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CLINICAL RESEARCH

Cytomegalovirus Viral Load and Virus-Specific Immune Reconstitution after Peripheral Blood Stem Cell versus Bone Marrow Transplantation

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Peripheral blood stem cell (PBSC) products contain more T cells and monocytes when compared with bone marrow (BM), leading to fewer bacterial and fungal infections. Cytomegelovirus (CMV) viral load and disease as well as CMV-specific immune reconstitution were compared in patients enrolled in a randomized trial comparing PSBC and BM transplantation. There was a higher rate of CMV infection and disease during the first 100 days after transplantation among PBSC recipients (any antigenemia/DNAemia: PBSC, 63% vs BM, 42%, $P = .04$; CMV disease: PBSC, 17% vs BM, 4%, $P = .03$). By 2 years, CMV disease rates were similar. The early increase in CMV events correlated temporarily with lower CMV-specific $CD4^+$ T helper and $CD8^+$ cytotoxic T lymphocyte function at 30 days after transplantation in PBSC recipients. By 3 months after transplantation and thereafter, CMV-specific immune responses were similar between BM and PBSC recipients. In conclusion, higher CMV infection and disease rates occurred in PBSC transplant recipients early after transplantation. These differences may be because of a transient delay in CMV-specific immune reconstitution following PBSC transplantation.

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KEY WORDS: Cytomegalovirus, Transplantation, T cells, Immune reconstitution

INTRODUCTION

The use of peripheral blood stem cells (PBSC) for transplantation improves survival in patients with high-risk hematologic malignancies compared with the use of bone marrow (BM) as a stem cell source [1-3]. PBSC products from donors who receive granulocyte-colony stimulating factor (G-CSF) contain at least 1 $\log_{10}$ more T cells and monocytes than BM, and absolute $CD4^+$ and $CD8^+$ lymphocyte numbers are higher early after PBSC transplant [4-6]. Overall, recipients of PBSC grafts have less frequent severe infections, especially those of bacterial and fungal etiologies [4].

The impact of PBSC transplantation on CMV-specific immune reconstitution, clinical infection, and disease is difficult to predict because of the unique properties of CMV. Active CMV infection after transplantation is controlled by CMV-specific $CD4^+$ T helper (Th) and $CD8^+$ cytotoxic T lymphocyte (CTL) responses [7,8]. There is evidence for the transfer of donor CMV-specific immunity with the graft, because CMV seropositive recipients of seropositive BM reconstitute CMV-specific T cell immunity earlier than recipients of seronegative BM [9,10]. Although greater numbers of T cells are transferred with PBSC grafts, CMV infects early and committed hematopoietic progenitors [11,12], which may result in transmission of a greater virus load with PBSC products. Moreover, the BM and blood contain different ratios of naïve, memory, and regulatory T cell subsets that could influence the recovery of functional CMV-specific T cells after transplantation [13-15]. Thus, differences in the cellular composition of BM and PBSC products may lead to different rates of CMV infection after transplantation both by influencing immune recovery and virus reactivation.

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$CD8^+$ and $CD4^+$ effector T cells have traditionally been characterized by functional assays that measure production of a single cytokine, such as interferon-gamma (IFN-γ), or by cytotoxicity or proliferation assays. Recently, multiparameter flow cytometry has made it possible to simultaneously evaluate multiple qualitative properties of T cells and enabled additional characterization of T cell function based on the production of combinations of cytokines [16,17]. In a prior study, polyfunctional $CD8^+$ CMV-specific T cells have been demonstrated to reconstitute differently in CMV-positive recipients of allogeneic hematopoietic cell transplantation (HCT) depending on the serostatus of the donor, and patients with lower numbers of polyfunctional $CD8^+$ CMV-specific T cells were taking antiviral drugs longer than patients with a higher number of polyfunctional $CD8^+$ CMV-specific T cells [18]. The purpose of this study was to compare quantitative virologic outcomes and to undertake exploratory analyses of CMV-specific T cell immune reconstitution in transplant recipients who participated in a randomized clinical trial of allogeneic BM versus PBSC as a stem cell source for HCT.

PATIENTS AND METHODS

Study Subjects

Patients participating in a multicenter randomized trial comparing PBSC or BM as a stem cell source for allogeneic transplant were analyzed for CMV infection and CMV-specific immune reconstitution [2]. Virologic analysis was done in all subjects who participated in the randomized trial, whereas CMV immunity was assessed in a subset of subjects at the Fred Hutchinson Cancer Research Center (FHCRC) who signed an additional consent form (Supplemental Figure S1). Conditioning regimens and graft-verus-host disease (GVHD) prophylaxis were administered as described [19]. CMV immune reconstitution studies were performed at 1 site (FHCRC) in a subset of patients who agreed to provide additional blood samples. Some of the data on qualitative immune reconstitution have been previously published [4]. All participants had an HLA-matched related donor and were >12 years of age. The study was approved by the institutional review board at FHCRC, and all patients gave informed consent.

Virologic Testing

Pretransplantation CMV serostatus was determined by an ELISA at FHCRC and the Veterans Administration Medical Center (Premier CMV IgG, Meridian Diagnostic, Cincinnati, OH), indirect immunofluorescence assay at City of Hope National Medical Center, Duarte, CA (Virgo; Hemagen Diagnostics Inc., Columbia, MD), passive agglutination test (Becton Dickinson, Meylan, France), and microparticle enzyme immunoassay (IMX Systems, Abbot Laboratories, Abbot Park, IL) at Stanford University Medical Center, Stanford, CA. All patients were prospectively monitored for CMV reactivation by pp65 antigenemia and blood cultures (FHCRC, Veteran's Administration Hospital Seattle), plasma polymerase chain reaction (PCR) for CMV DNA and shell vial cultures (City of Hope National Medical Center), or plasma PCR for CMV DNA (Stanford University Medical Center). CMV pp65 antigenemia was quantified as the number of positive cells per slide; CMV DNA was quantified as copy number/mL [4,20].

Use of Acyclovir, Ganciclovir, Intravenous Immunoglobulin, and CMV Seronegative Blood Products

Acyclovir was given to herpes simplex virus (HSV) seropositive individuals at a dose of 250 mg/m^2 i.v. twice daily from the start of conditioning until day 30 after transplantation. Documented HSV infections were treated with acyclovir at a dose of 250 mg/m^2 i.v. 3 times daily or 400 mg orally 5 times daily for 1 week.

Ganciclovir was administered for any pp65 antigenemia, shell vial cultures, or PCR positivity and continued until day 100 as described [21]. Intravenous immunogloblin was administered to maintain IgG level above 400 μg/mL [22]. CMV seronegative recipients of a seropositive BM or PBSC product received seronegative or filtered blood products throughout the pre- and posttransplantation period [23].

Definitions

CMV disease was defined as the identification of a virus from 1 or more visceral sites (lung, gastrointestinal tissue, liver) by culture or histology, or in the blood alcohol level (BAL) by shell vial centrifugation culture, conventional culture, or staining with direct fluorescent antibodies combined with new or changing pulmonary infiltrates on radiologic exam [24]. CMV infection was defined as any pp65 antigenemia or plasma PCR positivity. CMV viremia was defined as detection of CMV by rapid or conventional culture. Acute and chronic GVHD (aGVHD, cGVHD) were defined as described [25].

CMV-Specific Immune Reconstitution

CMV immune reconstitution studies were done on fresh (LDA and CTL assay) or cryopreserved peripheral blood mononuclear cells (PBMC) (intracellular cytokine analysis); experiments using cryopreserved cells were performed only in the CMV D+/R+ subset. Denominators for each test differ because of availability of cells (Supplemental Figure S1).

CTL assay

Short-term CMV-specific CTL lines were expanded in vitro according to previously described methods [10]. Briefly, fibroblast lines were established from skin biopsies obtained from the recipient or the HLA identical donor. Recipient PBMC were stimulated twice 7 days apart with fibroblasts infected with CMV AD169, and cytolytic activity against HLA-identical and HLA class I-mismatched CMV-infected and mock-infected fibroblasts was then measured using a 5-hour chromium release assay. Specific lysis was calculated according to the standard formula as described [10]. A positive CTL response was defined as lysis of HLA-identical CMV-infected targets at a level at least 10% greater than lysis of HLA-identical mock-infected and HLA class I mismatched CMV- and mock-infected targets.

Limiting dilution assay (LDA)

The frequency of CMV-specific T cells was determined by LDA using methods and calculations previously described [26]. Briefly, fresh PBMC were plated in 8 serial 2-fold dilutions starting from 10^5 cells per well in the presence of CMV antigen (24 replicates) or mock antigen (12 replicates). Each well also received 10^4 gamma irradiated (3300 rad) autologous PBMC to serve as antigen presenting cells. After 5 days, the wells were pulsed with 0.6 mCi ^{3}H thymidine and harvested after 18-24 hours. Wells were scored positive if the mean ^{3}H thymidine incorporation was >3 times that in the corresponding control wells. Precursor frequencies were calculated by the chi-square minimization method [27] with a computer program [28] written by L. Sirinek (provided by C. Orosz, both from Ohio State University, Columbus, OH). To compensate for differences between $CD4^+$ and total T cells between PBSC and BM recipients, results were normalized either with absolute lymphocyte count and to calculate the number of CMV-specific $CD4^+$ T cells/L or by the number of CMV-specific $CD4^+$ T cells per 1×10^6 PBMC [4].

Intracellular cytokine staining

Cryopreserved PBMC were thawed and rested overnight. The following day, PBMC were incubated with anti-CD107a PECy5 Ab for 10 minutes, followed by the addition of the costimulatory antibodies anti-CD28 (1 μg/mL; Becton Dickinson, San Jose, CA) and anti-CD49d (1 μg/mL). Cells were then stimulated with CMV pp65 protein (2 μg/mL), *Staphylococcus aureus* enterotoxin B (0.05 μg/mL), or medium for 6 hours at 37°C and 5% CO_2. Brefeldin A (10 μg/mL, Sigma)/Golgi stop (Becton Dickinson, San Jose, CA, USA) was added during the last 4 hours of incubation. Samples were held overnight at 4°C and then stained for flow cytometric analysis the following day.

After incubation with EDTA (20 mM), samples were incubated successively with FACSlyse and FACSperm (BD Biosciences & Pharmingen, San Diego, CA), washed, and then incubated for 30 minutes in the dark at room temperature with a cocktail of antibodies consisting of CD3 ECD (Beckman Coulter, Brea, CA), CD8 PerCypCy 5.5 (BD Biosciences & Pharmingen, San Diego, CA), IFN-γ APC, MIP-1β PE, and tumor necrosis factor-alpha (TNF-α) A700 (BD Bioscience & Pharmingen, San Jose, CA). Last, samples were fixed with 1% paraformaldehyde and cell acquisition (range 100,000 to 400,000) was done on an LSRII flow cytometer. Appropriate single color compensation and fluorescence minus 1 controls were run. Data were initially analyzed with FlowJo Version 8.8.6, and then further with PESTLE version 1.6.2 and SPICE version 5.0 (Simplified Presentation of Incredibly Complex Evaluations) software provided by M. Roederer of the National Institute of Allergy and Infectious Diseases.

Statistical Analysis

Time to CMV events was assessed by cumulative incidence curves and the log-rank test. The Student *t* test, Fisher exact test, and the Wilcoxon matched pairs test were done as appropriate on Prism software v5.0 (GraphPad Software, LA Jolla, CA).

RESULTS

Study Subjects

A summary of relevant patient characteristics is shown in Table 1, and additional characteristics have been published elsewhere [4] (Supplemental Figure S1). The 2 study arms (BM and PBSC) were

Table 1. Patient Characteristics*

Characteristic	BM N = 91	PBSC N = 81
Recipient age (median, range)	42 (12-55)	42 (15-55)
Recipient sex (male/female)	62/29	56/25
Underlying disease status		
Less advanced	51 (56%)	41 (51%)
More advanced	40 (44%)	40 (49%)
CMV serostatus		
Recipient positive/donor positive	36 (40%)	29 (36%)
Recipient positive/donor negative	14 (15%)	17 (21%)
Recipient negative/donor positive	14 (15%)	15 (18%)
Recipient negative/donor negative	27 (30%)	20 (25%)
Acute GVHD by day 100		
Grade 2-4	64%	57%
Grade 3-4	12%	15%
Chronic clinical extensive GVHD at 3 years	52%	63%

GVHD indicates graft-versus-host disease; CMV, cytomegalovirus; BM, bone marrow; PBSC, peripheral blood stem cell.
*Analysis of CMV virologic endpoints was performed in all randomized patients; analysis of CMV immune reconstitution was performed in a subset that agreed to additional blood draws and skin biopsies (Figure S1).

balanced for CMV serostatus and age. The subset of patients who were studied for CMV-specific T cell immune reconstitution was also balanced between the study arms.

CMV Infection and Disease

Seropositive recipients

The incidence of pp65 antigenemia or CMV DNA at any level before day 100 was higher in PBSC than in BM recipients ($P = .04$) (Figure 1A). There was no statistically significant difference between the 2 groups in the incidence of high (>100 cells/slide or >100,000 copies/μL) viral load by PCR or antigenemia (Figure 1B). CMV disease before day 100 was significantly higher in CMV seropositive recipients of PBSC ($P = .03$) compared with BM (Figure 1C). There was no difference in virologic parameters relative to donor CMV serostatus (Table 2).

Seronegative recipients

There was a trend toward more primary infection in patients who received PBSC than those who received BM (Table 2). In seronegative recipients of seronegative PBSC or BM, infection rates were low and not statistically different (Table 2). There was no CMV disease among seronegative recipients.

Time to cessation of antigenemia after ganciclovir early treatment

There was no statistically significant difference based on transplant type in the time to cessation of pp65 antigenemia or PCR positivity after the start of preemptive therapy in the subset of HCRC patients (data not shown).

Analysis of CMV-Specific T Cells in Donor PBMC Before and in the PBSC Product After G-CSF Treatment

We measured the number of CMV-specific T cells by LDA in the peripheral blood of PBSC donors before G-CSF treatment and in the stem product after G-CSF treatment. Interestingly, there were significantly fewer ($P = .04$) CMV-specific Th cells after G-CSF treatment (Figure 2). In addition, phytohemagglutin (PHA) stimulation also resulted in significantly less lymphocyte proliferation ($P = .005$, Wilcoxon matched pairs test) in the post-G-CSF stem cell product compared with pre-GCSF PBMC (data not shown). These results suggest that G-CSF mobilization either resulted in a decline in the number of CMV-specific $CD4^+$ T helper cells in the stem cell product or altered their ability to respond to antigen stimulation, either of which could contribute to delayed recovery of CMV-specific immune responses observed in PBSC recipients early after transplantation.

Analysis of CMV-Specific Immune Reconstitution in BM and PBSC Recipients

$CD8^+$ and $CD4^+$ CMV-specific T cell reconstitution was evaluated at days 30, 80, 180, and 365 posttransplantation by cytotoxicity assay (CTL) and LDA. In addition, based on immunocompetent donors' (N = 41) LDA results, ≥33 (25th percentile) CMV-specific $CD4^+$ T cells per 1×10^6 PBMC was established as a threshold for LDA (range: 13.91-6422.61, median: 76.92, 75th percentile 258.61 per 1×10^6 PBMC).

The proportion of BM recipients who exhibited CMV-specific lysis >10% at day 30 after transplantation was higher than in PBSC recipients (Figure 3A). This difference did not reach statistical significance, however ($P = 0.17$, Fisher exact test), and the numerical difference was no longer present by day 80 after transplantation. A trend toward a higher proportion ($P = .24$, Fisher exact test) of patients with ≥33 CMV-specific $CD4^+$ T cells was documented by LDA at day 30 and day 80 in BM compared with PBSC recipients (Figure 3B). Collectively, these results suggest that despite the higher T cell dose administered to PBSC recipients, CMV-specific T cell responses as measured by T cell proliferation and cytolytic function assays may have been enhanced in BM recipients.

Cytokine Production and Degranulation by CMV-Specific $CD8^+$ T Cells after PBSC and BM Transplantation

We used multiparameter flow cytometry combined with cytokine staining to directly evaluate the presence and function of CMV-specific $CD8^+$ T cells in blood samples that were obtained at days 30 and 80 posttransplantation in a subset of PBSC and BM recipients (D+/R+) with available PBMC. Cryopreserved PBMC were thawed, stimulated with CMVpp65 antigen, and then evaluated for IFN-γ, MIP-1β, TNF-α, and CD107a expression by intracellular staining. No statistical differences were documented in the absolute number of CMV-specific $CD8^+$ T cells between BM and PBSC recipients at either day 30 or 80 posttransplantation (Figure 4A and B).

Comparison of Polyfunctional CMV-Specific $CD8^+$ T Cells in BM and PBSC Recipients

Analyses of intracellular cytokine data using PESTLE and SPICE software allows for the further characterization of T cells as polyfunctional based on concurrent expression of different combinations of cytokines. Sixteen combinations were possible for $CD8^+$ T cells based on the evaluation of 4 parameters: IFN-γ, MIP-1β, TNF-α, and CD107a in this study. We grouped polyfunctional T cells as T cells positive for 4, 3, 2, or only 1 of these markers.

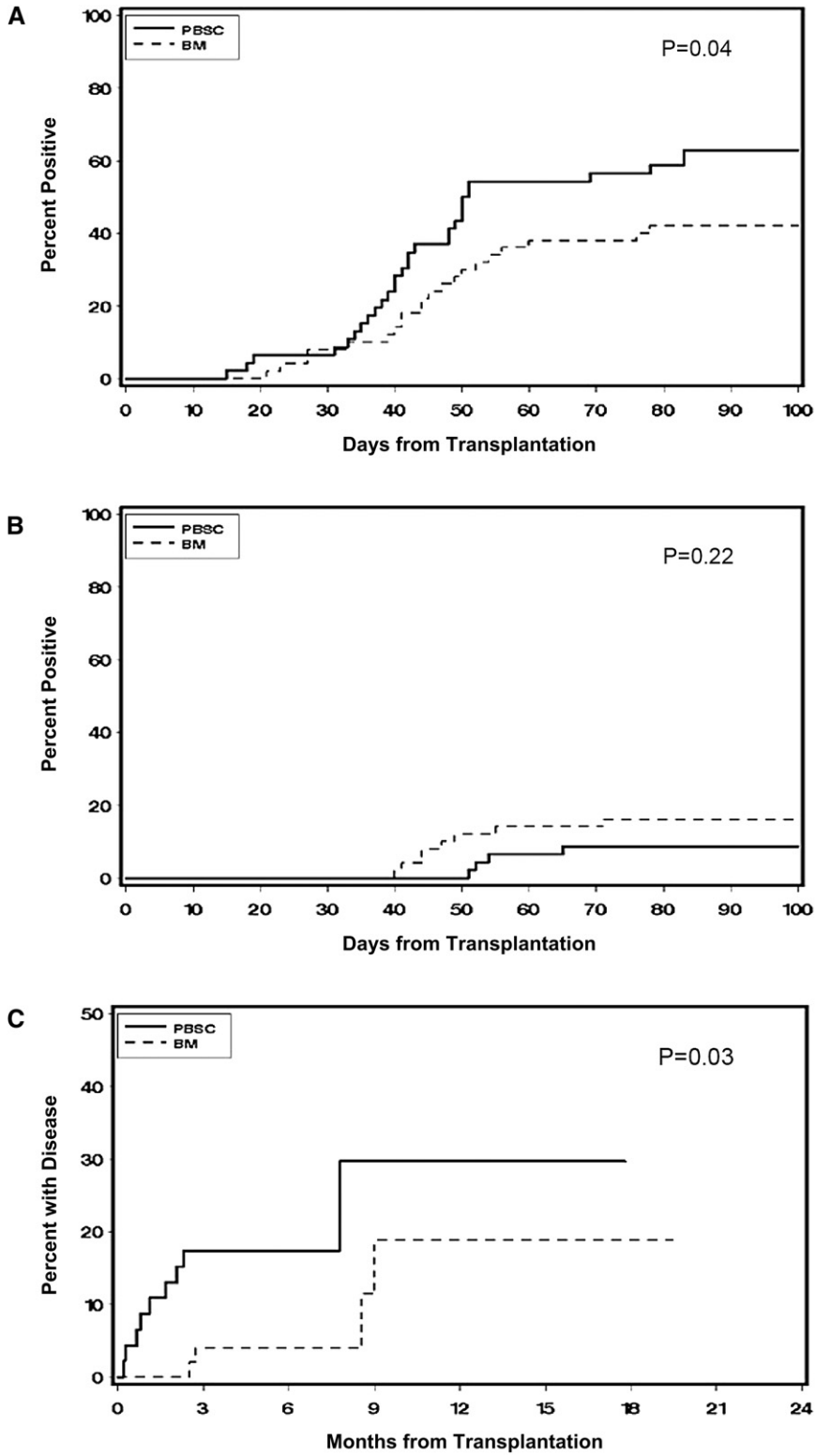

Figure 1. Differences in CMV infection and disease between BM and PBSC recipients. Time to positive antigenemia or PCR positivity at any level (A), antigenemia >100 positive cells per slide or CMV DNA >100,000 copies/mL plasma (B), and CMV disease (C) among CMV seropositive recipients (BM, n = 50; PBSC, n = 46).

At day 30 after transplantation, the overall proportion of the absolute number of polyfunctional CMV-specific $CD8^+$ T cells/L positive for 2, 3, and 4 markers in BM was 18%, 20%, and 27% compared with 6%, 8%, and 8%, respectively, for PBSC recipients. Single-cytokine-producing T cells encompassed the largest proportion of T cells in PBSC recipients at day 30 posttransplantation, and this fraction was

Table 2. Incidence of CMV Infection and Disease before 2 Years by CMV Serostatus

	BM					PBSC				
Manifestation of CMV	R+ N = 50	D+/R+ N = 36	D−/R+ N = 14	D+/R− N = 13	D−/R− N = 27	R+ N = 46	D+/R+ N = 29	D−/R+ N = 17	D+/R− N = 15	D−/R− N = 20
pp65 antigenemia or CMV DNA in plasma (day 100)										
Any level	42%*	42%	43%	0†	7%	63%*	59%	71%	19%†	10%
>10 + cells/slides or >1000 copies/mL	24%	25%	21%	0	0	26%	31%	18%	19%	5%
>50 + cells/slides or >10,000 copies/mL	16%	17%	14%	0	0	9%	14%	0	0	0%
CMV disease										
• Day 100 after HCT	4%‡	3%	7%	0	0	17%‡	14%	24%	0	0
• Two years after HCT	8%	6%	14%	0	0	20%	17%	24%	6%	0

R indicates recipient; D, donor; HCT, hematopoietic cell transplantation; CMV, cytomegalovirus; BM, bone marrow; PBSC, peripheral blood stem cell.
*P = .04, between BM and PBSC recipients who are R+.
†P = .03, between BM and PBSC recipients who are D+/R−.
‡P = .03, between BM and PBSC recipients who are R+.

significantly greater than the amount of single-cytokine-producing $CD8^+$ T cells per liter in BM recipients (PBSC, 77% [6.1×10^7 cells/L] vs BM, 36% [6.8×10^6 cells/L] (P = .006, Student test) (Figure 4C). However, by day 80, the proportion of polyfunctional CMV-specific $CD8^+$ T cells was similar between BM and PBSC recipients (Figure 4D).

Beyond single-cytokine-producing T cells, there was no difference in the absolute number of CMV-specific $CD8^+$ T cells/L positive for 2, 3, and 4 markers between BM and PBSC recipients (data not shown). This lack of quantitative difference prompted us to evaluate of the quality of T cell response rather than the quantity alone.

We analyzed the median fluorescence intensities (MFI) of each marker in quadruple-positive CMV-specific $CD8^+$ T cells to look for any differences in the capacity to produce cytokines or degranulate [29,30]. At day 30, we documented a trend toward higher MFI values for IFN-γ, MIP-1β, and CD107a, but not TNF-α, in BM compared with PBSC recipients. These differences were not significant, however, and were lost by day 80 (data not shown).

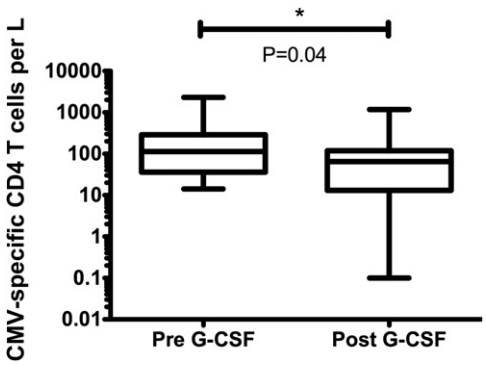

Figure 2. Effect of G-CSF on donor peripheral blood mononuclear cells (PBMC) and stem cell (SC) product. (A) Represents the number of CMV-specific $CD4^+$ T helper cells in donor PBMC and the stem cell product, pre- and post-G-CSF treatment (n = 12). P value is from the Wilcoxon matched pairs test.

We further analyzed the effect of polyfunctionality on the MFI values of IFN-γ based on the degree of decreasing polyfunctionality of CMV-specific $CD8^+$ T cells by the loss of 1 marker (TNF-α, MIP-1β, and CD107a). At day 30, the MFI values of IFN-γ in CMV-specific $CD8^+$ T cells from BM recipients decreased along with the loss of polyfunctionality compared with CMV-specific $CD8^+$ T cells from PBSC recipients, which increased along with the loss of polyfunctionality (Figure 4E). At day 80, MFI values of IFN-γ in CMV-specific $CD8^+$ T cells from both BM and PBSC recipients decreased with the loss of polyfunctionality (Figure 4F). Although not statistically significant, these differences in MFI values hint at the complexity of polyfunctional profiles in CMV-specific $CD8^+$ T cells isolated from BM and PBSC recipients. Overall, these observations support the notion that a delay in CMV immune reconstitution early posttransplantation in PBSC recipients is transient and associated not just with T cell quantity, but rather with the quality of T response.

Comparison of Polyfunctional T Cells between Patients with No Antigenemia and Those with Antigenemia

To determine the potential functional relevance of mono- versus polyfunctional CMV-specific T cells, we further compared the absolute number of polyfunctional CMV-specific $CD8^+$ T cells at day 30 in patients with and without subsequent antigenemia, regardless of transplant type. In this analysis, we defined polyfunctional T cells as any T cell positive for 2 or more markers. In patients with no antigenemia by day 80, the average number of polyfunctional T cells at day 30 was 2.14×10^7/L compared with 4.6×10^6/L in patients with any antigenemia (P = .09, Student test) (Figure 5A). In addition, a similar trend was documented between the average numbers of CMV-specific $CD4^+$ T cells in patients with no antigenemia (1.8×10^5 cells/L) compared with those with any antigenemia

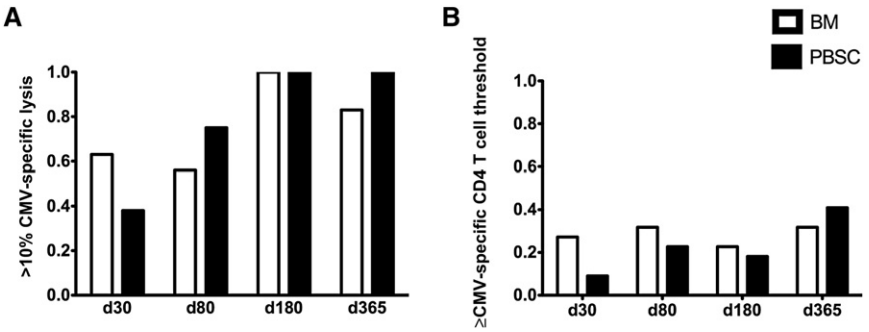

Figure 3. Differences in CMV-specific T cell immune reconstitution between BM and PBSC recipients. (A) Represents the proportion of patients with CMV-specific cell lysis greater than 10% between BM and PBSC recipients at day 30 (BM, n = 8; PBSC, n = 8), 80 (BM, n = 9; PBSC, n = 8), 180 (BM, n = 6; PBSC, n = 7), and 365 (BM, n = 6; PBSC, n = 4) posttransplantation. (B) Represents the proportion of patients with ≥33 CMV-specific $CD4^+$ T cells per 10^6 PBMC as determined by limiting dilution assay at day 30, 80, 180, and 365 (BM, n = 22; PBSC, n = 22) posttransplantation.

(5.7×10^3 cells/L) by LDA analysis ($P = .3$, Student t test) (Figure 4B). These results suggest that regardless of stem cell source, polyfunctional T cells are associated with protection from CMV reactivation.

DISCUSSION

In this study, we compared the temporal pattern of CMV reactivation, CMV disease, and CMV-specific immune reconstitution in patients enrolled in a randomized trial of BM or PBSC as a graft source for transplantation. We observed a higher incidence of CMV infection and CMV disease early after transplantation in PBSC recipients. We hypothesized that the differences in virologic data resulted from differences in the reconstitution of a CMV-specific immune response between BM and PBSC recipients after transplantation.

In contrast to the findings in nonrandomized studies, we observed a higher rate of CMV infection and disease in PBSC recipients. Walker et al. [31] showed a delay in the development of CMV antigenemia in PBSC recipients, but no overall difference in the incidence of antigenemia between BM and PBSC recipients. Trenschel et al. [32] reported a similar incidence of CMV antigenemia, but less persistent antigenemia and a trend toward less CMV disease in PBSC recipients. On the other hand, Manteiga et al. [33] found higher rates of CMV infection and disease in PBSC recipients, which is in agreement with our findings. Possible reasons for these differences include different proportions of unrelated donors, use of T cell depletion in some studies, high-dose acyclovir prophylaxis, and differences in the patient populations in terms of age, disease status, and prior therapy across these studies.

Our results stem from a randomized study with a well-balanced distribution of pre- and posttransplantation risk factors for CMV (Table 1). The increase in early CMV reactivation that we observed in PBSC recipients was not explained by earlier onset of GVHD or an increased use of corticosteroids. Therefore, it is theoretically possible that the increase of CMV reactivation resulted from higher rates of CMV transfer in the donor PBSC product, perhaps within granulocytes or hematopoietic progenitors. Granulocyte transfusions have been associated with an increased risk of CMV infection [34-36]. An alternative and not mutually exclusive explanation for the differences in CMV infection and disease is that G-CSF treatment promotes the emergence of CMV from latency, either by direct effects of G-CSF signaling in latently infected cells or by reducing the ability of T cells to limit reactivation [37]. Heightened transfer of CMV is partially supported by a trend toward a higher transmission rate in the CMV D+/R− setting, but not among R+ patients (Table 2). However, the number of D+/R− patients in this cohort was too small and the CMV event rate in the D+/R− BM recipients was unusually low [38], making it difficult to test this possibility statistically. No definitive conclusions can be drawn, and larger studies are needed to more thoroughly examine this question.

Mielcarek et al. [39,40] proposed that monocytes in G-CSF-treated PBMC are capable of inhibiting T cell proliferation, possibly resulting in lower GVHD following HSCT. We did not find any association between the number of monocytes and CMV-specific $CD4^+$ T cells after G-CSF treatment (data not shown). We did observe, however, that G-CSF treatment lowered the number of CMV-specific $CD4^+$ T cells in the donor product detected by a functional assay that requires T cell proliferation. Our proposal that a deficiency of functional CMV-specific $CD4^+$ T cells in PBSC contributes to the delayed recovery of immunity in PBSC recipients is supported by work done by Pourgheysari et al. [41], who demonstrated that early posttransplantation deficiencies in CMV-specific $CD4^+$ T cells are associated with a high risk of viral

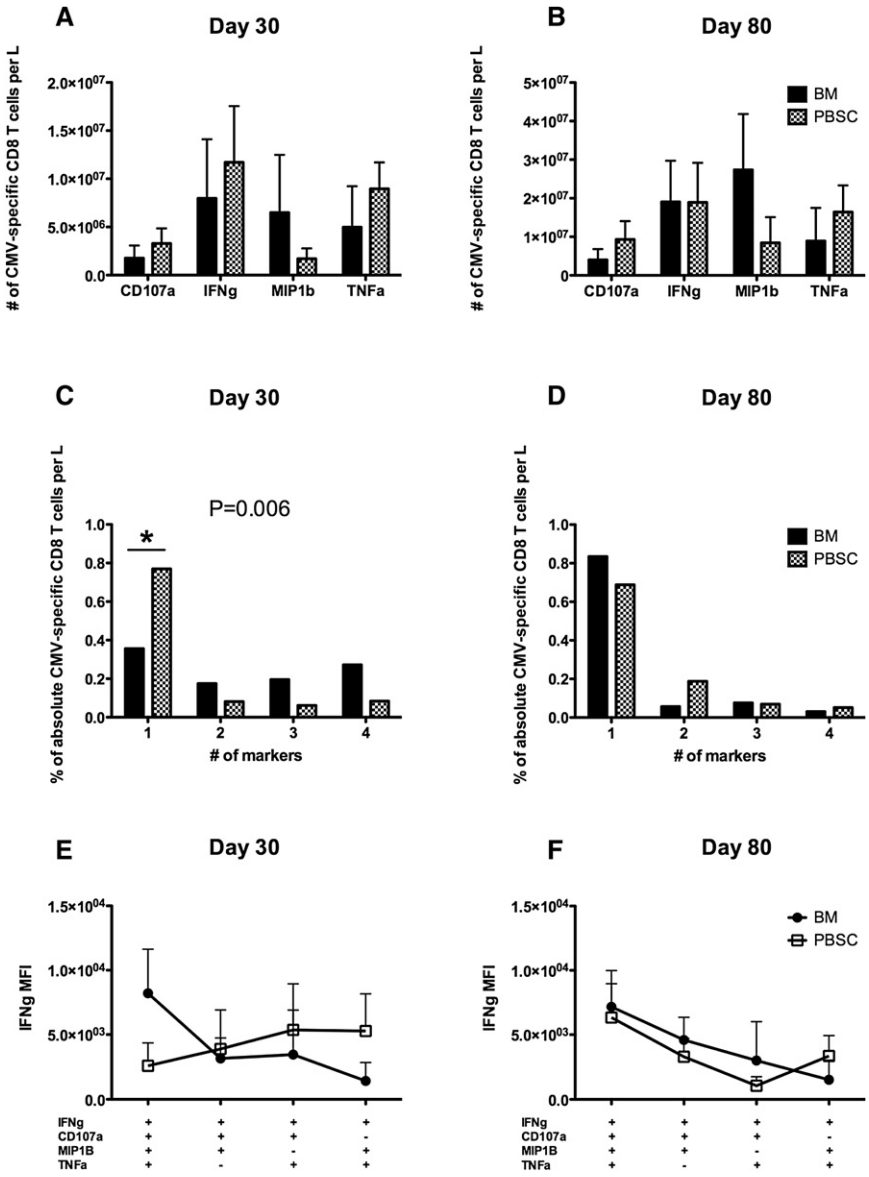

Figure 4. Differences in polyfunctional CMV-specific $CD8^+$ T cells between BM and PBSC recipients. (A, B) Represents the absolute number of $CD8^+$ T cells in BM ($n = 7^{\dagger}$) and PBSC ($n = 6^{\dagger}$) recipients positive for CD107a, IFN-γ, MIP-1β, and TNF-α at day 30 and day 80 posttransplantation. (C, D) Represents the absolute proportion of polyfunctional CMV-specific $CD8^+$ T cells within BM and PBSC recipients at day 30 and day 80. (E, F) Represents the MFI of IFN-γ based on the degree of decreasing polyfunctionality of CMV-specific $CD8^+$ T cells by the loss of one marker (CD107a, MIP-1β, and TNF-α). Polyfunctional T cells grouped by T cells positive for 1, 2, 3, or 4 of the markers based on the 16 possible combinations according to SPICE. The *P* value is a result of the Student test. Bars represent the mean value within each group, and error bars are the standard error of the mean.

reactivation in patients following allogeneic HSCT (90% of the patients in that study were PBSC recipients). Impairment of both $CD4^+$ and $CD8^+$ T cells before 100 days after transplantation was also associated with late viral reactivation in that study [41]. A plausible explanation for the virologic differences observed in our study is impairment of the quantity or quality of CMV-specific T cells early after PBSC transplantation. At day 30 posttransplantation in PBSC recipients, we documented (1) fewer CMV-specific $CD4^+$ T cells, (2) less CMV-specific cell lysis, (3) a greater proportion of $CD8^+$ CMV-specific T cells that mainly produced a single cytokine, and (4) a lower proportion of polyfunctional T cells together with a lower capacity for cytokine production and cytotoxicity.

The correlation of polyfunctional T cells with control of viral infection was first studied in HIV infection [42]. Betts et al. [42] demonstrated that individuals with nonprogressive HIV infection maintain polyfunctional HIV-specific $CD8^+$ T cells that correlate inversely with viral load. Similarly, Darrah et al. [30] demonstrated that protection against *Leishmania major* infection in mice is associated with polyfunctional $CD4^+$ T cells with enhanced effector function. Polyfunctional T cells are also known to have a higher capacity for cytokine production and cytotoxicity compared with

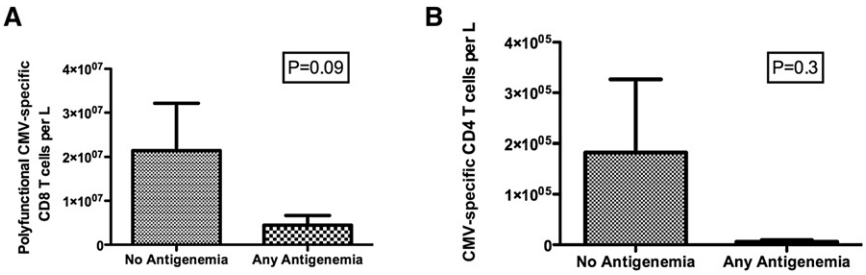

Figure 5. Comparison of CMV-specific polyfunctional $CD8^+$ T cells between patients with no antigenemia and those with antigenemia. (A) Represents the number of polyfunctional T cells/L at day 30 in patients with no (n = 5†) and any subsequent antigenemia (n = 7†) at day 30 regardless of stem cell source. (B) Represents the number of CMV-specific CD4 T cells/L (by LDA) at day 30 in patients without (n = 20†) and any subsequent antigenemia (n = 11†). †Includes patients who were eligible for randomization but did not participate in the randomized trial. Bars represent the mean value within each group, and error bars are the standard error of the mean.

single-cytokine-producing T cells [29]. When we compared CMV-specific T cell function between patients with no antigenemia versus those with antigenemia, we found a trend toward a larger number of polyfunctional T cells in patients with no antigenemia. These observations further support the association of polyfunctional T cells with protective immunity.

It is important to recognize that our polyfunctional studies were done on a small number of patients because of limited sample availability. Because of the resulting low statistical power for many of the comparisons, we primarily noted statistical trends. Our findings are consistent, however, with a prior study by Lilleri et al. [43], who also found that polyfunctional CMV-specific T cells producing IFN-γ and IL-2 were associated with protection. In addition, the increase of MFI values of IFN-γ, with decreased CMV-specific $CD8^+$ polyfunctionality in PBSC recipients at day 30 aligns with the work of Krol et al. [44], who suggested that production of IFN-γ alone may be a sign of T cell exhaustion during viral infection. We recently showed, using CMV-specific tetramers, that HCT patients with ≥7 positive tetramer cells/μL in at least 1 blood sample before day 65 posttransplantation were statistically protected from CMV infection [45], although the protection was not complete. Polyfunctional T cells may provide an improved functional marker of protection, but larger prospective studies are needed to demonstrate this conclusively.

In conclusion, PBSC recipients early after posttransplantation demonstrate more CMV reactivation and disease. These findings appear to be temporarily related to a transient delay in CMV-specific immune reconstitution, perhaps as a consequence of G-CSF treatment. Whether T cell polyfunctionality is a definitive marker of protection from CMV reactivation requires larger prospective studies.

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SUPPLEMENTARY DATA

Supplementary data related to this article can be found online at doi:10.1016/j.bbmt.2011.05.010.

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