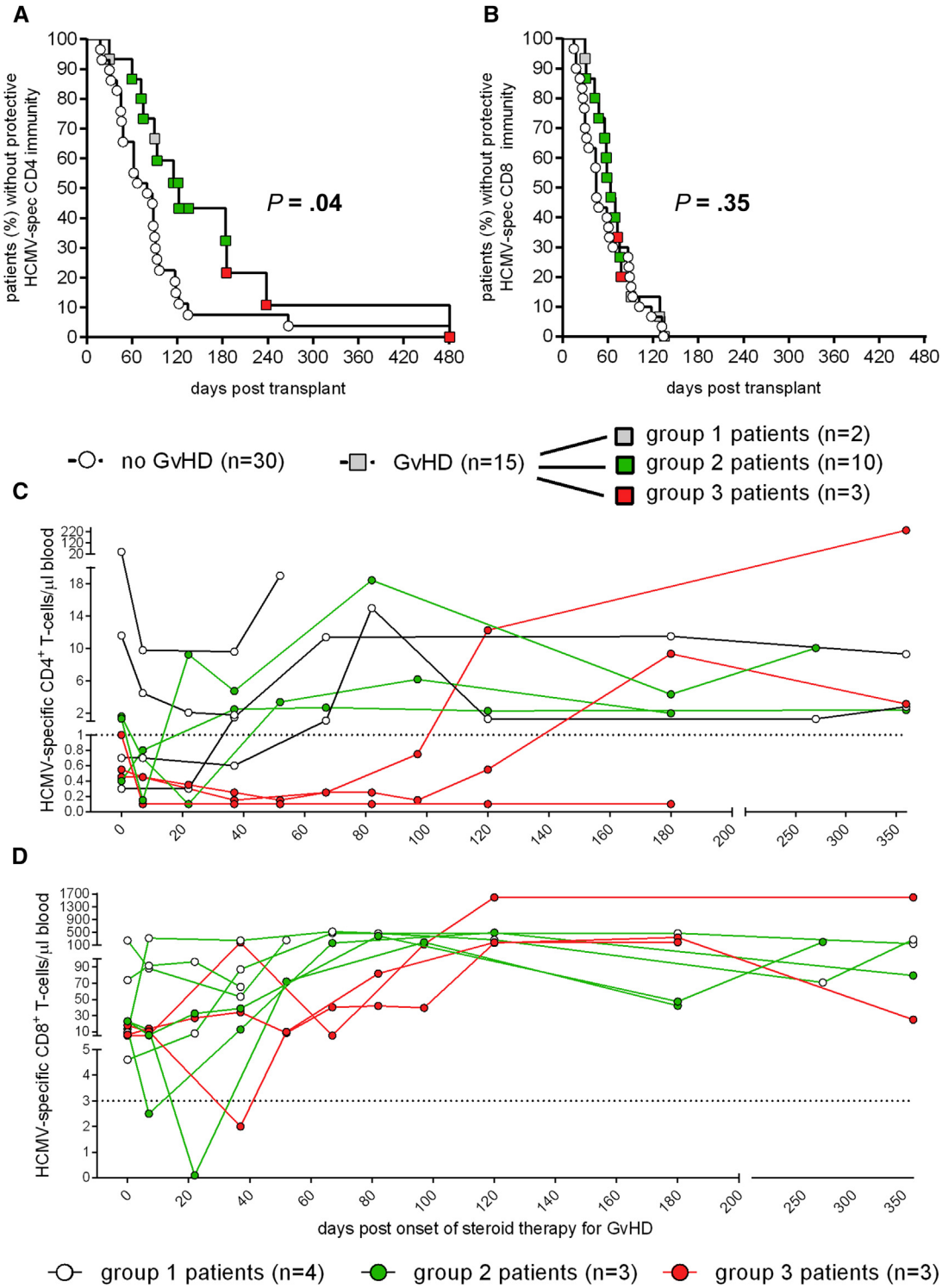

Figure 4. Kaplan-Meier curves represent time to reconstitution of HCMV-specific (A) $CD4^+$ and (B) $CD8^+$ T cells in 15 HSCTR with GVHD versus 30 without GVHD. Censored patients of the 3 groups are represented with different colors. *P* values of the log-rank test are shown. In panels (C) and (D) kinetics of HCMV-specific $CD4^+$ and $CD8^+$ T cells in 10 HSCTR patients steroid-treated for GVHD and with presteroid immunity, are reported.

HCMV-related symptoms and antiviral therapy duration. GID developed at a median time of 325 days (range, 144 to 387) after transplantation: at this time, nearly all patients (except patients number 14 and 38) had protective levels of HCMV-specific $CD4^+$ T cells (median value, 6.7 cells/µL blood) and HCMV-specific $CD8^+$ immunity was largely above the protective cut-off (median value, 123.8 cells/µL). At the time of organ infection diagnosis, viral DNA in blood was negative for 3 to 9 months before (6 patients) or reached very low levels after a recent reactivation. Moreover, in terms of cytokines and perforin production, we observed that HCMV-specific $CD4^+$ and $CD8^+$ T cells were as functional as in

Table 3
HSCTR with HCMV GID: Virological and Immunological Monitoring

Pt. ID (n = 8)	Type of GVHD, Localization, Grade	Onset of Anti-GVHD therapy, d after tx	Organ Infection			HCMV-Specific T cells		HCMV DNAemia (copies/mL blood)	HCMV-Related Symptoms	Antiviral Therapy Duration for GID, d	Outcome of Transplant at 360 d after tx
			HCMV Detection, d after tx	HCMV load (DNA copies/10^5 cells)	Organ Pathology	$CD4^+$	$CD8^+$				
1	C, intestinal and cutaneous	+100	+352	11,000 (cecum)	Weak chronic infl	7.9	74.1	0	Abdominal and epigastric pain, nausea	24	Alive
13	A, cutaneous, grade 1*	+59	+339	8000 (antrum)	Strong acute active infl with epithelial necrosis	10.01	200.1	0	Fever, abdominal pain	15	Dead†
14	A, intestinal, grade 2	+37	+144	8700 (antrum)	Weak chronic infl	**.1**	186.2	100	Nausea	42	Dead‡
31	A, cutaneous, grade 1	+36	+188	11,800 (antrum)	Weak chronic infl	1.3	266.8	0	Diarrhea, epigastric pain	27	Alive
38	C, intestinal	+107	+387	4000 (colon)	Weak chronic infl	**.4**	34.8	0	Diarrhea	16	Alive
49	C, hepatic	+225	+336	136,700 (antrum)	Mild chronic infl with mucosal damage	24.7	101.8	0	Nausea	17	Alive
55	A, cutaneous, grade 2*	+27	+314	56,000 (ileum)	Weak chronic and acute active infl	9.3	145.8	0	Diarrhea	51	Alive
56	C, hepatic	+150	+260	114,900 (ileum)	Mild chronic infl with mucosal damage	5.5	43.5	550	Diarrhea	20	Alive
Median		+79.5	+325	NA		6.7	123.8	0		22	
Range		27-225	144-387	NA		.1-24.7	34.8-266.8	0-550		15-51	

Numbers in bold indicate HCMV-specific CD4 < 1 cell/μL blood.
Pt indicates patient; tx, transplantation; A, indicates acute; C, de novo chronic; infl, inflammation; NA, not applicable.
* With progression to chronic GVHD.
† Died from intestinal surgery complications.
‡ Died from neurotoxoplasmosis.

patients who were HCMV disease-free (data not shown). At the time of GID, all HSCTR were receiving low-dosage methylprednisolone (.25 to 1 mg/kg/day); in 3 cases in association with FEC and MMF, in other 2 cases with FEC or MMF only. Five patients were still receiving cyclosporine A. All patients (except patient numbers 14 and 31) underwent this treatment for chronic GVHD (intestinal, cutaneous, or hepatic, either occurring de novo or after acute GVHD). However, the first diagnosis of GVHD occurred at a median time of 79 (range, 27 to 225) days after transplantation, between 4 and 10 months before detection of HCMV in organ biopsies (Table 3) and no sign of active GVHD (ie, presence of apoptotic bodies) were observed in GI biopsies at the time of diagnosis of HCMV-GID. In addition, 6 of 8 patients (75%) received HSCT from an HCMV-seronegative donor. All patients, except patient number 13 (died from intestinal surgery complications) and patient number 14 (died of neurotoxoplasmosis) recovered completely after antiviral therapy and were alive 1 year after transplantation.

DISCUSSION

In this study, we adopted a method involving the use of HCMV-infected autologous dendritic cells (DC) for T cell stimulation, and enabled the simultaneous and comprehensive determination of HCMV-specific $CD4^+$ and $CD8^+$ T cells [6], to investigate the contribution provided by both arms of T cell immunity to the control of HCMV infection in adult HSCTR. This method allows the detection of T cells specific for multiple HCMV antigens as naturally presented by infected DC. Instead, the more common use of peptide pools from individual viral proteins (such as pp65 and IE-1), although permitting a rapid and easier monitoring of HCMV-specific T cells, underestimates the actual T cell immune response against the virus [12]. On the other hand, we observed a similar polyfunctional profile in healthy donors' T cells stimulated by infected DC, infected cell lysate, or peptide pool [13]. Thus, it is possible to compare the quality of the T cell response obtained with infected DC or other methods.

Indeed, some authors consider $CD8^+$ T cells to play a major role in the protection against HCMV reactivation [14,15], while a few others indicate that HCMV-specific $CD4^+$ T cells are required to provide protection [16,17]. The present study demonstrates the following: (1) HCMV-specific $CD8^+$ T cell reconstitution after HSCT is not sufficient to protect against HCMV reactivation, if not associated with HCMV-specific $CD4^+$ T cell reconstitution; (2) control of HCMV infection is better achieved in the presence of polyfunctional HCMV-specific $CD4^+$ T cells simultaneously producing IFN-γ, TNFα, and IL-2; (3) GVHD occurrence and the relevant high-dose steroid treatment, as well as transplantation from a HCMV-seronegative or unrelated donor (the latter is associated with ATG treatment), are risk factors for HCMV reactivation that have a major impact on reconstitution of HCMV-specific $CD4^+$ rather than $CD8^+$ T cells; and (4) reconstitution of systemic HCMV-specific T cell immunity, though required for control of HCMV reactivation, does not prevent late-onset GID in patients with previous GVHD.

As for the first point (predominant role of $CD4^+$ T cells in the control of HCMV infection), 63 HCMV-seropositive adult HSCT recipients were divided into 3 groups on the basis of severity of HCMV reactivation and the need for pre-emptive antiviral treatment. Group 1 included patients able to control HCMV reactivation without antiviral therapy. Almost all of these HSCTR rapidly recovered (within 2 months after

transplantation) levels of HCMV-specific $CD4^+$ T cells considered as protective (>1 cell/μL blood). This cut-off level was previously determined by our group in both young and adult HSCTR [7,11]. Similar cut-off levels have been reported by others [18].

Group 2 patients did not control HCMV infection spontaneously but required a cycle of antiviral therapy when reaching or approaching the established HCMV DNA cut-off level during the second month after HSCT. In the majority of these patients, recovery of HCMV-specific $CD4^+$ T cells was delayed until 3 to 4 months after transplantation.

Group 3 included a minority of patients who underwent multiple courses of antiviral therapy because of relapsing systemic HCMV infection, which occurred as a first episode at about the same time as in patients of the other groups. In many group 3 patients, HCMV-specific $CD4^+$ T cells were not yet reconstituted 1 year after transplantation.

It should be noted that the kinetics of HCMV-specific $CD8^+$ T cell recovery after transplantation was very rapid, as $CD8^+$ T cells exceeded the previously supposed [7,11] protective cut-off level of 3 cells/μL blood within 2 months after transplantation. Moreover, HCMV-specific $CD8^+$ T cell levels were not significantly different among the 3 groups of patients at any time point, although a trend towards a lower levels of HCMV-specific $CD8^+$ T cells at day +60 was observed in group 3 patients with severe relapsing infection. This suggests that, in the studied population, the major actors in the protection against HCMV reactivation were HCMV-specific $CD4^+$ T cells and that HCMV-specific $CD8^+$ T cells alone did not confer sustained immune control of virus reactivation. This finding is in accordance with a recent study by our group showing that, in a population of seropositive solid-organ transplant recipients, long-term protection from HCMV reactivation was not conferred in the absence of HCMV-specific $CD4^+$ T cells [19]. Similarly, levels of specific $CD4^+$, but not $CD8^+$ T cells, were found to inversely correlate with reactivation episodes in allogeneic HSCT recipients in a study by Widmann et al. [20]. Moreover, a deficient HCMV-specific $CD4^+$ T cell immune response within the first 30 to 50 days after transplantation was found to be associated with high risk of viral reactivation after allogeneic HSCT [21].

As for the second point (better protection conferred by polyfunctional $CD4^+$ T cells), as many as 7 patients belonging to group 2 developed HCMV infection requiring antiviral treatment in the presence of $CD4^+$ T cell levels defined as protective. In a previous study by our group, the predictive value of this cut-off was found to be around 80%, indicating that 20% of patients may still develop severe HCMV infection in the presence of specific $CD4^+$ T cell levels above the cut-off [7]. Thus, it was investigated whether HCMV-specific $CD4^+$ T cells of these patients were functionally different from $CD4^+$ T cells of group 1 (self-resolving infection) patients. It was observed that these nonprotected patients had a lower number of $CD4^+$ T cells producing IL-2 and TNFα together with IFN-γ. These results are in accordance with the previous study, in which a lack of IL-2 was observed in HCMV-specific $CD4^+$ T cells of nonprotected patients [7]. In the current study, no differences in the HCMV-specific $CD8^+$ T cell cytokine profile were observed between protected and nonprotected patients, as well as in perforin production by the same cells (data not shown). As a next step, the analysis of the expression of exhaustion markers, or the direct evaluation of the ability to kill HCMV-infected cells could help in the search for parameters of protection in $CD8^+$ T cells.

In our high-risk seropositive population [21,22], we studied the impact of different factors on both HCMV reactivation severity and HCMV-specific $CD4^+$ and $CD8^+$ T cell reconstitution. Among factors analyzed, donor HCMV seronegativity (and, thus, lack of transfer of memory HCMV-specific T cells with the graft) was found to play a major role: all patients able to spontaneously control virus infection (group 1) received a transplant from HCMV-seropositive donors. A correlation with the severity of infection was also found in patients receiving a transplant from unrelated donors (all receiving ATG in the conditioning regimen), and in patients receiving high-dose corticosteroids for acute GVHD treatment, as already reported [23,24]. Instead, patient age and stem cell source (peripheral blood or bone marrow) had no affect on the virological outcome. Conflicting results have been provided by other studies [25,26]; however, the number of patients analyzed in the present study is too low to draw robust conclusions on this point.

We observed that the same factors influencing virological outcome have an impact on reconstitution of HCMV-specific $CD4^+$ T cells. From previous studies it is not clear to what extent these risk factors, particularly high-dose steroid treatment, could inhibit/delay immune reconstitution of HCMV-specific $CD4^+$ rather than $CD8^+$ T cells [4]. One study reported a negative correlation between the intensity of immunosuppressive therapy and levels of HCMV-specific immunity that appeared to be much more impaired for $CD4^+$ than $CD8^+$ T cells [16]. On the other hand, in a previous study on pediatric HSCTR, some patients receiving high-dose steroids did not show a reduction in the number of HCMV-specific $CD4^+$ or $CD8^+$ T cells, although developing a severe SI [11]. In the current study, treatment with high-dose corticosteroids for acute GVHD determined a rapid and long-lasting depletion in HCMV-specific $CD4^+$ T cells in patients who subsequently developed severe recurrent infections, whereas $CD8^+$ T cells were only slightly decreased and rapidly returned to the presteroid level. Whether the effect of steroids was related to T cell depletion or their functional inhibition is not clear. Glucocorticoids were found to block IL-2 through inhibition of IL-1 and IL-6 genes [27]. In our study, GVHD treatment also induced a reduction in total $CD4^+$ T cells (data not shown).

Among the 63 HSCT recipients, 8 patients (13%) suffered from late-onset HCMV GID, diagnosed at a median of 325 days after transplantation, which was successfully resolved after prompt antiviral treatment. All of these patients reactivated HCMV in the first weeks after transplantation at variable levels: 3 of them had low HCMV DNAemia and spontaneously resolved the infection, whereas the remaining 5 underwent 1 or more courses of antiviral therapy. At the time of organ infection diagnosis, 6 patients (75%) showed HCMV-specific $CD4^+$ T cells in peripheral blood and all of them had very high levels of HCMV-specific $CD8^+$ T cells. Concomitantly, HCMV DNA in blood was undetectable or detected at a very low level. The diagnosis of HCMV GID is often challenging, and the use of additional techniques would corroborate the diagnosis by PCR. Although immunohistochemistry was performed, HCMV nuclear inclusion bodies were not detected in almost all samples examined. However, as no other possible cause of GID was detected (ie, active GVHD or other infections), the high level of virus in the tissue versus its absence or very low level in blood, and the ex juvantibus proof that GCV resolved clinical symptoms, indicate HCMV as the major pathogen involved. We already observed, in patients receiving

solid-organ transplantation, that HCMV end-organ disease may be associated with high and also with low systemic viral load [28], and, in lung transplant recipients, we showed that systemic T cell response may not be sufficient to provide protection against HCMV pneumonia [29]. Also in this report, despite the pivotal role of HCMV-specific $CD4^+$ T cells in the control of HCMV reactivation, the presence of these cells in blood was not found to be sufficient in some cases to confer protection against late-onset end-organ HCMV GID. Interestingly, all patients developing HCMV GID were treated some months before with high-dose steroids for acute or chronic GVHD, and the majority of them received a transplant from a HCMV-seronegative donor. Occurrence of GVHD has been reported to be an important risk factor for the development not only of HCMV viremia but also of HCMV GID [30]. Whether this is the consequence of an impaired T cell response due to prolonged steroid therapy or a local effect of allogeneic stimulation during GVHD leading to HCMV reactivation [31,32] still needs to be clarified. However, we did not observe a significant impairment of systemic immunity in patients suffering from HCMV GID disease. Investigation of local (tissue) T cell immunity would be of help in understanding the pathogenesis of GID in these patients.

In conclusion, our results suggest that reconstitution of HCMV-specific $CD4^+$ T cells is pivotal for control of HCMV reactivation after HSCT and that $CD8^+$ T cells alone do not confer sustained immune control of HCMV infection. This implies that measurement of HCMV-specific $CD4^+$ T cells alone might be sufficient to predict whether a patient is at risk of HCMV reactivation or not, whereas testing levels of HCMV-specific $CD8^+$ T cells alone appears to be prognostically inadequate. Thus, monitoring of HCMV-specific $CD4^+$ T cell reconstitution can help in assessing the time lapse after HSCT in which the patient is at risk for HCMV infection/disease and should be monitored for HCMV in blood to start timely pre-emptive treatment, when required. After specific $CD4^+$ recovery, HCMV monitoring in blood could be discontinued. At the same time, specific $CD4^+$ T cells are more susceptible than $CD8^+$ to high-dose steroid treatment for GVHD. Thus, strict virological/immunological monitoring is further required in these patients, continuing after immune reconstitution has occurred. Patients showing a sustained lack of HCMV-specific $CD4^+$ T cells could take advantage of adoptive HCMV-specific T cell immunotherapy to avoid repeated HCMV recurrences.

Finally, patients with GVHD (intestinal or not) should also be considered high-risk for developing late HCMV GID, even in the presence of protective systemic HCMV-specific $CD4^+$ T cell immunity and with the absence of HCMV DNA in blood. Thus, with currently available tools, virological and immunological monitoring in blood are not useful in the case of HCMV GID and GI biopsies should be considered for the differential diagnosis of HCMV GID in HSCTR with GI clinical symptoms. Moreover, the ex vivo study of the anti-HCMV activity of tissue-infiltrating T cells obtained from organ biopsies will represent a step forward in our understanding of HCMV control by the reconstituting immune system in HSCT.

ACKNOWLEDGMENTS

The authors thank Daniela Sartori for editing the manuscript and Laurene Kelly for revising the English. We also thank the technical staff of the virology laboratory for performing viral assays and the nurses of the hematology division for taking samples from the patients.

Financial disclosure: This work was partially supported by the Fondazione Carlo Denegri, Torino, Italy.

Conflict of interest statement: There are no conflicts of interest to report.

SUPPLEMENTARY DATA

Supplementary data related to this article can be found online at http://dx.doi.org/10.1016/j.bbmt.2015.08.002.

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