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Rapid Acquisition of Cytomegalovirus-Specific T Cells with a Differentiated Phenotype, in Nonviremic Hematopoietic Stem Transplant Recipients Vaccinated with CMVPepVax

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Early cytomegalovirus (CMV) reactivation remains a significant cause of morbidity and mortality in allogeneic hematopoietic cell transplant (HCT) recipients. CMVPepVax is an investigational peptide vaccine designed to control CMV infection in HCT recipients seropositive for CMV by stimulating the expansion of T cell subsets that target the CMV tegument protein pp65. In a randomized Phase Ib pilot trial (ClinicalTrials.gov NCT01588015), two injections of CMVPepVax (at days 28 and 56 post-HCT) demonstrated safety, immunogenicity, increased relapse-free survival, and reduced CMV reactivation and use of antivirals. In the present study, we assessed the phenotypes and time courses of the pp65-specific CD8 T cell subsets that expanded in response to CMVPepVax vaccination. The functionality and antiviral role of CMV-specific T cells have been linked to immune reconstitution profiles characterized predominantly by differentiated effector memory T (TEM) subsets that have lost membrane expression of the costimulatory molecule CD28 and often reexpress the RA isoform of CD45 (TEMRA). Major histocompatibility complex class I $pp65_{495\text{-}503}$ multimers, as well as CD28 and CD45 memory markers, were used to detect immune reconstitution in blood specimens from HCT recipients enrolled in the Phase Ib clinical trial. Specimens from the 10 (out of 18) vaccinated patients who had adequate (≥.2%) multimer binding to allow for memory analysis showed highly differentiated TEM and TEMRA phenotypes for $pp65_{495\text{-}503}$-specific CD8 T cells during the first 100 days post-transplantation. In particular, by day 70, during the period of highest risk for CMV reactivation, combined TEM and TEMRA phenotypes constituted a median of 90% of $pp65_{495\text{-}503}$-specific CD8 T cells in these vaccinated patients. CMV viremia was not detectable in the patients who received CMVPepVax, although their $pp65_{495\text{-}503}$-specific CD8 T cell profiles were strikingly similar to those observed in viremic patients who did not receive the vaccine. Collectively, our findings indicate that in the absence of clinically relevant viremia, CMVPepVax reconstituted significant levels of differentiated $pp65_{495\text{-}503}$-specific CD8 TEMs early post-HCT. Our data indicate that the rapid reconstitution of CMV-specific T cells with marked levels of effector phenotypes may have been key to the favorable outcomes of the CMVPepVax clinical trial.

INTRODUCTION

Cytomegalovirus (CMV) is one of the largest and most complex of all known viruses, with a genome encoding approximately 165 genes. CMV is widely prevalent globally but is immunologically controlled in healthy individuals with an intact immune system. The immune effector mechanisms involved do not eliminate the virus or preclude transmission but can control viral replication and prevent disease. High frequencies of CMV-specific CD8 T cells are detectable in the peripheral blood of healthy individuals [1]. This suggests that a significant proportion of the T cell repertoire is devoted to the control of this persistent virus. In particular, CMV infection maintains high frequencies of highly functional effector memory T cells in both lymphoid and extralymphoid sites. These effector T cells control viral replication mainly through cytokine secretion and direct cytotoxicity [2].

Early immune reconstitution of CMV-specific T cells is critical for viral control after allogeneic hematopoietic cell transplantation (HCT) [3,4]. Even with preemptive antiviral therapy,

Corinna La Rosa and Jeff Longmate contributed equally to this investigation, and should be considered co-first authors.

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CMV reactivation and uncontrolled viremia frequently occur in CMV seropositive patients within the first 100 days post-HCT, due to the immunosuppressive regimens required for the procedure [3]. CMV viremia remains associated with profound defects in immune reconstitution and increased transplantation-related mortality [5,6]. Stimulating viral immunity and increasing the magnitude of functional CMV-specific T cells early post-transplantation by vaccination may promote CMV viremia control [7]. The compromised immune system of HCT recipients is still able to mount an adaptive response to CMV, despite effective immunosuppression of allospecific T cell-mediated graft rejection [1]. In this context, the goal of a protective CMV vaccine is to quantitatively and qualitatively enhance the nascent immune response early post-HCT in CMV-seropositive recipients [5]. A safe and protective vaccine that enables the patient's immune system to control CMV reactivation is highly desirable in view of the potential positive impact on HCT outcomes, reduction of antiviral drug use, and decreased healthcare costs [7].

The pp65 tegument protein is among the most frequently immunologically recognized CMV antigens in CMV-seropositive healthy adults [8]. Reconstitution of cytotoxic CD8 T cells targeting the pp65 tegument protein of CMV after HCT correlates with decreased frequency of early CMV reactivation and improved outcomes of CMV disease [9-13]. CMVPepVax, one of few promising vaccine candidates for CMV-seropositive HCT recipients is a chimeric peptide composed of a cytotoxic HLA-A*0201-restricted CD8 T cell epitope from pp65 [14,15]. The $pp65_{495\text{-}503}$ epitope contained in CMVPepVax is fused with the P2 epitope of tetanus toxin, which provides T helper function. Formulated with the adjuvant PF03512676 (Pfizer, New York, NY), a Toll-like receptor 9 agonist that augments cellular immunity [16], CMVPepVax was first evaluated in healthy adults. An acceptable safety profile and vaccine-driven expansion of pp65 T cells when used with PF03512676 adjuvant supported its further evaluation in HCT recipients [15].

In a randomized pilot trial (ClinicalTrials.gov identifier NCT01588015), CMVPepVax was safely administered on days +28 and +56 post-HCT to a cohort of 18 CMV-seropositive HCT patients, who are at the highest risk for CMV reactivation. The primary outcome was safety; secondary outcomes included immunogenicity, prevention of CMV reactivation, and clinical outcomes. PepVax was well tolerated and did not induce any adverse effect on HCT or cases of acute graft-versus-host disease, and no unexpected adverse events. Compared with an observational cohort (n = 18) who did not receive the investigational vaccine treatment, PepVax-vaccinated HCT recipients achieved 3 major outcomes: a rise in average pp65-specific absolute CD8 T cell counts (cells/μL) during the critical first 100 days post-HCT (3.5-fold versus 1.4-fold; $P = .025$); reduced incidence of CMV reactivation (1 event versus 6 events; $P = .039$) necessitating the use of antivirals (15 versus 263 days; $P = .03$); and increased relapse-free survival (1 event versus 7 events; $P = .015$) [16]. This was the first vaccine approach in the HCT setting to demonstrate the feasibility of expanding CMV-specific T cells early post-HCT. Based on these favorable outcomes, PepVax is currently being tested in a placebo-controlled Phase II multicenter trial (NCT02396134).

Several aspects of the dynamic relationship between CMV reactivation and the immune response remain to be clarified. Recognition of CMV antigens by the immune system leads to multiple changes in lymphocyte functions, cell surface phenotypes, and ability to expand and migrate to different tissues [17]. The capacity of CMV-specific T cells to control the virus depends on both the magnitude of the T cell response and the functional memory phenotype of the expanded CMV-specific T cell subsets [18,19]. Multifunctional and actively proliferating CMV-specific T cells with antiviral activity have a predominantly differentiated memory phenotype characterized by the loss of membrane expression of the costimulatory molecule CD28 (TEMs; $CD28^-$). The effector memory T cell population includes a large subset that reexpress the RA isoform of CD45 (TEMRAs; $CD28^-CD45RA^+$) [2,20-22]. In healthy CMV-seropositive individuals, high frequencies of activated CMV-specific TEMs and TEMRAs are associated with a lack of virus detection in the blood, whereas more quiescent $CD28^+CD45^-$ central memory (TCM) and naïve $CD28^+CD45^+$ subsets of CMV-specific T cells are found in lung tissue along with detectable virus [2]. Interestingly, when CMV reactivation occurs post-HCT, almost all responding CD8 T cells exhibit a more differentiated TEM and TEMRA phenotype, which is consistent with the recruitment of effector T cells to immunologically control CMV viremia [18]. Therefore, an effective CMV vaccine should elicit the expansion of CMV-specific T cells with an effector phenotype to achieve viral containment early post-HCT, when the risk of CMV reactivation is greatest [23]. To date, only a few studies in healthy adults have analyzed the differentiation pattern of memory T cells following immunization with a CMV candidate vaccine [24,25].

The present study was designed to investigate this aspect in the context of transplantation by longitudinally investigating the memory repertoire of CMV specific T cells, in CMVPepVax-vaccinated HCT patients. We assessed blood specimens collected in the Phase Ib trial to monitor the impact of the CMVPepVax vaccination on the phenotype and time course of $pp65_{495\text{-}503}$-specific CD8 T cells expanded in vaccinated CMV-seropositive HCT patients, in which CMV viremia was controlled. We aimed to characterize the frequency and type of memory profiles of the patients in whom a protective immune response developed to better understand the role of the CMVPepVax in preventing CMV viremia.

METHODS

Phase Ib Clinical Trial: Design and Procedures

This longitudinal immune phenotype analysis was performed using blood specimens from patients participating in a single-site, randomized, open-label, parallel-arm, Phase Ib trial conducted at the City of Hope Comprehensive Cancer Center [16]. Enrolled patients, who underwent allogeneic HCT for hematologic malignancies, provided written informed consent in accordance with the Declaration of Helsinki. Eligible participants were CMV-seropositive, positive for HLA-A*0201, age 18 to 75 years, willing to be monitored for at least 6 months, and had either a related or unrelated donor with 8/8 or 7/8 (HLA-A, -B, -C, and -DRB1) high-resolution donor HLA allele matching. Per protocol, T cell depletion with T cell-depleting antibodies (alemtuzumab or antithymocyte globulin) was not allowed. Further details on the patients and transplantation regimens have been fully reported elsewhere [16]. On day +28 post-HCT, a computer-generated blocked randomization, stratified by CMV donor serostatus, assigned each eligible recipient to the vaccine arm (VA; n = 18) or to the noninterventional, observation arm (OA; n = 18). In the VA, eligible patients were injected s.c. twice (on days +28 and +56 post-HCT) with CMVPepVax. The cGMP-grade peptide vaccine (NSC721434) consists of the immunodominant HLA-A*0201-specific $pp65_{495\text{-}503}$ (NLVPMVATV) CD8 T cell epitope fused with the P2 epitope of tetanus toxin ($tt_{830\text{-}843}$: QYIKANSKFIGITE) [14,15]. The adjuvant, PF03512676, is a synthetic, 24-nucleotide, single-stranded phosphorothioate DNA-containing CpG motifs. PF03512676, supplied by Pfizer, is classified as an investigational agent. The CMVPepVax formulation contains 2.5 mg of NSC-721434 peptide vaccine solution and 1.08 mg of PF03512676 in a final injection volume of 1 mL [15].

Blood draws for immune monitoring were performed in all enrolled patients (n = 36) every 2 weeks on post-HCT days +28 to +100, then at days +130, +160, and +180. Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood using standard Ficoll-Paque techniques and cryopreserved in centrally monitored liquid nitrogen tanks [16]. CMV-specific immunogenicity was monitored by measuring levels of CD8 T cells binding to MHC class I $pp65_{495\text{-}503}$ and HIV$gag_{77\text{-}85}$ pentamers (ProImmune, Oxford, UK) [16]. CMV viremia was observed by quantitative PCR twice weekly on post-HCT days +21 to +100 and subsequently as necessary. Quantitative results were reported between 1250 and 1.25×10^6 IU/mL. Patients received

preemptive anti-CMV therapy at CMV PCR values ≥3750 IU/mL. Preemptive therapy was recommended for CMV PCR values <3750 IU/mL only when patients were considered at high risk for CMV disease, such as when receiving high-dose steroids.

Patient Characteristics

The present study included available specimens from HCT recipients enrolled in the CMVPepVax Phase Ib clinical trial [16] who developed ≥.2% of pp65-specific CD8 T cells between post-HCT days +28 and +100, when patients are at greatest risk of CMV reactivation and uncontrolled viremia [3] (Table 1). This was also the time frame in which patients in the VA exhibited a significantly higher increase in CMV-specific CD8 T cells and lower rates of CMV reactivation compared with patients in the OA [16]. A T cell value ≥.2% is considered an adequate functional sensitivity threshold for gating strategies in multimer binding assays and memory immune phenotyping analyses. Therefore, memory phenotype analyses of CMV-specific responses with a frequency <.2% could not be performed, because they were lower than the assay's limit of detection [20,21,25,26].

MHC Class I Multimer Binding and Memory Membrane Marker Staining

PBMC labeling was performed as described previously [25], with HLA-A*0201-specific MHC class I $pp65_{495-503}$ dextramers (APC; ImmuDex, Copenhagen, Denmark) in combination with conjugated antibodies (BD Biosciences, San Jose, CA) specific for CD3 (PerCP Cy5.5), CD8 (V450), CD28 (FITC), and CD45RA (PE) at the time points indicated in Table 1 for each of the evaluated patients. HLA-A*0201-specific HIVgag_{77-85} dextramers were used for background binding control. (All HCT recipients enrolled in the PepVax clinical trial were HIV-negative.) For each sample, 1×10^6 PBMCs were used, and the intensity of the bound fluorescent labels was measured using multiparameter (5-color) fluorescence-activated cytometry (FC). Approximately 300,000 events per sample were acquired by FC on a Gallios flow cytometer with Kaluza software (Beckman Coulter, Brea, CA). A primary lymphocyte gate was set on forward and side scatter of PBMCs, and subsequent gates were set on $CD3^+$ and $CD8^+$ populations. The amount of T cells specific for the $pp65_{495-503}$ epitope was expressed as a percentage of the respective $CD3^+CD8^+$ reference population. The analysis of CD28 and CD45RA memory membrane markers was conducted when $CD3^+CD8^+$ T cell binding to the $pp65_{495-503}$ dextramer was ≥.2%, following previously reported gating strategies [20,21,25]. Figure 1 illustrates our gating strategy and the subsequent identification of memory markers.

4-1BB (CD137) Surface Marker and Memory Phenotype Staining

PBMCs were stimulated for 24 hours with the entire pp65 peptide library (HCMV pp65 Peptide Pool; National Institutes of Health AIDS Reagent Program), the immediate early 1 (IE1) exon 4 library prepared in house [27], or medium (background control) [28]. Cells were then stained with CD3 (PerCP Cy5.5), CD8 (V450), CD4 (PE Cy7), CD137 (APC), CD28 (FITC), or CD45RA (PE) antibodies (BD Biosciences). The pp65- or IE1-specific $CD137^+$ T cell content was expressed as a percentage of the $CD3^+CD8^+$ or $CD3^+CD4^+$ T cell populations. When the pp65- or IE1-specific T cell percentage was ≥.2%, further analysis for CD28 and CD45RA memory membrane markers was performed, as described for the dextramer binding and memory analyses [20,21,25]. For each sample, $\sim 1 \times 10^6$ PBMCs were used, and 200,000 events were acquired and analyzed by 6-color FC.

Statistical Analysis

The present study assessing the immune response in patients enrolled in a randomized trial was observational in nature. The longitudinal patterns of CMV-specific T cell phenotypes are reported when the relevant T cell levels were sufficient for a phenotypic classification. The analysis relies largely on graphical displays to broadly illustrate the comparison of CMV-specific immunity observed following vaccination, to that arising following CMV reactivation in unvaccinated OA patients. T cell phenotype proportions were recorded for each of 4 mutually exclusive categories (naive, TCM, TEM, and TEMRA), with proportions summing to 1. The 2 categories of effector memory cells, TEM and TEMRA, were combined for graphical and statistical summaries. T cell concentrations are shown on individual longitudinal trajectories, with the combined TEM and TEMRA proportions as thermometer glyphs. Fisher's exact test was used to compare study arms in terms of the number of patients with measurable T cell levels. Wilcoxon rank-sum tests, with 95% confidence intervals for a shift parameter (difference in percentages), were used to compare phenotype percentages between study arms, conditional on measurability. For this purpose, individuals were represented by the value at day +100 or nearest to day +100, to avoid stochastic dependency and give equal weight to each patient. Statistical analyses were performed using R version 3.4.3 (R Foundation for Statistical Computing, Vienna Austria), and GraphPad Prism version 7.01 for Windows (GraphPad Software, San Diego, CA).

RESULTS

Patient Cohort

As detailed in Table 1, 12 VA patients and 8 OA trial patients (out of 18 for each arm) developed levels of CMV-specific T cells evaluable for memory phenotyping in at least 1 of the assays performed, with each assay evaluable in at least 16 patients. The remaining 6 VA and 10 nonviremic OA trial patients had insufficient pp65 or IE1 recognition to perform memory analysis during the first 100 days post-HCT and thus were not included in Table 1. The 20 patients included in Table 1 includes 10 patients with a matched related donor (MRD) equally distributed between the 2 arms and 10 patients with a matched unrelated donor, 7 of whom were assigned to the VA. A total of 8 patients, 4 in each arm (representing 33% of the VA versus 50% of the OA) received a transplant from a CMV-seronegative donor (D^- versus D^+, seropositive). Of note, CMV donor serostatus did not significantly impact CMV-specific T cell levels in the CMVPepVax trial [16]. Eleven of the 12 VA patients in Table 1 did not reactivate CMV during the 6-month study period. Five of the OA patients (unique patient numbers [UPNs] 6, 12, 15, 27, and 28) and 1 VA patient (UPN 7) developed viremia necessitating antiviral treatment. UPN 19 exhibited late CMV reactivation (at day +147) and was the only patient in the trial whose viremia was controlled without antivirals. In this viremic OA patient, a level of CMV-specific T cells ≥.2% was detected starting at day +130. Nonetheless, UPN 19 was included in the study to assess the role of memory phenotype in viral containment. UPN 17 was the only nonviremic OA patient who had ≥.2% $pp65_{495-503}$ dextramer binding within 100 days post-HCT. UPNs 2, 3, 7, and 12 had minimal (∼.1%) $pp65_{495-503}$-specific T cells, whereas recognition of pp65 and/or IE1 peptide libraries was >.2% at the same time points; thus, memory analysis phenotyping was performed for the CD137 CMV peptide library-based assay. In the remaining 16 patients, memory phenotype analysis was conducted concomitantly using cell-surface staining of $pp65_{495-503}$- and CMV peptide library-specific T cells.

Quantification of CMV-Specific CD8 T Cells by Multimers

In the present study, the frequency (%) of CMV-specific T cells was quantified using MHC class I dextramers, which have been described as a suitable and effective multimer tool for enumerating CMV-specific T cells in the transplantation setting [29,30]. The initial immunogenicity evaluation was carried out with pentamers, as reported previously [16], with the dextramer used when T cell levels appeared to be adequate at the initial assessment. In the present analysis, dextramers were capable of enumerating CMV-positive cells equally across PBMC samples with varying magnitudes of $pp65_{495-503}$-dextramer binding, as shown in Supplementary Figure 1. Background binding to the control HLA-A*0201-specific HIVgag_{77-85} (<.1%; data not shown) was comparable in the two dextramer and pentamer multimers.

There was wide variability in $pp65_{495-503}$-specific T cell levels in HCT recipients in both trial arms before randomization and in the absence of clinically detectable viremia, as we detailed in a previous report [16]. This finding of marked variability in CMV-specific T cell responses has been commonly observed in CMV-seropositive healthy adults and HCT recipients [6,15,25]. The median percentages of $pp65_{495-503}$ dextramer binding were similar in both arms: 5% (range, .1% to 13%) in the OA arm and 4% (range, .1% to 14%) in the VA arm.

Phenotypic Characterization of CMV $pp65_{495-503}$-Specific T Cells

The differentiation status of CMV-specific $CD3^+$ $CD8^+$ T cells, identified using dextramers bound to $pp65_{495-503}$ epitopes,

Table 1
Characteristics of the Study Cohort

UPN*	CMV Serostatus†	Arm‡	Type of HCT§	CMV Viremia‖	First Viremic Day‖	Memory Analysis: Days Post-HCT Evaluated				
						$pp65_{495-503}$-Specific CD8 T Cells¶	pp65-Specific CD8 T Cells#	IE1-Specific CD8 T Cells**	pp65-Specific CD4 T Cells#	IE1-Specific CD4 T Cells**
2	D-	OA	MUD	NO	NA	NA	NA	42, 100	NA	NA
17	D+	OA	MRD	NO	NA	70, 84, 130	NA	NA	70, 84, 130	84
6	D+	OA	MRD	YES	33	28, 42, 56, 84	42, 56, 84	42, 56, 84	42, 84	42
12	D-	OA	MUD	YES	38	NA	28, 70, 84, 100, 160	28, 70, 84, 100, 160, 180	70, 84, 100, 160, 180	100, 160, 180
15	D+	OA	MRD	YES	63	28, 70, 84, 180	28, 70, 84, 180	28, 70, 84, 180	28, 70, 84, 180	28, 70, 180
19	D-	OA	MRD	YES	147	130, 160, 180	130, 160, 180	130, 160, 180	130, 180	130, 160, 180
27	D-	OA	MUD	YES	41	100, 130	42, 100, 130	NA	42,100, 130	100
28	D-	OA	MRD	YES	36	42, 100, 160	42, 100, 160	100, 160	42, 100, 160	100, 160
3	D+	VA	MUD	NO	NA	NA	42, 84, 100	42, 84, 100	42	NA
8	D+	VA	MRD	NO	NA	28, 70, 100, 180	28, 70, 100, 180	28, 70, 100, 180	28, 100	100
14	D+	VA	MRD	NO	NA	28, 56, 70, 84, 100, 130, 160, 180	28, 56, 70, 84, 100, 130, 160, 180	28, 56, 84, 100, 130, 160, 180	28, 56, 130, 160, 180	56, 130, 180
16	D+	VA	MRD	NO	NA	70, 100, 180	100, 180	70, 100, 180	70, 100, 180	70, 100, 180
18	D+	VA	MRD	NO	NA	28, 56, 70, 84	56, 84	NA	56, 84	NA
26	D-	VA	MUD	NO	NA	70, 100, 180	70, 100, 180	70, 100, 180	70, 100, 180	70, 100, 180
30	D+	VA	MUD	NO	NA	56, 70, 100, 160	56, 70, 100, 160	NA	56, 70, 100, 160	56, 70, 100, 160
32	D-	VA	MUD	NO	NA	84, 100, 130, 160, 180	84, 100, 130, 160, 180	84, 100, 130, 160, 180	84, 100, 130, 160, 180	84, 100, 130, 180
33	D+	VA	MUD	NO	NA	70, 100, 130, 160, 180	70, 100, 130, 160, 180	70, 100, 130, 160, 180	70, 100, 130, 160, 180	100, 130, 160, 180
35	D+	VA	MUD	NO	NA	28, 42, 56, 70, 84, 100, 130, 160	28, 42, 56, 70, 84, 100, 130, 160	28, 42, 56, 70, 84, 100, 130, 160	28, 42, 56, 70, 84, 100, 130, 160	28, 70, 130, 160
36	D-	VA	MRD	NO	NA	70, 84, 100, 130	70, 84, 100, 130	70, 84, 100, 130	70, 84, 100, 130	70, 84, 100, 130
7	D-	VA	MUD	YES	42	NA	42	NA	42, 70, 84	NA

MUD indicates matched unrelated donor; NA, not assessed because <.2% of T cells were CMV-specific.

* UPN in the Phase Ib CMVPepVax clinical trial [18], sorted by study arm (VA or OA) and CMV viremia status (yes/no).

† CMV serostatus of the donor (D).

‡ Trial arm to which the patient was randomly assigned: VA or OA.

§ Type of allogeneic HCT: MRD or MUD.

‖ CMV viremia ≥1250 IU/mL.

¶ Post-HCT days at which memory phenotypes were assessed with $pp65_{495-503}$ multimer binding.

Post-HCT days at which memory phenotypes were assessed with the pp65 peptide library in the CD137 assay.

** Post-HCT days at which memory phenotypes were assessed with the IE1 peptide library in the CD137 assay.

was determined by quantifying the percentages of CD28 and CD45RA cell-surface phenotypic markers by polychromatic flow cytometry panels [20,21,25]. After gating of $CD3^+CD8^+$ T cells, and subsequently $pp65_{495-503}$-specific $CD3^+CD8^+$ T cells, 4 subpopulations were identified according to the expression of CD28 and CD45RA (Figure 1). $CD45RA^+CD28^+$ cells were classified as naïve, $CD45RA^-CD28^+$ cells were classified as central memory (TCM), and $CD28^-$ cells were classified as effector T cells. Within the effector T cell group, 2 subpopulations were identified: $CD45RA^-CD28^-$ cells (TEM) and $CD45RA^+CD28^-$ effector "revertant" T cells, reexpressing the RA isoform of the CD45 surface marker (TEMRA) [22]. Representative analysis of a VA HCT patient who did not develop viremia (D^+ MRD UPN 8, at day 70 post-HCT) is reported in Figure 1. This shows a large population of $CD3^+CD8^+pp65^+$ T cells, with massive accumulation of effector T cells with a $CD28^-$ phenotype (Figure 1B, right). The highly preferential effector phenotype of the pp65-specific T cells contrasts with the overall CD8 T cell population (Figure 1C, right), which, as expected, is composed more equally of $CD28^+$ and $CD28^-$ T cells [4,6].

Patterns of Memory Types for $pp65_{495-503}$-Specific CD8 T Cells

Memory phenotype frequencies of $CD3^+$ $CD8^+$ T cells specific for the $pp65_{495-503}$ epitope were longitudinally analyzed in 10 nonviremic VA patients and 6 OA patients, 5 of whom were viremic (see Table 1). The VA patients more often achieved a ≥.2% level of $pp65_{495-503}$-specific CD8 T cells in the absence of clinically detectable CMV viremia (10 nonreactivating patients of 17 in the VA versus 1 nonreactivating patient of 12 in the OA; $P = .008$, Fisher's exact test). Phenotype proportions for each arm are displayed on stacked bar plots in Figure 2. Proportions of naïve, TCM, and terminally differentiated effector (both TEM and TEMRA) $pp65_{495-503}$-specific CD8 T cells are plotted for all patient specimens in which memory phenotypes were measurable (Table 1). As shown in Figure 2, most patients who developed ≥.2% $pp65_{495-503}$-specific CD8 T cells had very high proportions of effector T cells (median, >84%), as soon as the threshold for phenotyping was reached. A substantial overlap in the proportion of TEMRA and TEM phenotypes between nonviremic VA and viremic OA patients was observed ($P = .22$, Wilcoxon rank-sum test, day +100 data); though the confidence interval (CI) was consistent with higher percentages in the non viremic VA (95% CI for shift, -5% to 27%). In fact, medians of TEMRA and TEM phenotypes were 92.5% in the VA arm and 84.1% in the OA arm. The only apparent difference between CMVPepVax vaccinated and nonvaccinated patients, aside from the observed CMV reactivation, was a high proportion of the naive phenotype in 2 OA patients who were among the few who did not immediately exhibit an effector phenotype. In summary, in the absence of detectable viremia, approximately one-half (10 out of 18) of the vaccinated patients reached a ≥.2% level of $pp65_{495-503}$-specific CD8 T cells, strongly favoring the effector phenotype. CMV-specific T cells exhibiting a marked effector memory repertoire are typically found during episodes of CMV viremia [18] and were detected, as expected, in the 5 viremic OA patients in whom memory phenotypes were measurable.

Longitudinal Dynamic of $pp65_{495-503}$-Specific CD8 T Cells and Their Memory Profiles

Figure 3A shows 2 examples of $pp65_{495-503}$-specific CD8 T cell profiles from a patient who did not develop viremia (VA

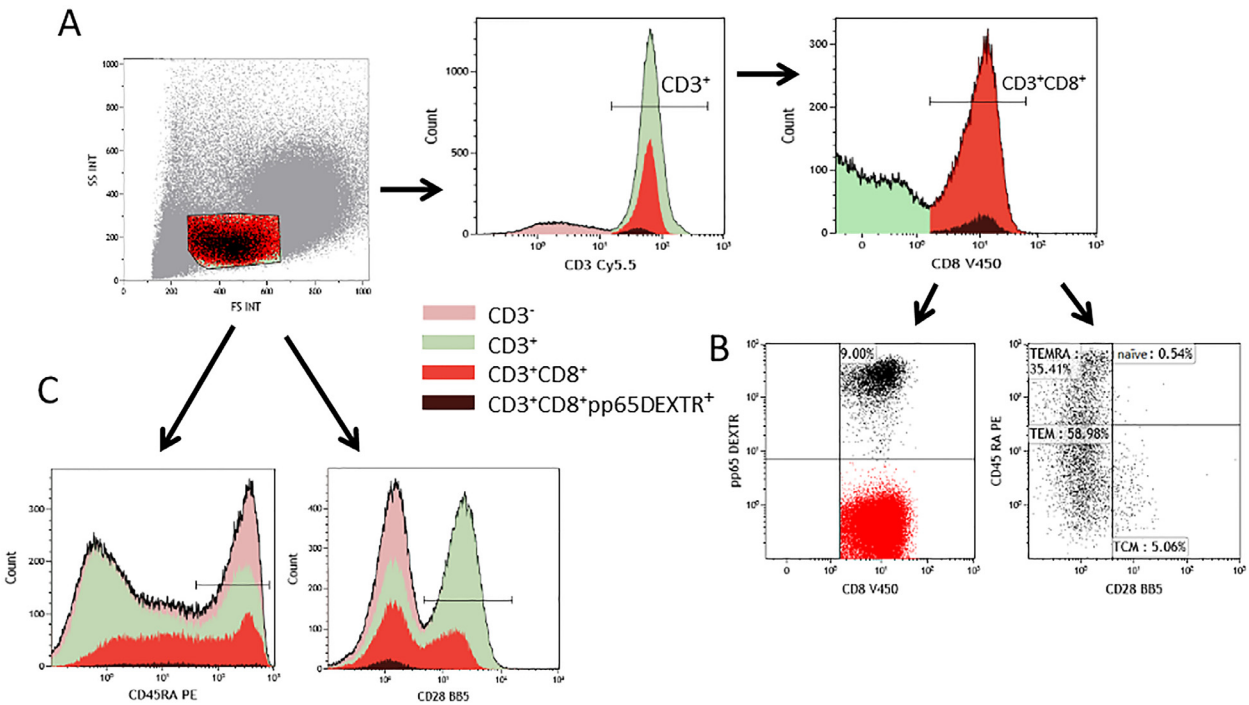

Figure 1. Gating strategy for identifying the memory phenotype of pp65-specific T cells. Representative data plots for VA nonviremic UPN 8 at day 70 post-HCT, analyzed with Kaluza software 1.5, are shown. (A) A primary lymphocyte gate was set on forward and side scatter (FS INT versus SS INT), and subsequent gates were set on $CD3^+$ and $CD8^+$ populations. (B) (Left) Quantification of $pp65_{495-503}$-specific $CD3^+$ $CD8^+$ T cells based on binding to APC-conjugated dextramers incorporating the $pp65_{495-503}$ peptide (pp65 DEXT). (Right) T cell memory populations defined according to CD28 and CD45RA expression as follows: naïve T cells ($CD28^+CD45RA^+$, upper right quadrant), TCMs ($CD28^+CD45RA^-$, lower right quadrant), TEMs ($CD28^-CD45RA^-$, lower left quadrant), and TEMRAs ($CD28^-CD45RA^+$, upper left quadrant). (C) Histogram plots showing the levels of CD45RA and CD28 expression of the gated lymphocyte populations. The T cell populations are color-coded as follows: $CD3^-$ in pink, $CD3^+$ in green, $CD3^+CD8^+$ in red, and $CD3^+CD8^+$ $pp65_{495-503}$ dextramer$^+$ in black.

D⁻ MRD UPN 36) and a patient with spontaneously controlled late viremia (1670 IU/mL; OA D⁻ MRD UPN 19). These profiles illustrate the observation that pp65-specific CD8 T cells ≥.2% was accompanied by high frequencies of effector memory cells within the CMV-specific population. Figure 3B shows the post-HCT reconstitution profiles and memory kinetics for the only 2 patients in whom the predominance of a memory effector profile was not immediately detected. In nonviremic VA D⁺ MRD UPN 14 (left plot), marked TCM profiles were detected on days +28 and +56; however, a sustained increase in TEMs followed soon after, peaking on day +84 when the level of pp65$_{495\text{-}503}$-specific T cells was highest. In OA D⁻ MRD UPN 28 (right plot), when CMV viremia (7600 IU/mL) developed and antiviral treatment was administered, approximately 30% of the pp65$_{495\text{-}503}$-specific T cells still had a naïve phenotype. At later time points when viremia was under control, this patient developed marked levels of pp65$_{495-503}$-specific T cells with an effector profile pattern.

Figure 4 shows the pp65$_{495-503}$ multimer binding percentages for all VA and OA patients enrolled in the Phase Ib clinical trial, including those who had <.2% pp65$_{495\text{-}503}$-specific CD8 T cells [16]. In addition, thermometer glyphs were superimposed to the longitudinal pp65$_{495-503}$ dextramer percentage profiles to indicate the levels of pp65$_{495-503}$-specific CD8 TEM and TEMRA cells in samples from patients in whom a memory analysis was feasible (≥.2% pp65$_{495\text{-}503}$-specific CD8 T cells). The effector memory phenotype typically predominated as soon as memory phenotypes were measurable. Specifically, pp65$_{495\text{-}503}$-specific T cells in the evaluable viremic OA patients had a median of 91% effector phenotype profiles (range, 31% to 99%) as soon as they were measurable. These levels are similar to those of the evaluable nonviremic VA patients, in whom the median percentage of effector phenotype pp65$_{495\text{-}503}$-specific T cells was 93% (range, 13% to 100%). Importantly, at 2 weeks after the second CMVPepVax vaccination, >80% of pp65$_{495\text{-}503}$-specific T cells were TEM and TEMRA cells in 9 of the 10 evaluable

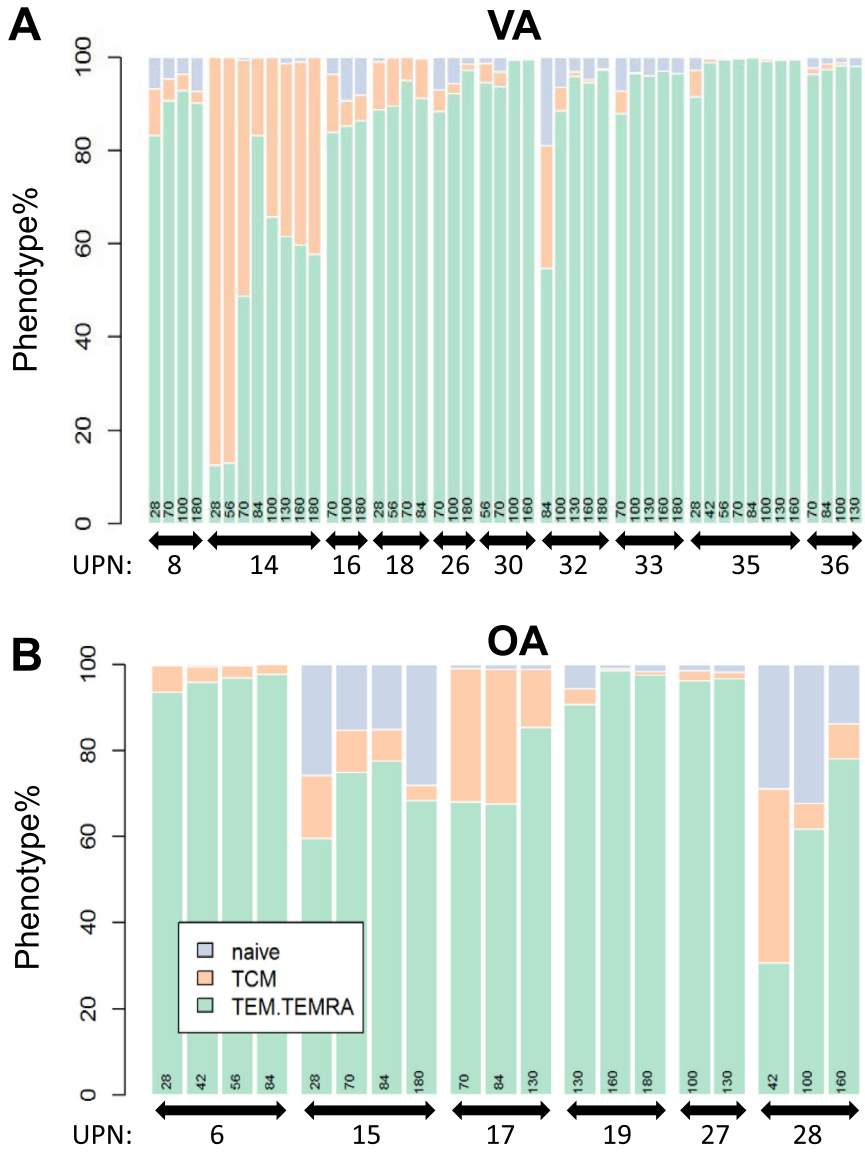

Figure 2. Memory phenotypes in pp65$_{495-503}$-specific T cells. The stacked bar plots show the proportions of 3 T cell phenotypes: naïve (purple), TCMs (orange), and TEMs and TEMRAs (green). The number on each stacked bar shows the post-HCT time point at which the phenotype analysis was performed. On the *x*-axes, UPNs are reported below the arrow lines. (A) Ten nonviremic vaccinated patients. (B) Five viremic and 1 nonviremic OA patients (Table 1).

VA patients. Thus, in these HCT recipients who did not develop clinically detectable CMV viremia, CMVPepVax rapidly expanded CMV-specific T cells with substantial effector memory profiles, which have been associated with antiviral activity and might have prevented CMV reactivation in these patients [2,19,22]. In contrast, in the only VA patient in whom CMV was reactivated (red line in Figure 4A), levels of $pp65_{495-503}$ dextramer binding remained <.1%, and T cell memory could not be analyzed. As shown in Table 1, the availability of earlier post-HCT vials was scattered among the VA patients and thus were inadequate for data interpretation.

Functional Activation Markers and T Cell Memory Profiles for pp65- and IE1-Specific T Cells

The $pp65_{495-503}$ sequence contained in CMVPepVax is an immunodominant HLA-A*0201-restricted CD8 T cell cytotoxic epitope from the CMV pp65 tegument protein epitope [14,15]. To study the cellular response to the entire pp65 protein in HCT recipients, we measured expression (%) of the 4-1BB (CD137) functional activation marker on $CD3^+CD8^+$ and $CD3^+CD4^+$ T cells stimulated for 24 hours with a peptide library encompassing the full sequence of the pp65 protein, including the $pp65_{495-503}$ epitope [25]. The IE1 protein is also highly recognized in CMV-seropositive HCT recipients, and both pp65 and IE1 CMV antigens are considered to play prominent roles in protective immunity [27,31-35]. Thus, we also monitored the CD137 surface marker on $CD3^+CD8^+$ and $CD3^+CD4^+$ T cells stimulated with the IE1 peptide library, following previously described techniques [25].

Memory phenotypes were analyzed in combination with all CD137 assays to measure the level of functional activation of pp65- and IE1-specific T cells [20,21,25]. Figure 5 illustrates the gating strategy used to quantify the pp65 and IE1 responsive T cells and their memory phenotype percentages. In contrast to the predominant effector phenotype observed for the $pp65_{495-503}$-specific T cell populations among nonviremic VA and viremic OA patients (Figure 2), the distribution of memory markers in CD8 T cells specific for the entire pp65 library was more scattered, with marked variation in T cell phenotype proportions among the patients (Figure 6A and B). No significant difference in TEM+TEMRA percentage between arms was observed ($P = 0.25$, Wilcoxon rank-sum test; 95% CI, -15,50). In response to pp65 library stimulation, the overall median percentage of TEM and TEMRA cells for pp65-specifc CD8 T cells was lower than that for $pp65_{495-503}$-specific CD8 T cells. The median percentage was 43% in the OA patients (Figure 6A) and 66% in the VA patients (Figure 6B). A marked variability in the proportions of effector T cells without any clear difference between arms was also observed in response to IE1 library stimulation ($P = 0.61$, Wilcoxon rank-sum test, day 100 data;

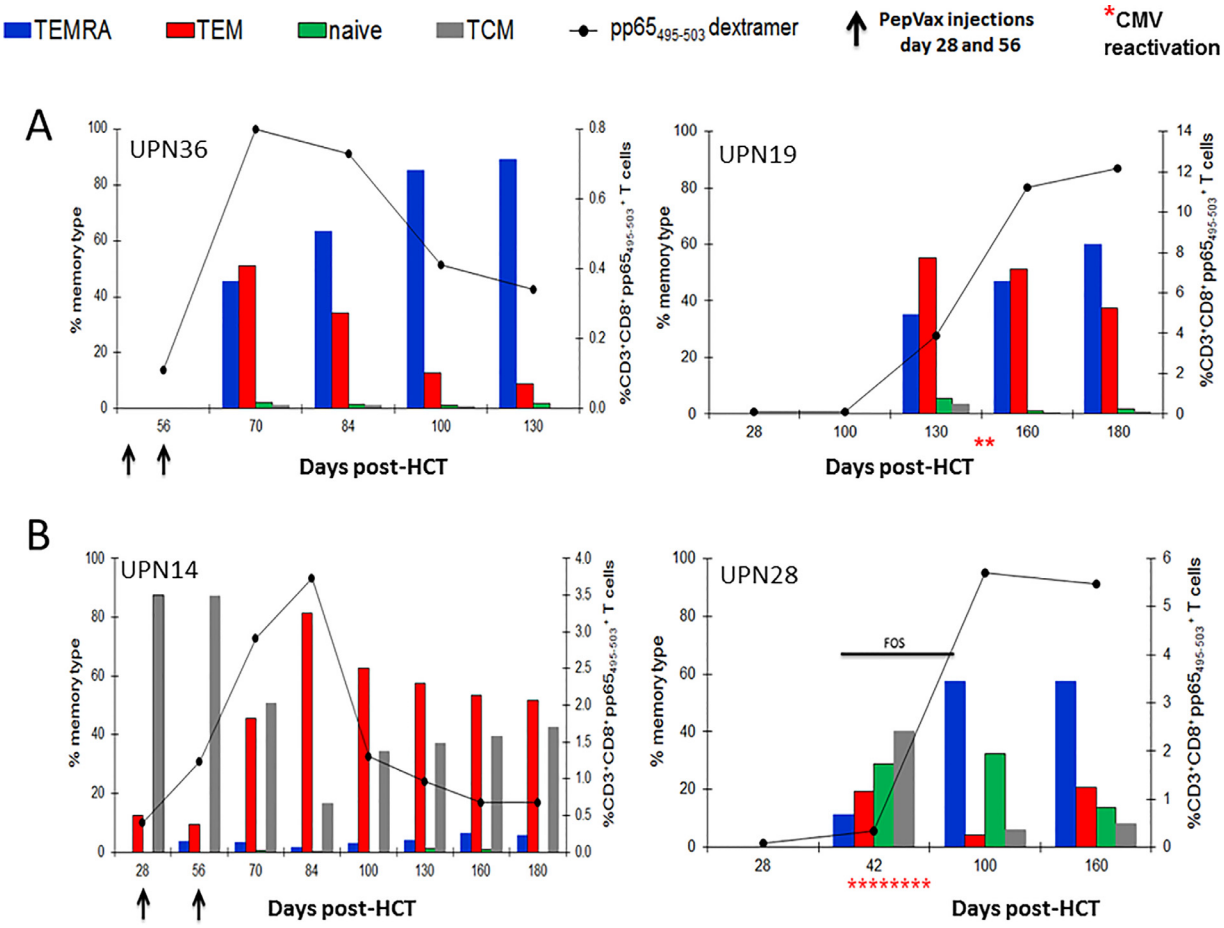

Figure 3. Time course of $pp65_{495-503}$-specific T cell reconstitution and memory phenotypes. Line graphs indicate the post-HCT reconstitution pattern of $pp65_{495-503}$-specific T cells (expressed as % binding to $pp65_{495-503}$ dextramers; right axes) over time. Histograms show the kinetics of the corresponding percentages of memory types (left axes). The left panels show data for 2 patients treated with CMVPepVax (arrows indicate the day of vaccination) who did not develop viremia. The right panels show data for 2 unvaccinated patients who developed viremia at the time points indicated by asterisks. The black bar indicates the timing and the type of antiviral administered. FOS, foscarnet.

95% CI for shift: −24,41) (Figure 6C and D), with a median of 39% for the OA patients (Figure 6C) and 48% for VA patients (Figure 6D). These data point to the heterogeneity of the CMV peptide epitopes contained in the pp65 and IE1 peptide libraries, which triggered different subsets and diversified memory repertoires of CMV-specific CD8 T cells [17].

As for pp65-specific $CD3^+CD4^+CD137^+$ T cells (data not shown), memory profiles were predominantly TCM in both the VA patients (median, 95%) and the viremic OA patients (median, 93%). Similar patterns were observed for IE1-specific $CD3^+CD4^+CD137^+$ T cells in both the VA patients (median, 83%) and the viremic OA patients (median, 91%; data not shown). The median percentage of effector T cells was ~1% to 2% in all HCT recipients; these low levels may be due to delayed reconstitution of $CD4^+$ T cells [36,37].

Reconstitution of Post-HCT pp65- and IE1-Specific T Cells

There was no clear increase in the percentages of pp65-specific CD8 T cells in samples from the evaluable patients within the first 100 days post-HCT (Figure 7A and B, left plots). The average levels of pp65-specific CD8 T cells starting from day +42, the first postvaccination time point, were markedly lower in the viremic OA patients (mean, .8%; range, .01% to 3.1%) compared with the VA patients (mean, 3.4%; range, .07% to 23.18%; Figure 7B, left plot). These data indicate that CMVPepVax-vaccinated patients underwent a more conspicuous immune reconstitution of pp65 CD8 T cells than viremic OA patients.

In contrast, more variable profiles and marginally higher average levels of IE1-specific T cells were observed in samples from viremic OA patients (starting from day +42: mean, 1.2%; range, .03% to 5.1%) compared with samples from nonviremic VA patients (starting from day +42: mean, .9%; range, .03% to 6.7%), whose IE1-specific T cells profiles showed limited variation over time (Figure 7B, right plot). T cell responses to IE1 are a critical indicator of viral reactivation and have a major role in resolving acute infection [19,31].

As for pp65- and IE1-specific $CD3^+CD4^+$ T cells, levels were variable among patients and generally lower than those observed for the corresponding $CD3^+CD8^+$ T cells responses (data not shown).

DISCUSSION

In healthy persons, CMV infection and periodic reactivation are generally well controlled by the CMV-specific T cell response. Although persistent infection is asymptomatic, the impact of CMV on the memory T cell compartment is substantial in an immunocompetent host. CMV infection results in accumulation of late differentiated T cells and an increased ratio of memory to naïve T cells, with approximately 10% of total T cell responses dedicated to CMV antigens in seropositive individuals [8,20,38]. CMV-specific T cells are distributed in the quiescent $CD28^+CD45RA^-$ TCM subsets, as well as in the terminally differentiated $CD28^-CD45RA^-$ TEM and $CD28^-CD45RA^+$ TEMRA effector subpopulations. These late effector T cells generally show no evidence of T cell exhaustion and retain functionality [20,25,39]. The proportions of various memory subsets generally vary across age groups, but levels of CMV-specific T cells and the predominance of terminally differentiated effector T cells increase markedly with age. CMV-specific T cells produce mainly IFN-γ, less IL-2, and no IL-4, and their function is unaffected by aging [20,25,39]. In fact, clinically relevant viremia in

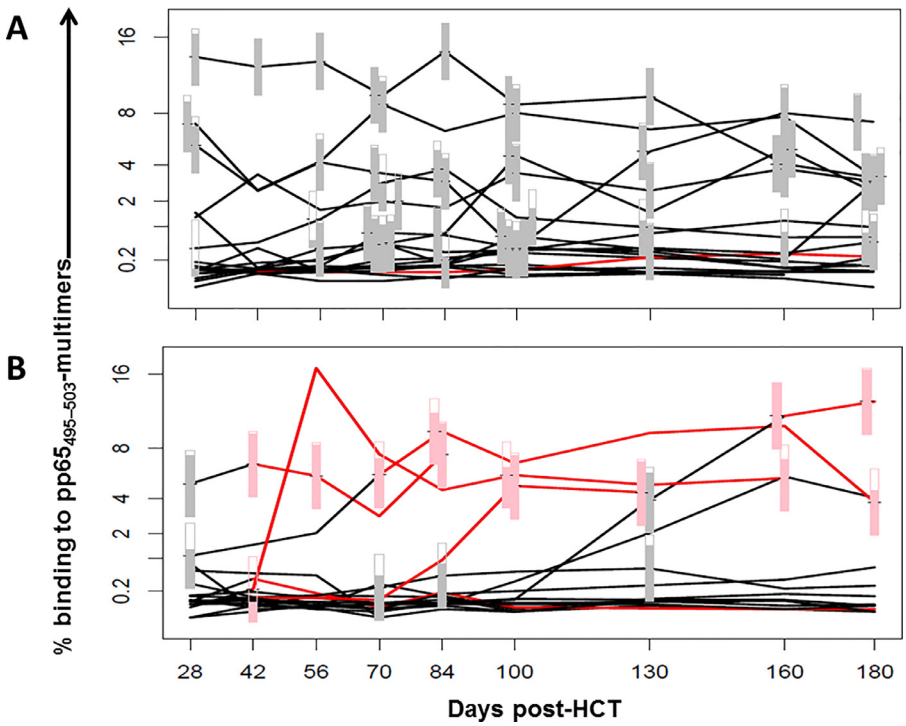

Figure 4. Longitudinal analysis of $pp65_{495-503}$-specific T cell responses. Percentages of CD8 T cells binding to $pp65_{495-503}$ multimers at different time points post-HCT are shown for 18 CMVPepVax vaccinated patients (A) and 18 unvaccinated patients (B). Available patient specimens from the primary trial showing <.2% binding to pentamers between day 28 and day 100 were not evaluable for the memory assessment, but lines have been inserted as a reference in the graph for completeness. An arcsine scale was used for % binding on the *y*-axes. Thermometer symbols indicate the fraction of $pp65_{495-503}$ dextramer binding CD8 T cells with an effector TEM and TEMRA memory phenotype. The color of the line changes from black to red when patients exhibited the first episode of CMV reactivation (defined as ≥1250 IU/mL CMV viremia).

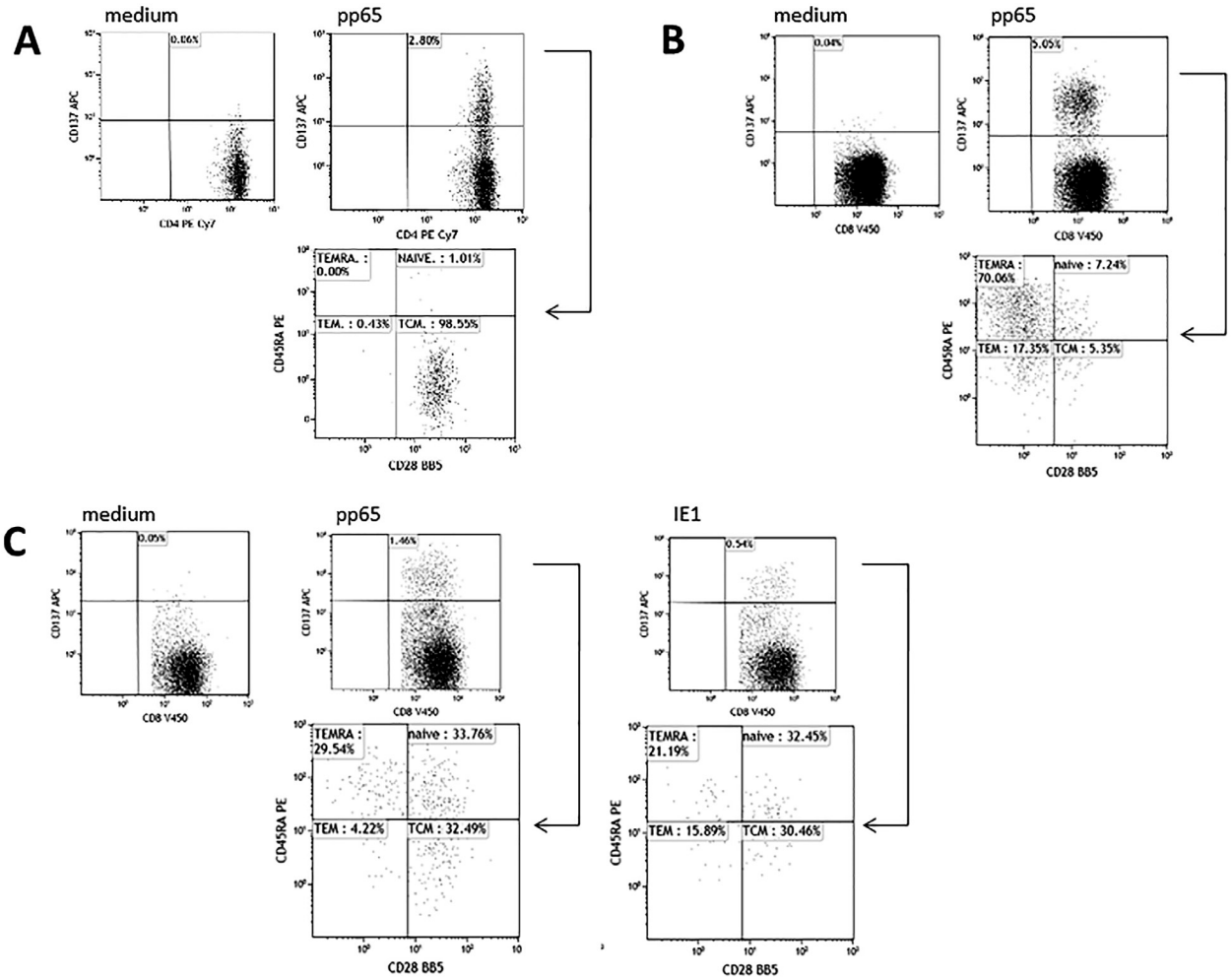

Figure 5. pp65- and IE1-specific T cell responses and memory markers. The gating strategy for the phenotype analysis of CMV-specific T cells is shown with 3 representative examples. CD137 versus CD8 dot plots show the T cell response to cell medium (background control) or the pp65 and IE1 libraries. The corresponding dot plots below show the relative memory marker percentages of the activated $CD3^+CD8^+CD137^+$ T cell populations after CMV peptide stimulation. Memory phenotypes were not analyzed for medium-only stimulations. (A) $CD4^+$ T cell response to cell medium and the pp65 library in a nonviremic VA patient (UPN 35) at day +130 post-HCT. (B) $CD8^+$ T cell response to cell medium and the pp65 library in a viremic OA patient (UPN 12) at day 180 post-HCT. (C) $CD8^+$ T cell response to cell medium, the pp65 library, and the IE1 library in a nonviremic VA patient (UPN 32) at day +84 post-HCT.

elderly persons is rarely found despite the frequency and severity of other infections, such as influenza [40].

Reconstitution of CMV-specific CD8 T cells after HCT is necessary to bring CMV reactivation under control. However, the immune correlates that define protective CMV-specific T cell reconstitution remain unclear. Detection of antigen-specific CD8 T cells by MHC class I peptide multimers is widely used to study immune reconstitution in HCT recipients and to demonstrate the responses to and effects of vaccination [10,16,20,25,26,41]. Nevertheless, the direct quantification of CMV-specific CD8 T cells by multimers might not be sufficient to define a protective anti-CMV immune response. Because the functionality of CMV-specific CD8 T cells has been linked to phenotypic markers describing the level of T cell differentiation [42,43], more detailed analyses of the memory differentiation profiles of these cells may provide better predictive insight into their function [18-20]. To our knowledge, this is the first study describing the impact of a CMV vaccine on the HCT patient memory compartment.

Data from the present study indicate that the recently developed CMVPepVax vaccine [16] rapidly expands CMV-specific T cells with a marked memory effector phenotype, which is typically found during and after episodes of CMV reactivation in nonvaccinated viremic HCT recipients [18,19]. Substantial frequencies of predominantly effector-type CD8 T cells specific for the $pp65_{495-503}$ epitope, which is contained in CMVPepVax, developed in vaccinated HCT recipients in the absence of clinically detectable viremia. Several studies have shown the high cytotoxic potential of these $pp65_{495-503}$-specific CD8 T cells in HLA-A*0201 individuals [14,44-46]. Thus, antiviral cytotoxicity, which is a critical mediator of protection against CMV, could have been the prominent function of these terminally differentiated effector CD8 T cells. These properties make the CMVPepVax vaccine a promising candidate for further clinical evaluation.

Nonetheless, CMVPepVax can be administered only to HLA-A*0201 recipients (~40% of the population), which is a significant limitation of this vaccine. Studies have suggested that 90% coverage of all major ethnic groups is attainable with 15 uniquely defined HLA-restricted epitopes [47]. CMV-pp65 epitopes restricted to other major HLA types have been well characterized, and these data can be used for the production of a universal multiepitope pp65 vaccine [47,48].

In a Phase Ib study of live recombinant CMV, Towne/Toledo chimera vaccines did not affect the proportion of effector

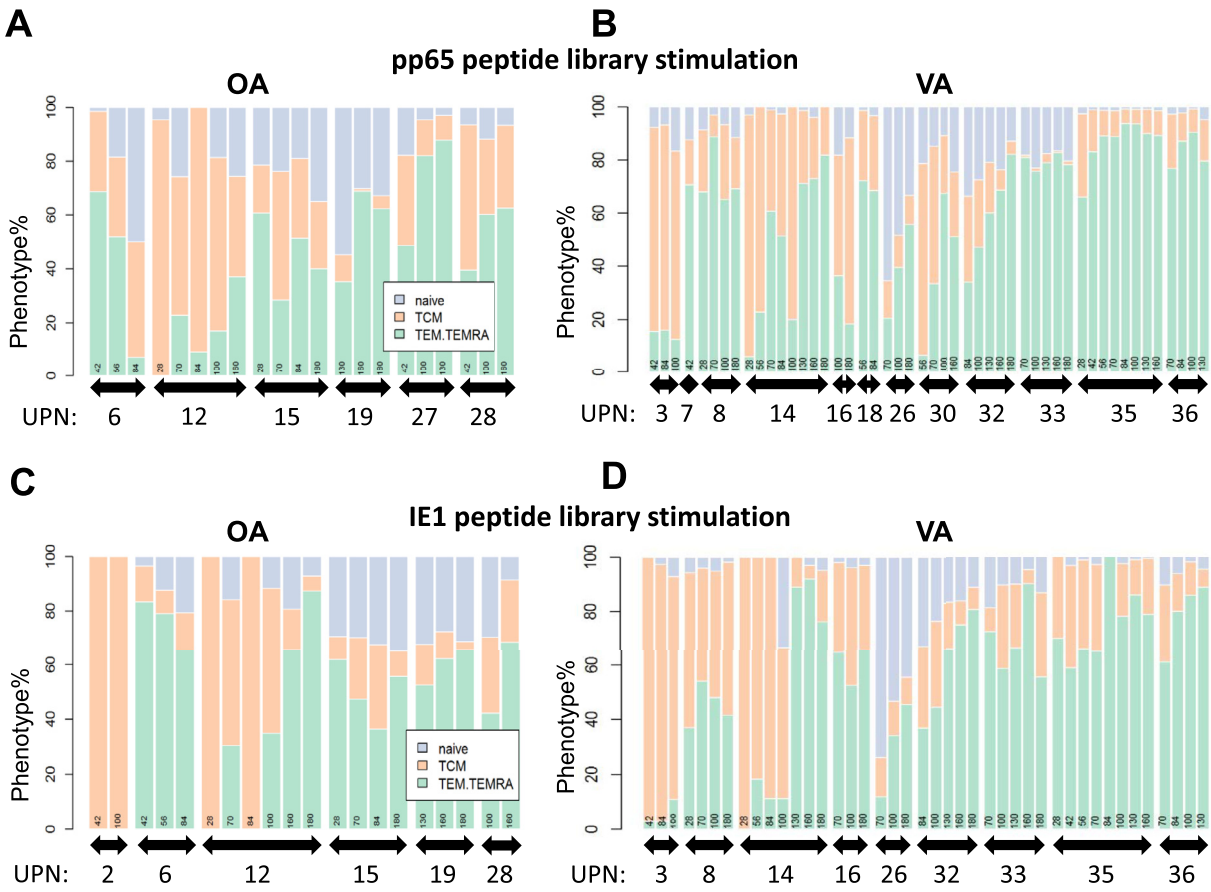

Figure 6. Stacked bar plots showing memory phenotypes of pp65- and IE1-specific T cells. The distribution of memory phenotypes percentages is represented and determined, as detailed in Figure 2. (A and C) Five viremic and 1 non viremic OA patients (Table 1). (B and D) Ten nonviremic vaccinated patients (Table 1). In A and B, the bars indicate the percentage of memory phenotypes of pp65-specific $CD3^+CD8^+$ T cells; in C and D, the bars indicate the percentage of memory phenotypes of IE1-specific $CD3^+CD8^+$ T cells.

memory cells in the CD8 T cell compartment in CMV-seronegative men [24]. The results presented here were obtained in a subgroup of vaccinated patients enrolled in the CMVPepVax trial [16] in whom a T cell memory study was feasible and specimens were available (Table 1). Although this is a limitation of this study, the data indicate that levels of $pp65_{495\text{-}503}$ dextramer-binding CD8 T cells with marked effector profiles rapidly developed in CMVPepVax- vaccinated HCT recipients to levels similar to those seen in the CMV viremic OA patients, despite a lack of CMV reactivation and regardless of donor CMV serostatus (Figure 3 and Figure 4, upper plots) [16]. Importantly, the occurrence of viremia in the entire Phase Ib population was significantly different between the 2 cohorts, with 6 OA patients, developing clinically detectable viremia compared with a single VA patient, as reported previously [16]. In the present study, elevated levels of $pp65_{495\text{-}503}$-specific TEMs developed in 9 of 18 VA patients, none of whom became viremic, and in 6 OA patients, 5 of whom became viremic.

In HCT recipients who develop CMV viremia, the magnitude of CMV-specific T cell expansion and the accumulation of cells with a late effector phenotype can be massive. Nevertheless, despite the presence of CMV, specific T cell reactivation may occur due to delayed immune recovery and qualitative disorders, rather than to quantitative defects of the immune response [1]. In line with these findings, the viremic OA patients in our study had high levels of $pp65_{495\text{-}503}$ dextramer-binding CD8 T cells (median, 5%). In addition, elevated levels (median, >90%) of terminally differentiated $pp65_{495\text{-}503}$-specific T cells (Figure 2) were detected as soon as memory was measurable ($pp65_{495\text{-}503}$ dextramer binding ≥.2%) in the viremic OA patients. This suggests that CMV poses a constant challenge to the immune system, which leads to the expedited reconstitution of CMV-specific immunity [10]. None of the patients in whom CMV reactivation occurred experienced recurrence during the 6-month trial follow-up, in agreement with findings showing that a lack of CMV-specific T cells after the first episode of reactivation was associated with multiple subsequent reactivations in HCT recipients [19].

In CMV-seronegative recipients of a solid organ transplant (SOT) from a CMV-seronegative donor (D+R- recipients), programmed death (PD)-1 up-regulation on CMV-specific CD8 T cells was significantly associated with incipient and overt CMV disease and with viremia [49] In contrast, we did not identify a trend toward PD-1 elevation among patients enrolled in the CMVPepVax trial, who had measurable levels of $pp65_{495\text{-}503}$-specific and developed viremia (data not shown). Prophylactically treated SOT recipients are diagnosed when they become symptomatic. Thus, the elevated expression of the coinhibitory receptor PD-1 detected on CMV pp65-specific CD8 T cells may reflect the exhaustion of T cells during prolonged CMV viremia, in which they eventually become anergic and unable to limit viremia and late end-organ disease. Preemptively treated HCT recipients are frequently monitored for CMV viremia early post-HCT, and promptly treated with antivirals. The difference in antiviral regimen could explain the

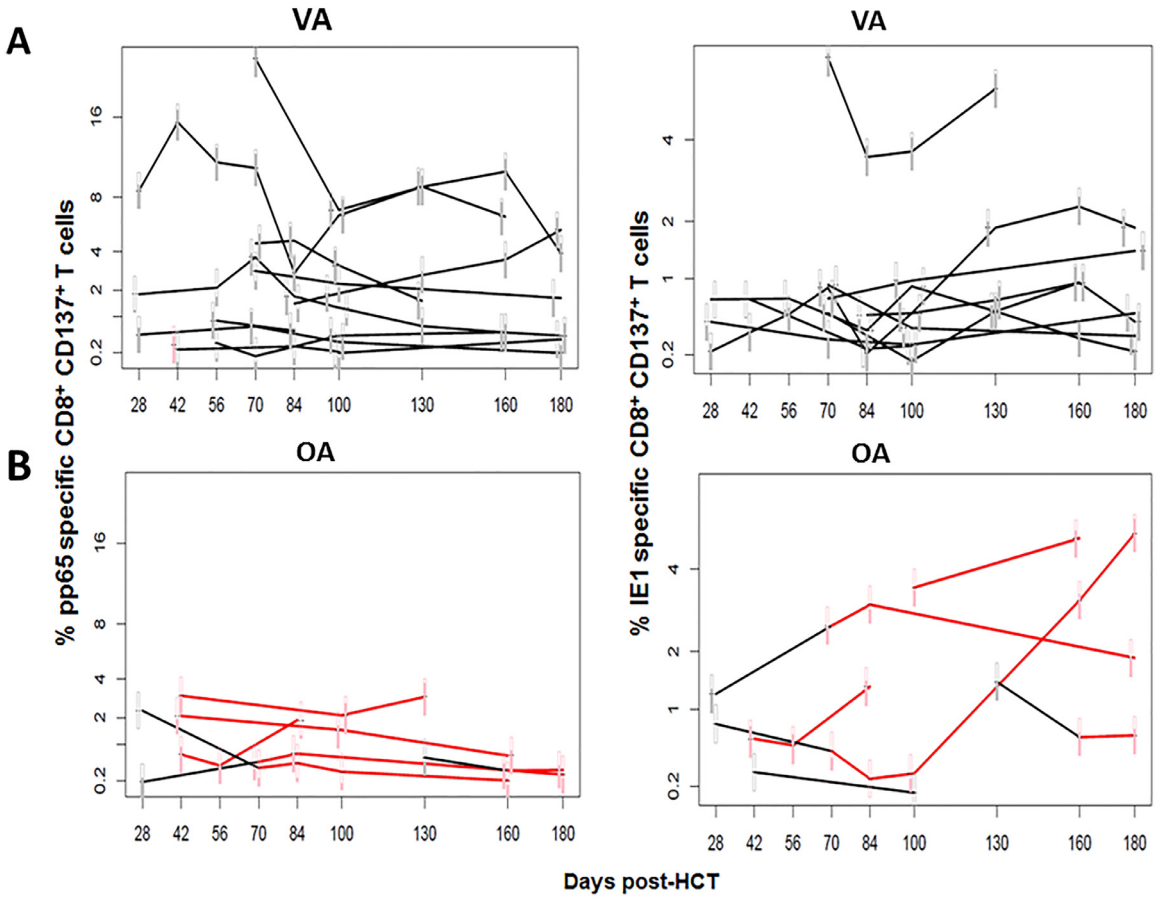

Figure 7. Longitudinal analysis of pp65- and IE-specific CD8 T cell responses. PBMCs collected at different time points post-HCT were analyzed for functional responses against 2 CMV peptide libraries. Detection of the surface marker CD137 was used as a measure of T cell activation. (A) Data from all the evaluated vaccinated patients (black lines). (B) Data from all the evaluated unvaccinated patients (red after reactivation). The left plots show the percentages of $CD8^+CD137^+$ T cells detectable after stimulation with the pp65 peptide library, and the right plots show the percentages of $CD8^+CD137^+$ T cells after stimulation with the IE1 library. Axes, glyphs, and line colors are as described in Figure 4.

lack of elevated levels of PD-1 on CMV specific T cells in our cohort of viremic HCT patients, all of whom were able to control CMV viremia on administration of antiviral agents. The roles of other coinhibitory receptors, such as CTLA-4, Lag-3, Tim-3, and TIGIT, in down-regulating T cell effector responses and their functions during the immune reconstitution of the CMV response merit investigation in future studies [50].

UPN 19 was the only OA patient in this study who was able to control clinically detectable viremia without the use of antivirals (Figure 3A, right plot). Late but rapidly increasing frequencies of $pp65_{495\text{-}503}$-specific CD8 TEMs, both before and after CMV reactivation, may be key to this patient's spontaneously controlled viremia. However, elevated percentages of $pp65_{495\text{-}503}$-specific CD8 TEMs were also found in 4 other OA patients who required antiviral treatment to control CMV viremia [19,51]. In just 1 patient (UPN 28; Figure 3**,** lower right plot), $pp65_{495\text{-}503}$-specific CD8 T cells did not precede viremia, and when they were detected at viremia onset, their memory phenotype was predominantly quiescent TCMs and naïve T cells rather than functional effector cells. The variability in memory phenotype observed among viremic patients requiring antivirals might have been due to differing patterns of immune reconstitution after HCT. Immune reconstitution is a dynamic and complex process that depends on recipient and donor matching characteristics, the modalities of transplantation, including conditioning regimen intensity, and the post-HCT occurrence of graft-versus-host disease [36,52]. Further studies in large cohorts of HCT recipients are needed to fully understand the degree of impairment and the impact of immunosuppressive therapy on the functionality of $pp65_{495\text{-}503}$-specific CD8 T cells in HCT recipients [1].

We monitored the memory phenotypes of T cells expanded in response to full-length CMV pp65 and IE1 antigens, both of which are highly recognized in HCT recipients and play important roles in protective immunity [31,32,35]. In particular, the immune recognition of IE1 in HCT recipients is an important indicator of viral reactivation [19,31]. Great emphasis has been placed on the frequency of IFN-γ-producing CD8 T cells for the purposes of immune monitoring in HCT recipients [53,54]. However, single cytokine measurements are limited as predictors of immunologic and clinical outcomes after HCT [18,55]. Thus, in the present study, we incorporated monitoring of CD137 expression into our longitudinal assessment of memory surface markers. CD137 is a functional marker of activation that is uniformly up-regulated by 24 hours after antigen stimulation on the surface of all T cells, regardless of their differentiation stage or profile of cytokine secretion [25,56]. The cellular CMV-specific response and memory phenotype evaluation were performed by measuring the coexpression of CD137 and the memory subset markers CD28 and CD45RA on $CD3^+CD8^+$ and $CD3^+CD4^+$ T cells stimulated for 24 hours with either pp65 or IE1 peptide libraries [21,25]. The phenotypes observed for

both pp65- and IE1-specific CD8 T cells in the examined patients varied greatly, as expected [20,25]. In fact, the short-term stimulation was performed with peptide libraries expressing many CMV antigens with various properties and characteristics, able to elicit T cell subsets with multiple functions and a diversified memory repertoire [17].

The sustained CD8 T cell response to pp65 observed in VA patients indicates that CMVPepVax-vaccinated patients achieved enhanced immune recovery with T cells showing a higher average of CD137 functionality marker compared with the viremic OA patients (Figure 7, left panels). The use of the CMVPepVax adjuvant PF03512676, designed to specifically agonize Toll-like receptor 9, may have played a role in increasing the CD8 T cell response to pp65, because this immunomodulating synthetic oligonucleotide has been shown to independently stimulate potent functional cellular responses when administered with a wide range of antigens [57,58].

In response to the IE1 library, the viremic OA patients showed greater longitudinal variation in CD137 levels compared with the nonviremic VA patients (Figure 7, right plots). This finding confirms the role of the IE1 response during acute infection [19,31].

Memory markers in the CD4 compartment of T cells specific for both pp65 and IE1 were composed primarily of cells of the quiescent TCM phenotype, with minimal levels of effector T cells. In HCT recipients, the reconstitution of $CD4^+$ and $CD8^+$ T cell subsets is not synchronized. In fact, peripheral homeostatic expansion is strongly enhanced for $CD8^+$ cells, which often exceeds normal levels within 2 to 8 months post-HCT [36,59]. The emergence of $CD4^+$ T cells is delayed compared with $CD8^+$ T cells, and $CD4^+$ T cells are the last to recover post-HCT. Thus, the predominance of CMV-specific TCM cells, as well as the concomitantly reduced percentages of differentiated effector $CD4^+$ T cells, in the HCT recipients during the 6-month study follow-up might have resulted from the delayed and impaired reconstitution of these T cell subsets [36,37].

Reconstitution of CMV-specific $CD3^+CD8^+$ T cells after HCT is necessary to bring CMV reactivation under control. However, the parameters determining protective CMV-specific T cell reconstitution remain unclear. In the CMVPepVax trial, 10 OA patients and 6 VA patients (out of 18 in both arms) did not develop viremia, though they showed minimal (<.2%) recognition levels of both pp65 and IE1 antigens, precluding further memory kinetic studies [16]. It is possible that those HCT recipients responded at a higher frequency to CMV antigens, such as pp50 and IE2, that also have been considered protective in the context of HCT but were not tested in this study [8,27,60]. Alternative effector mechanisms also could have protected those patients from CMV viremia reactivation. Some studies have indicated that $\gamma\delta$ T cells produce IFN-γ and TNF-α that may synergize to inhibit CMV replication and kill CMV-infected cells [61]. Moreover, natural killer (NK) and adaptive NK ($NKG2C^+$) cells also have been associated with protection against CMV reactivation [62,63]. NK cells are the first lymphocyte subset to recover after HCT [36,37]. Interestingly, the CMVPepVax adjuvant PF03512676 is known to induce early NK immune activation [64].

In conclusion, immune activation by the adjuvant combined with the rapid expansion of effector $pp65_{495\text{-}503}$-CD8 T cells driven by the peptide portion of the CMVPepVax vaccine may have contributed to the favorable outcome of the pilot trial [16]. CMVPepVax vaccine efficacy is currently being examined in an ongoing multicenter, placebo-controlled Phase II trial (NCT02396134), which will further define the role and the impact of this anti-CMV vaccine strategy in the setting of HCT.

Finally, new treatments and safer therapeutic options are urgently needed to protect vulnerable HCT recipients from the risk of CMV reactivation [65,66]. Recently, a Phase III trial showed that letermovir prophylaxis prevents clinically significant CMV infection from developing in CMV-seropositive recipients without causing a major myelotoxic adverse event [67]. However, CMV reactivation and late CMV disease can occur on discontinuation of prophylaxis, negatively impacting the outcome and reducing the benefit of the lifesaving HCT procedure. A CMV vaccine administered during letermovir prophylaxis could be a powerful tool to induce protective CMV immunity and expedite an effective immune reconstitution, preventing serious clinical sequelae and maximizing the benefit of this novel antiviral.

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Conflict of interest statement: D.J.D. is chair of the scientific advisory board of and receives personal service fees from Helocyte. The other authors have no conflicts of interest to report.

Authorship statement: C.L.R., J.L., and D.J.D. developed the study design. R.N. and I.A. contributed to the clinical implementation of the study and supervision for scheduling of the patients. J.L. designed, performed, and verified the accuracy of the final statistical analysis. C.R.L., Q.Z., and C.L.R. conducted the laboratory experiments. C.L.R. conducted the immunological analyses. C.L.R., J.L., N.H., and D.J.D. wrote and all authors critically revised, reviewed, and approved the final version of this manuscript.

SUPPLEMENTARY MATERIAL

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.bbmt.2018.12.070.

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