TRANSPLANTATION

CMV reactivation drives posttransplant T-cell reconstitution and results in defects in the underlying TCRβ repertoire

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Key Points

- CMV reactivation fundamentally resets posttransplant CD8 reconstitution, resulting in massive expansion of CMV-specific CD8 Tem.
- CMV reactivation is associated with defects in the underlying TCRβ immune repertoire.

Although cytomegalovirus (CMV) reactivation has long been implicated in posttransplant immune dysfunction, the molecular mechanisms that drive this phenomenon remain undetermined. To address this, we combined multiparameter flow cytometric analysis and T-cell subpopulation sorting with high-throughput sequencing of the T-cell repertoire, to produce a thorough evaluation of the impact of CMV reactivation on T-cell reconstitution after unrelated-donor hematopoietic stem cell transplant. We observed that CMV reactivation drove a >50-fold specific expansion of Granzyme B^{high}/$CD28^{low}$/$CD57^{high}$/$CD8^{+}$ effector memory T cells (Tem) and resulted in a linked contraction of all naive T cells, including $CD31^{+}$/$CD4^{+}$ putative thymic emigrants. T-cell receptor β (TCRβ) deep sequencing revealed a striking contraction of $CD8^{+}$ Tem diversity due to CMV-specific clonal expansions in reactivating patients. In addition to querying the topography of the expanding CMV-specific T-cell clones, deep sequencing allowed us, for the first time, to exhaustively evaluate the underlying TCR repertoire. Our results reveal new evidence for significant defects in the underlying CD8 Tem TCR repertoire in patients who reactivate CMV, providing the first molecular evidence that, in addition to driving expansion of virus-specific cells, CMV reactivation has a detrimental impact on the integrity and heterogeneity of the rest of the T-cell repertoire. This trial was registered at www.clinicaltrials.gov as #NCT01012492. (*Blood*. 2015;125(25):3835-3850)

Introduction

One of the major obstacles associated with hematopoietic stem cell transplant (HSCT) is the fact that this treatment, although curative for many diseases, is associated with significant toxicity and resultant transplant-related mortality (TRM).[1-4] TRM takes on many forms, but is often related to dysfunctional immune reconstitution after HSCT. The extent to which cytomegalovirus (CMV) influences posttransplant immune dysfunction has been the subject of intense interest,[1,5-9] with several studies documenting the increased risk of TRM based on CMV serostatus, infection, and expansion of CMV-tetramer–positive cells.[10-22] However, although the phenomenology of CMV's impact on TRM is well documented, the causative molecular immunologic mechanisms remain unknown. To address these questions, we have undertaken a detailed assessment of immunologic reconstitution after HSCT using new deep-sequencing technologies that have allowed us to investigate this issue at a level of molecular detail not previously possible. We present evidence that, in a cohort of patients undergoing unrelated-donor transplantation, CMV reactivation is capable of resetting CD8 T-cell homeostasis, resulting in the clonal expansion of CMV-specific CD8 effector memory T cells (Tem). Importantly, T-cell receptor β (TCRβ) deep-sequencing analysis allowed us, for the first time, to look beyond the clonal expansions, and to evaluate the remainder of the TCR repertoire and, thereby, to assess the impact of CMV reactivation on the integrity of the repertoire as a whole. This analysis has revealed that CMV reactivation was associated with the development of defects in the underlying Tem TCR repertoire, providing the strongest

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Table 1. Patient characteristics and clinical outcomes

UPN	Recip. age, y	Donor age, y	Sex, M/F	Disease, status	HLA typing		Prep. reg.	GVHD prop. reg.	Graft source	CMV status, D/R	CMV react., Y/N	Grade II-IV aGVHD to day +100, Y/N (day)	Grade III-IV aGVHD to day +100, Y/N (day)	Late aGVHD (day)	Systemic aGVHD Tx	Mild cGVHD, Y/N	Mod/ Sev cGVHD, Y/N	Systemic cGVHD Tx through day 365	Non-CMV infections	Relapse Y/N, day post-Tx	Alive day 365 post-Tx, Y/N (day of death)
001-001	46	24	M	AML, CR2	A: 01/03; B: 08/52	Cw: 1202*/0701; DRB1: 0301/ 0404* Recip. Cw: 1216	Bu/ Cy	CNI/ MTX/ AB	PBSC	+/+	Y	N	N	Y (220)	Steroids	N	N	N	Bacteremia	N	Y
001-002	61	20	F	AML, CR1	A: 02/68; B: 18*/27* Recip. B: 08	Cw: 0701/0704; DRB1: 1104/ 1501	Flu/ Mel	CNI/ MTX/ AB	PBSC	−/−	N	N	N	N	N	Y	N	Short-course steroids	None	N	Y
001-004	74	33	F	AML, CR2	A: 03/68; B: 07/18	Cw: 0701*/0702; DRB1: 1401/ 1501* Recip. Cw: 0501	Flu/ Mel	CNI/ MTX/ AB	PBSC	−/+	Y	N	N	N	N	N	N	N	Bacteremia, parainflu.	Y, day +98	N (121)
001-005	59	19	F	MDS → AML	A: 01/29; B: 08/44	Cw: 0701/1601; DRB1: 0301/ 0701	Flu/ Mel	CNI/ MTX/ AB	PBSC	−/−	N	N	N	N	N	N	Y	Steroids, siro.	Rhinovirus	N	Y
001-006	43	21	M	Biphen. Ph^+ leukemia, CR1	A: 03/11; B: 07/35	Cw: 0401/0702; DRB1: 0101/ 1501	TBI/ Cy	CNI/ MTX/ AB	PBSC	−/+	N	N	N	N	N	N	Y	Steroids, cyclo.	None	N	Y
001-007	46	45	M	ALL, CR1	A: 01/24*; B: 08/18* Recip. A: 26	Cw: 0701/1203; DRB1: 0701/ 1104	TBI/ Cy	CNI/ MTX/ AB	PBSC	+/+	N	N	N	N	N	N	N	N	None	Y, day + 121	N (147)
001-008	39	36	M	AML, CR1	A: 24/32; B: 27/40	C: 0102/0202; DRB1: 0101/ 1601	Bu/ Cy	CNI/ MTX/ AB	PBSC	+/+	Y	Y (84)	Y	N	Steroids	N	Y	Steroids, ritux.	None	N	Y
001-009	40	27	M	CML, EMD	A: 02/24*; B: 14/45* Recip. A: 68	Cw: 0802/1601; DRB1: 1001/ 1303	Bu/ Cy	CNI/ MTX/ AB	PBSC	−/+	Y	N, did develop grade I skin (49)	N	N	Steroids	N	Y	Steroids, cyclo., MMF	None	N	Y
002-001	22	27	F	ALL, CR3	A: 02/68; B: 40/51	Cw: 0304/1402; DRB1: 0401/ 1101	TBI/ Cy	CNI/ MTX/ AB	BM	+/+	N	N	N	N	N	Y	N	N	None	N	Y
002-002	17	30	F	ALL, IF	A: 02/03; B: 07/39	Cw: 0701/0702; DRB1: 1401*/ 1303* Recip. DRB1: 1001	TBI/ Cy	CNI/ MTX/ AB	BM	−/+	Y	Y (29)	N	N	Steroids	N	Y	Steroids, cyclo.	Cellulitis, BK virus, LPD	N	Y
003-001	30	40	M	ALL, PIF	A: 02/02; B: 07/49	Cw: 0701/0702; DRB1: 0401/ 0405	Bu/ Cy	CNI/ MTX	PBSC	−/+	N	N	N	N	N	Y	N	N	Y	Y, day + 562	Y
003-002	35	45	F	MF	A: 23/30*; B: 07/78* Recip. A: 33	Cw: 0702/1601; DRB1: 0701/ 0901	Bu/ Cy	CNI/ MTX	PBSC	+/−	Y	Y (48)	N	N	Steroids	N	Y	Steroids, tacro.	N	N	Y

AB, abatacept; aGVHD, acute GVHD; ALL, acute lymphoblastic leukemia; AML, acute myelogenous leukemia; ATG, antithymocyte globulin; Biphen., biphenotypic; BM, bone marrow; Bu, busulfan; cGVHD, chronic GVHD; CML, chronic myelogenous leukemia; CNI, calcineurin inhibitor; CP, chronic phase; CR, complete remission; Cy, cyclophosphamide; cyclo., cyclosporine; D/R, donor/recipient; EMD, extramedullary disease; F, female; Flu, fludarabine; GVHD, graft-versus-host disease; IF, induction failure; inflix., infliximab; LPD, lymphoproliferative disease; M, male; MDS, myelodysplastic syndrome; Mel, melphalan; MF, myelofibrosis; MMF, mycophenolate mofetil; Mod/Sev, moderate/severe; MTX, methotrexate; N, no; N/A, not applicable; parainflu., parainfluenza; PBSC, peripheral blood stem cell; Ph^+, Philadelphia chromosome positive; PIF, primary induction failure; Prep. reg., preparative regimen; prop. reg., prophylaxis regimen; react., reactivation; Recip., recipient; R/I, resistant/intolerant; ritux., rituximab; siro., sirolimus; tacro., tacrolimus; TBI, total body irradiation; TKI, receptor-tyrosine kinase inhibitor; Tx, treatment; UPN, unidentified patient number; VP16, etoposide; Y, yes.

Table 1. (continued)

UPN	Recip. age, y	Donor age, y	Sex, M/F	Disease, status	HLA typing	Prep. reg.	GVHD prop. reg.	Graft source	CMV status, D/R	CMV react., Y/N	Grade II-IV aGVHD to day +100, Y/N (day)	Grade III-IV aGVHD to day +100, Y/N (day)	Late aGVHD (day)	Systemic aGVHD Tx	Mild cGVHD, Y/N	Mod/ Sev cGVHD, Y/N	Systemic cGVHD Tx through day 365	Non-CMV infections	Relapse Y/N, day post-Tx	Alive day 365 post-Tx, Y/N (day of death)
003-003	66	19	M	MDS	A: 02; B: 15/44 Cw: 0304/0501; DRB1: 0401	Flu/ Mel	CNI/ MTX	PBSC	+/+	N	Y (35)	Y (35)	N	Steroids inflix.	N	N	N	Y	N	Y
003-004	48	25	F	CP CML, TKI R/I	A: 01/29; B: 08/44 Cw: 0501*/0701; DRB1: 0301/ 1201* Recip. Cw: 0102	Bu/ Cy	CNI/ MTX	PBSC	+/−	N	Y (11)	Y (11)	N/A	Steroids inflix.	N/A	N/A	N/A	N	N	N (46)
003-005	64	19	F	ALL, CR2	A: 02; B: 07/18 Cw: 0501/0702; DRB1: 0301/ 1501	TBI/ Cy	CNI/ MTX	PBSC	+/−	Y	N	N	N/A	N	N/A	N/A	N/A	N	N	N (80)
003-006	41	55	M	MDS	A: 02/03; B: 44*/40* Recip. B: 1302 Cw: 0304/0501; DRB1: 0403/ 39	Bu/ Cy/ ATG	CNI/ MTX	PBSC	+/−	N	Y (44)	Y (44)	N	Steroids inflix.	N/A	N/A	N/A	N	N	N (95)
004-001	12	29	M	ALL, CR1	A: 02/11; B: 15/44 Cw: 0304/1601; DRB1: 0401/ 0701	TBI/ Cy/ VP16	CNI/ MTX	BM	−/+	N	N	N	N	N	N	N	N	N	N	Y

AB, abatacept; aGVHD, acute GVHD; ALL, acute lymphoblastic leukemia; AML, acute myelogenous leukemia; ATG, antithymocyte globulin; Biphen., biphenotypic; BM, bone marrow; Bu, busulfan; cGVHD, chronic GVHD; CML, chronic myelogenous leukemia; CNI, calcineurin inhibitor; CP, chronic phase; CR, complete remission; Cy, cyclophosphamide; cyclo., cyclosporine; D/R, donor/recipient; EMD, extramedullary disease; F, female; Flu, fludarabine; GVHD, graft-versus-host disease; IF, induction failure; infix., infliximab; LPD, lymphoproliferative disease; M, male; MDS, myelodysplastic syndrome; Mel, melphalan; MF, myelofibrosis; MMF, mycophenolate mofetil; Mod/Sev, moderate/severe; MTX, methotrexate; N, no; N/A, not applicable; parainflu., parainfluenza; PBSC, peripheral blood stem cell; Ph$^+$, Philadelphia chromosome positive; PIF, primary induction failure; Prep. reg., preparative regimen; prop. reg., prophylaxis regimen; read., reactivation; Recip., recipient; R/I, resistant/intolerant; rflux., rituximab; siro., sirolimus; tacro., tacrolimus; TBI, total body irradiation; TKI, receptor-tyrosine kinase inhibitor; Tx, treatment; UPN, unidentified patient number; VP16, etoposide; Y, yes.

molecular evidence to date linking CMV reactivation and quantitative immune dysregulation after HSCT.

Methods

Study design

Seventeen patients underwent prospective, calendar-based clinical and immune monitoring after enrollment on 2 contemporaneous clinical trials: (1) The Bone Marrow Immune Monitoring Protocol and (2) The Abatacept Feasibility Study. Both studies were institutional review board–approved and conducted between 2010 and 2013, as previously described.[23] Patient and transplant characteristics are shown in Table 1. Patients were analyzed prospectively for CMV reactivation by polymerase chain reaction (PCR), and those that reactivated CMV (>300 copies per mL whole blood) were treated with antiviral therapy according to institutional standards. All patients reactivating CMV developed viremia that was responsive to either valganciclovir or ganciclovir. No patient developed CMV disease. In addition to patient analyses, 10 healthy adult controls also underwent single time-point immune analysis and 7 healthy adult controls underwent TCRβ repertoire analysis.

Human studies ethics statement

The patients and controls described in this manuscript were enrolled in clinical trials that were conducted according to the principles set forth in the Declaration of Helsinki, and which were approved by the institutional review boards. Written informed consent was received from all participants.

Immunologic analysis

Patients underwent longitudinal multiparameter flow cytometric analysis of immune reconstitution, CMV functional response, tetramer analysis, and flow cytometric sorting of CD8$^+$ naive T cells (Tnaive), Tem, and Tetramer$^+$ cells as described in supplemental Methods (available on the *Blood* Web site) and supplemental Tables 1-2.

TCRβ receptor diversity analysis by deep sequencing

Total genomic DNA was extracted and quantified (Qiagen) from unsorted peripheral blood mononuclear cells (PBMCs) and from sorted CD8$^+$ Tnaive, Tem, and CMV-tetramer$^+$ cells; TCRβ chain sequencing was performed at Adaptive Biotechnologies using the ImmunoSEQ platform[24-26] with primers specific for all 54 known expressed Vβ regions and all 13 Jβ regions. TCRβ deep-sequencing depth was sufficient to achieve at least fivefold coverage of every original template, sufficient to prevent sampling effects.[25] An average of $3.3 \times 10^6 \pm 3.4 \times 10^5$ reads were generated for the PBMC, Tnaive, and Tem samples (supplemental Table 3).

Bioinformatic analysis was performed on the sequencing data using the ImmunoSEQ platform, which included a determination of: the number and sequence of each of the productive unique Vβ and Jβ genes identified within each sample, the degree of clone sharing between samples, and the identity of the shared clones. In addition, clonality and TCRβ repertoire hole analysis was performed as described in detail in the supplemental Methods.

Statistical analysis

To identify predictors of high CD8$^+$ Tem status, 2 statistical tests were used: for binary variables, we calculated the nonparametric Kendall τ-b statistic using the cor.test function in R (http://www.R-project.org/). For the 2 continuous variables (recipient age and donor age), we carried out Spearman rank correlation, also using R. Additional statistical analyses were calculated with the Mann-Whitney *t* test with 95% confidence intervals, using GraphPad Inc software, Prism version 5.0.

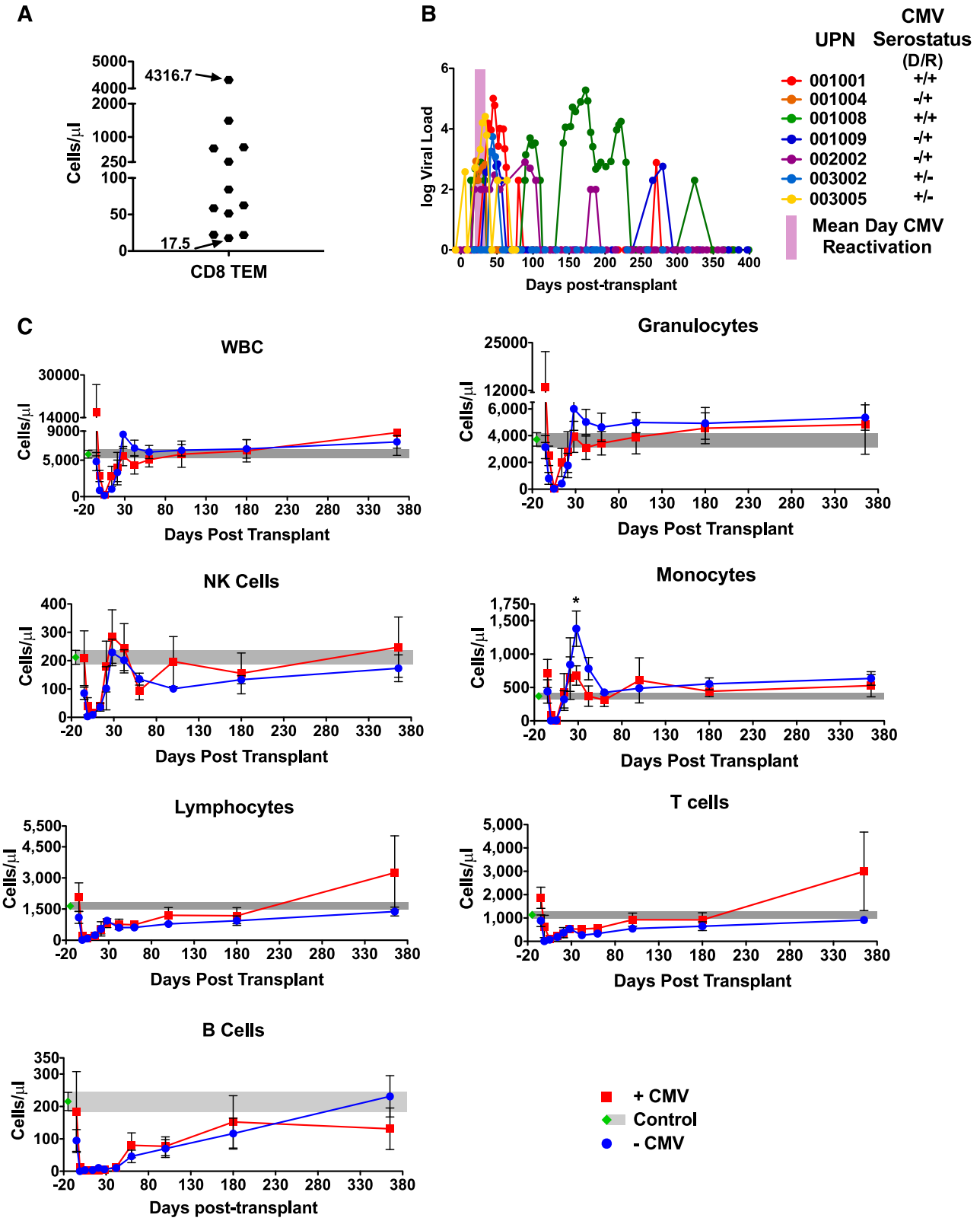

Figure 1. Viral reactivation and T-cell reconstitution in CMV-reactivating and nonreactivating patients. (A) $CD8^+$ Tem counts at day +365 posttransplant. (B) CMV viral load measured longitudinally. Each CMV-reactivating patient is shown as a unique colored line. Donor/recipient (D/R) pretransplant CMV serostatus is also indicated. Also shown is the mean day of CMV reactivation (±standard error of the mean [SEM]), depicted as a purple bar. (C) Longitudinal analysis of WBC, granulocytes, NK cells, monocytes, lymphocytes, total T cells, and total B cells posttransplant. Data are mean ± SEM in +CMV patients (red traces, n = 7), −CMV patients (blue traces, n = 10), and healthy controls (green diamond longitudinally extended as gray area, n = 10). *$P \leq .05$; Wilcoxon rank-sum test. (D) Longitudinal analysis of absolute numbers ± SEM of $CD4^+$ and $CD8^+$ T-cell reconstitution (+CMV [n = 7] and −CMV [n = 10]). Also shown are the means ± SEM of 10 healthy controls (green diamond extended longitudinally as gray area). (E) Analysis of CD4:CD8 ratio at baseline and days 100, 180, and 365 posttransplant as mean ± SEM in +CMV, −CMV, and healthy controls (green diamond). *$P \leq .05$; **$P \leq .01$; ***$P \leq .001$; NS, nonsignificant, Wilcoxon rank-sum test. (F) Analysis of CD4:CD8 ratio at day 100 of an extended cohort of 46 HSCT patients: +CMV (n = 26) and −CMV (n = 20).

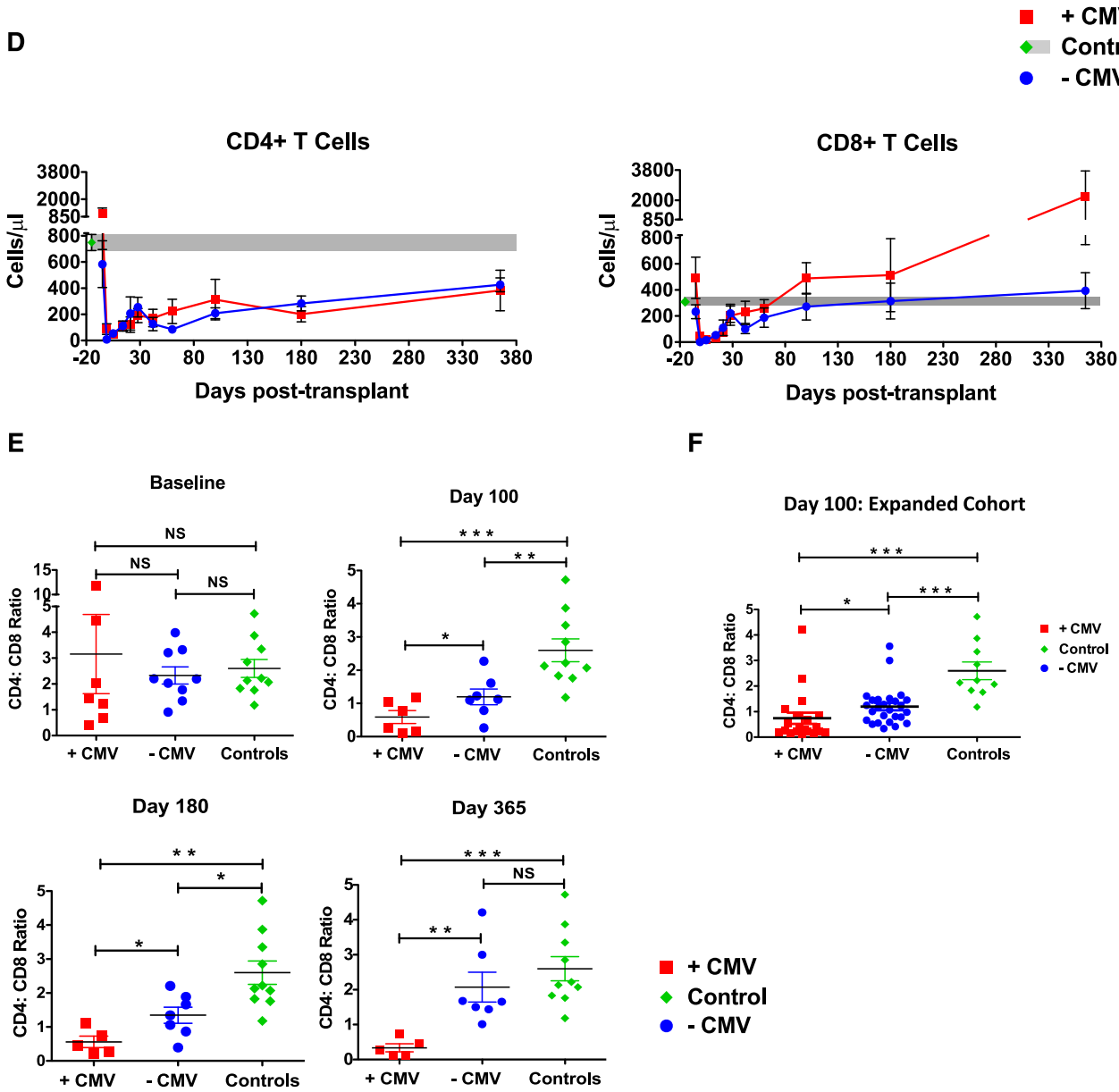

Figure 1. (Continued).

Results

Patient characteristics and statistical analysis

This study was based upon prospective, calendar-based clinical and immune monitoring of HSCT recipients enrolled on 2 contemporaneous clinical trials (Table 1). All patients received myeloablative pretransplant conditioning and unmanipulated unrelated-donor allografts.[23] Immune reconstitution was monitored in detail in all patients on-study, and revealed an extremely wide variability in the pace and character of their immune reconstitution, for which the heterogeneity was most prominent in the $CD8^+$ Tem compartment, with over 200-fold variability in the first year posttransplant (Figure 1A). To determine the factors that were contributing to this variability, we performed univariate analysis of the following patient characteristics and posttransplant complications (Table 2): patient sex, age, donor age, degree of donor/recipient HLA matching, relapse, posttransplant GVHD prophylaxis regimen (cyclosporine/methotrexate vs cyclosporine/methotrexate/abatacept),[23] grade II-IV aGVHD, moderate-severe cGVHD, infections (including respiratory viruses and bacterial infections), and CMV reactivation. Univariate analysis with the Kendall τ-b test (for binary variables) or with the Spearman rank test (for continuous variables) was used to determine which variables were positively correlated with elevated $CD8^+$ Tem counts. This analysis identified only CMV reactivation as predictive of day +365 $CD8^+$ Tem count ($P = .009$ for CMV reactivation, $P =$ not significant for all other parameters, Table 2). Of note, none of the clinical variables listed in Table 2 were predictive of total $CD3^+$, $CD4^+$, or $CD8^+$ T-cell counts (not shown). Although this study was not sufficiently large to build a full multivariate model, we did perform partial least squares (PLS) discriminant analysis to determine the primary predictors of high CD8 Tem counts,[27]

Table 2. Univariate analysis of contributors to day +365 CD8$^+$ Tem counts

Possible contributors to the day 365 CD8 Tem count	*P* value
CMV reactivation (Y/N)	.009
Sex (M/F)	.24
Moderate/severe cGVHD (Y/N)	.82
Grade II-IV aGVHD to day +100 (Y/N)	.87
Relapse (Y/N)	.92
Posttransplant immunosuppression + abatacept (Y/N)	.87
Non-CMV infections (Y/N)	.43
Recipient age	.89
Donor age	.1
8/8 vs 7/8 HLA match	.19

and this analysis also identified CMV reactivation as the primary driver of CD8 Tem expansion (not shown).

CMV reactivation kinetics

Figure 1B shows the kinetics of CMV reactivation in the +CMV cohort. The remaining patients (−CMV) did not demonstrate any CMV reactivation during the 1-year period of follow-up. As shown in the figure (purple bar), the mean day of first CMV reactivation in the +CMV cohort was 28 ± 4 days.

CMV reactivation resets CD8 Tem homeostasis

Although CMV reactivation did not impact the reconstitution kinetics of total white blood cells (WBCs), granulocytes, monocytes, total natural killer (NK) cells, lymphocytes, total T cells, B cells (Figure 1C), or total CD4$^+$ T cells (Figure 1D), CMV reactivation was associated with significant expansion of CD8$^+$ T cells, with a total CD8$^+$ T-cell count at 1 year posttransplant that was more than fivefold higher in the +CMV compared with –CMV cohort (Figure 1D). Indeed, as shown in Figure 1E, the canonical inversion of the CD4:CD8 ratio occurred exclusively in CMV reactivating patients, with an increasingly inverted ratio observed in the +CMV cohort throughout the first year posttransplant, whereas –CMV patients progressively normalized this ratio. The inversion of the CD4:CD8 ratio in CMV-reactivating patients was validated in a larger cohort of 46 patients who underwent unrelated donor HSCT at Emory University and who received CD4 and CD8 T-cell counts at day +100 as a part of their clinical care (Figure 1F).

Figure 2A-C demonstrates that the reconstitution of total CD8$^+$ T cells in the +CMV cohort was driven by a massive expansion of CD8$^+$ Tem and, unexpectedly, with a contraction of CD8$^+$ Tnaive. As shown in the figure, the reciprocal expansion/contraction of these 2 CD8 subpopulations occurred both in their relative proportions (Figure 2B) and in their absolute numbers (Figure 2C). This resulted in an average of >10-fold more CD8$^+$ Tem in the +CMV cohort at day +365 compared with the −CMV cohort ($P = .03$) and fivefold fewer CD8$^+$ Tnaive in the +CMV cohort at day +365 compared with the −CMV cohort ($P = .02$ at day +365). Importantly, as shown in Figure 2B, the inflection point of the CD8$^+$ Tem expansion and the concomitant CD8$^+$ Tnaive contraction closely correlated with the mean time to initial CMV reactivation (28 ± 4 days; purple bar). Indeed, as shown in this figure, the reconstitution of CD8 Tem was indistinguishable between the 2 cohorts prior to day +28, with the resetting of CD8 T-cell subpopulation homeostasis occurring in concert with the mean time to first CMV reactivation. The expanding CD8$^+$ Tem in the +CMV cohort expressed many of the canonical markers of viral-reactive effector memory cells, including loss of CD28, gain of Granzyme B (Figure 2D), and expression of CD57 (Figure 2E).

CMV reactivation drives antigen-specific T-cell clonal expansion

To determine whether the massive expansion of CD8 Tem in +CMV patients was due to viral reactivation, we first determined whether these T cells were derived from T-cell clonal expansions (TCEs).[28,29] To accomplish this, we used TCRβ deep sequencing, and compared the clonality of T cells purified from +CMV and −CMV patients. Given the dichotomous effect that CMV reactivation had on the size of CD8$^+$ T naive vs CD8$^+$ Tem subpopulations (Figure 2B-C), it was critical to examine the TCR repertoire and clonality of purified naive and Tem subsets individually (Figure 3A), rather than determining the repertoire in unfractionated T cells, as has been previously performed.[30-32] As shown in Figure 3, TCR deep-sequence analysis of the purified Tnaive and Tem samples enabled us to develop novel insights into the impact of CMV reactivation on posttransplant TCR repertoire dynamics. Thus, as shown in the representative TCR landscape (Figure 3B) and in Figure 3C-E, the clonality of the TCR repertoire of each sample was calculated using both normalized Shannon entropy number[33] and Gini coefficients.[34] This analysis confirmed that CMV reactivation was accompanied by a striking contraction of the TCR repertoire diversity in the CD8$^+$ Tem population, which was directly linked to the global expansion of the Tem compartment: thus, as shown in Figure 3D, a linear relationship was observed between the CD8 Tem clonality and the percentage of Tem for the +CMV cohort. Taken together, these data provide strong evidence for TCEs in the +CMV cohort.

Having established that +CMV patients developed TCEs posttransplant, we then used 3 assays of increasing sensitivity to probe the degree to which CMV-specific T cells accounted for these TCEs: first, we enumerated CMV-tetramer$^+$ cells in both the +CMV and −CMV cohorts (Figure 4A-B). Second, we measured the proportion of polyfunctional (interferon γ [IFNγ] + tumor necrosis factor [TNF]) CMV-peptide–responsive CD4$^+$ and CD8$^+$ T cells after transplant[35] in each cohort (Figure 4C-D). These 2 assays demonstrated that CMV-reactivating patients possessed significantly more CMV-specific and CMV-responsive T cells compared with nonreactivating patients.

To most rigorously define the degree to which Tem clonal expansion was driven by CMV-specific T cells, we performed a tetramer-specific sorting experiment, followed by high-throughput TCRβ repertoire sequencing of these highly purified CMV-specific populations (supplemental Table 4; Figure 5A-B). Tetramer$^+$ cells were sorted from day +365 samples from 4 CMV-reactivating patients (no cells were available from 1 patient): 001-001 (B*08 tetramer), 001-008 (A*24 tetramer), 0001-009 (A*02 tetramer), and 002-002 (A*02 + B*07 tetramers) (supplemental Table 4; supplemental Table 2). The analysis demonstrated that although each patient expanded a highly unique set of CMV-specific T-cell clones (with little clone sharing between patients) for each individual patient, there was an extremely high degree of overlap between the expanded Tem and tetramer$^+$ clones (Figure 5A). Moreover, as shown in Figure 5B, enrichment for the Tetramer$^+$ TCRβ clones in the most highly expanded Tem clones occurred. To further delineate this overlap, we mapped the 10 most highly expanded Tem clones onto the 10 most highly expanded tetramer$^+$ clones (Table 3). This analysis also documented high clone sharing, which is perhaps best illustrated for patient 001-008, in which 7 of the 10 most highly

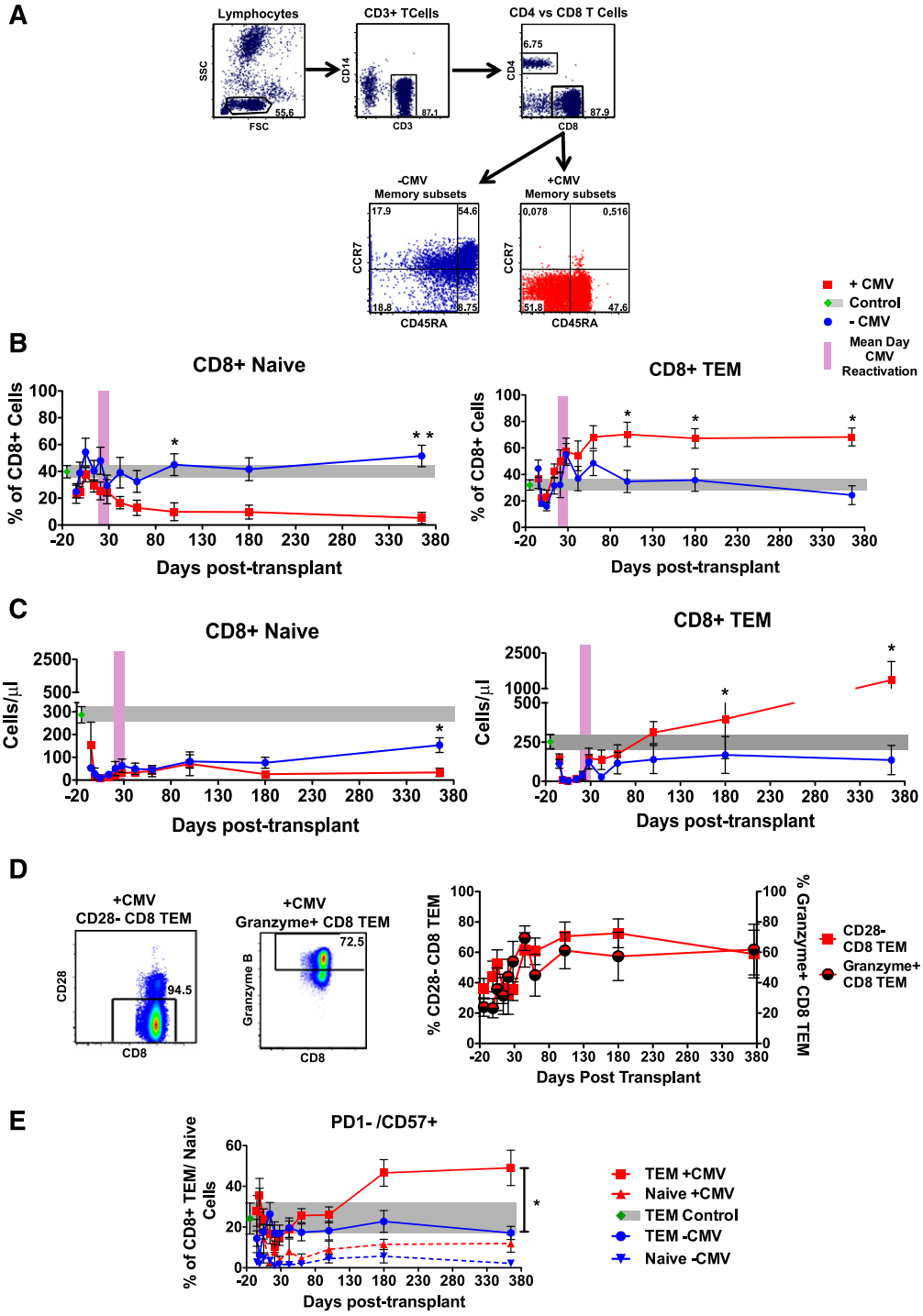

Figure 2. Longitudinal analysis of CD8 naive and memory T-cell subpopulation reconstitution in +CMV and −CMV patients. (A) Representative flow cytometry analysis of memory subset marker expression in $CD8^+$ T cells. Cells were gated as follows: lymphocytes were identified by FSC and SSC, $CD14^-CD3^+$ T lymphocytes were identified, further distinguished into $CD8^+$ and $CD4^+$ T cells, and analyzed for their expression of CCR7 and CD45RA memory markers. Tnaive were identified as $CCR7^+$ /$CD45RA^+$, TCM: $CCR7^+$/$CD45RA^-$, TEM: $CCR7^-$/$CD45RA^-$, TEMRA: $CCR7^-$/$CD45RA^+$. (B-C) Longitudinal analysis of $CD8^+$ naive and Tem subsets depicted in percentage frequency (B) or absolute cell numbers (C). Data are mean ± SEM in +CMV patients (n = 7), −CMV patients (n = 10), and healthy controls (n = 10). Also shown is the mean day of CMV reactivation (±SEM), depicted as a purple bar. (D) Left, Representative flow cytometry analysis at day +365 shows CD28 expression (left panel) and Granzyme B expression (right panel) on $CD8^+$ Tem. Right, Longitudinal analysis of mean ± SEM $CD28^-CD8^+$ Tem (left y-axis) and Granzyme B^+CD8^+ Tem (right y-axis). (E) Longitudinal analysis of PD-1^-/$CD57^+CD8^+$ Tem (solid lines) and naive T cells (dotted lines) of +CMV (n = 7), −CMV (n = 10) patients, and healthy controls. All data are mean ± SEM. *$P \leq .05$; **$P \leq .01$ Wilcoxon rank-sum test. CCR, C-C chemokine receptor; FSC, forward scatter; SSC, side scatter; TCM, central memory T cells; TEMRA, effector memory-RA T cells.

expanded Tem TCRβ clones were also tetramer+. Taken together, these data provide strong molecular evidence that the expansion of $CD8^+$ Tem in +CMV patients was driven by the clonal expansion of CMV-specific T cells. Moreover, the TCRβ sequences of the expanded and shared Tem and $Tetramer^+$ T-cell clones from each patient (Table 3), identified 17 novel TCRβ sequences for which this

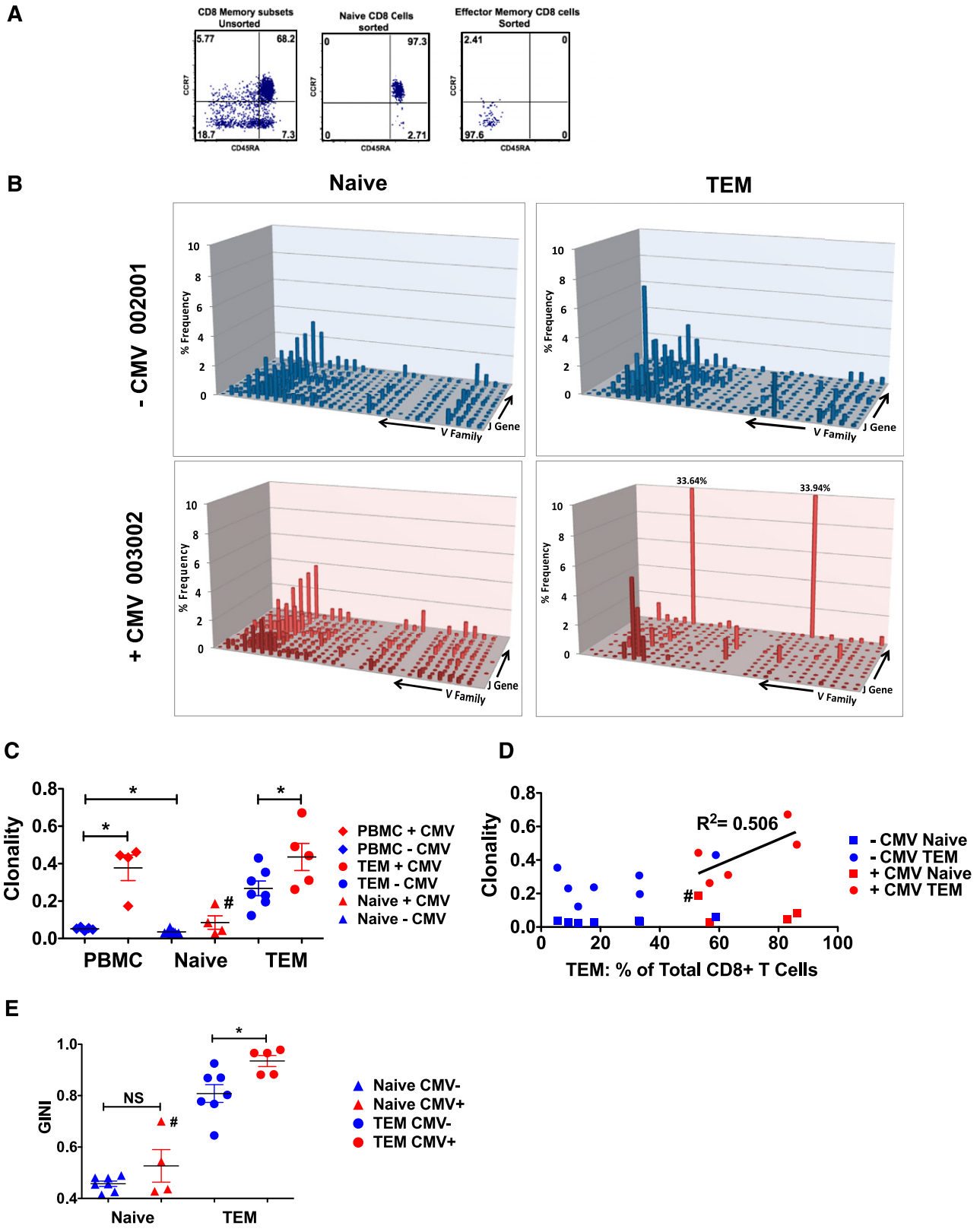

Figure 3. Contraction of Tem TCR diversity but not Tnaive TCR diversity in CMV-reactivating patients. (A) Representative flow cytometry analysis illustrates the purity of CD8$^+$ Tnaive and CD8$^+$ Tem cells before (left plot) and after (middle and right plots) cell sorting. (B) Shown are representative graphs of the CD8$^+$ naive (left column) and CD8$^+$ Tem (right column) TCR landscape (showing frequencies of V and J gene combinations detected through deep sequencing) from 1 −CMV (blue) and 1 +CMV (red) patient. (C) Clonality of PBMC, CD8$^+$ Tnaive, and CD8$^+$ Tem are shown for −CMV and +CMV patients as measured by the inverse of the normalized Shannon entropy number. All data are mean ± SEM. *$P \leq .05$; Wilcoxon rank-sum test. (D) The clonality of Tnaive and Tem CD8$^+$ cells for each patient is compared with the percentage of Tem CD8$^+$ cells detected via flow cytometry in each patient. Linear regression ($R^2 = 0.506$) shows a correlation between expansion of Tem and increased Tem clonality in +CMV patients (red). No such relationship could be detected in −CMV patients (blue). (E) TCR diversity of CD8$^+$ Tnaive and CD8$^+$ Tem are shown for −CMV and +CMV patients as measured by the Gini coefficient.[44] All data are mean ± SEM. *$P \leq .05$; Wilcoxon rank-sum test. #Sorting purity of Tnaive from patient 001-008 could not be confirmed due to low yield.

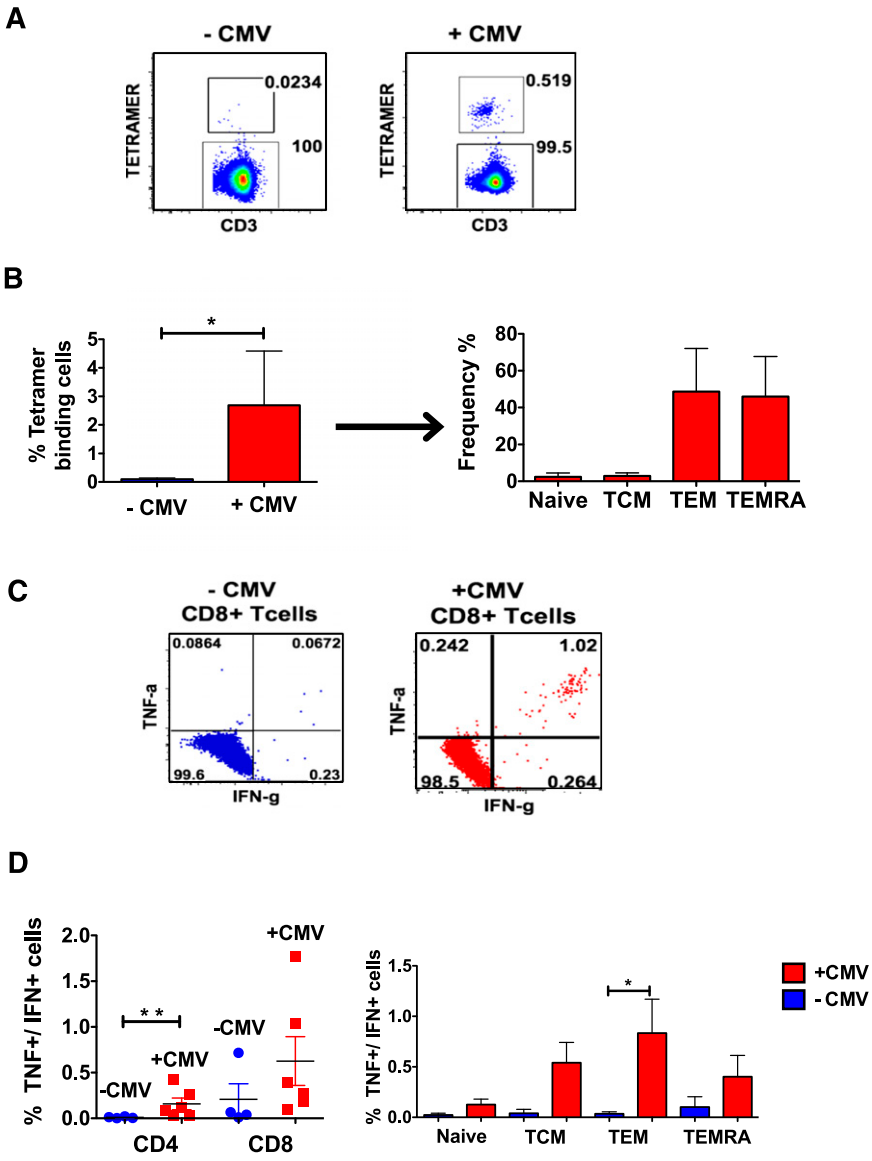

Figure 4. Analysis of CMV-specific CD8$^+$ T cells using peptide stimulation and tetramer binding. (A) Patients who were positive for HLA-A2, -B7, or -B8 were analyzed for the frequency with which they bound to appropriate CMV-specific tetramers. Representative flow cytometry analysis is shown for −CMV (left) and +CMV (right) patients. Cells were gated as follows: lymphocytes were identified in a FSC/SSC plot, out of these, singlets were isolated. From these, CD14$^-$/CD20$^-$/CD3$^+$ T cells were identified and further gated on CD8$^+$ T cells, which were analyzed for their binding to matching tetramers. (B) Left, bar graph illustrating the frequency of tetramer-binding cells from −CMV (blue) and +CMV (red) patients. Right graph, Tetramer$^+$ cells from +CMV patients were further separated into their respective memory subsets. (C) Representative flow cytometry analysis from −CMV and +CMV patients of intracellular cytokine expression after stimulation with CMV-specific peptides. CD8$^+$ T cells were gated as described in Figure 2A and then analyzed for the production of IFNγ and TNF. (D) Frequency of IFNγ$^+$/TNF$^+$ CD4$^+$ and CD8$^+$ T cells of +CMV (n = 6) and −CMV (n = 4) patients (left). Also the frequency of IFNγ$^+$/TNF$^+$ CD8 T cells in each of the 4 memory subsets is shown for +CMV and −CMV patients (right). *$P \leq .05$; **$P \leq .01$ Wilcoxon rank-sum test.

study provides new evidence for linked functional expansion and CMV specificity. Although these clones likely represent only a portion of the entire CMV-specific repertoire,[36,37] to our knowledge, this comparative TCRβ deep-sequencing data constitutes the first quantitative molecular link between posttransplant CD8 Tem reconstitution and CMV-specific T cells, and strongly supports a model of oligoclonal T-cell expansion in the setting of CMV reactivation.

CMV reactivation is associated with holes in the underlying TCR repertoire, despite the CMV-specific TCEs

Although previously available technologies were able to document the impact of CMV reactivation on the clonality of the T-cell repertoire,[38,39] these methods (including flow cytometry–based V-J tracking and spectratyping) are not sensitive or specific enough to interrogate the TCR landscape that underlies the highly clonally expanded CMV-specific T-cell clones. In the current study, we were able to intensively probe, for the first time, the impact of CMV reactivation on the CD8 Tem TCR landscape in toto and, specifically, to determine whether CMV reactivation was associated with specific deficits or "holes" in the repertoire. To test this hypothesis, we first created 2 reference V-J family distributions: (1) a reference repertoire derived from unique CD8 Tnaive clones of 7 healthy adults between the ages of 26 and 57 years (supplemental Table 5) which was termed the VJ^r and (2) a reference derived from naive CD8 T cells of the transplanted patients at day +365 (which was termed the VJ^r-transplant). The rationale for the choice of Tnaive to create the VJ^{r}s was that a healthy adult Tnaive repertoire would be expected to be the most representative of the breadth of V-J fractions (rather than deriving the VJ^r from memory subsets, which, even in normal individuals, would be expected to display skewing for selected V-J fractions given previous antigen experience). To estimate the number of holes in the Tem compartment of each patient, we subtracted the V-J family distribution for each of the patient-specific query repertoires (VJ^q, see supplemental Methods) from either the VJ^r or the VJ^r-transplant. This exposed deficiencies in the former relative to each reference, and holes were deemed to exist in V-J families where such deficiencies exceeded the mean for VJ^r. Hole analysis was confined to the Tem compartment (instead of the Tnaive) because it was only in the CD8 Tem that TCEs occurred (Figures 3 and 5; Table 3) and, thus, it was in this compartment that the issue of holes was most

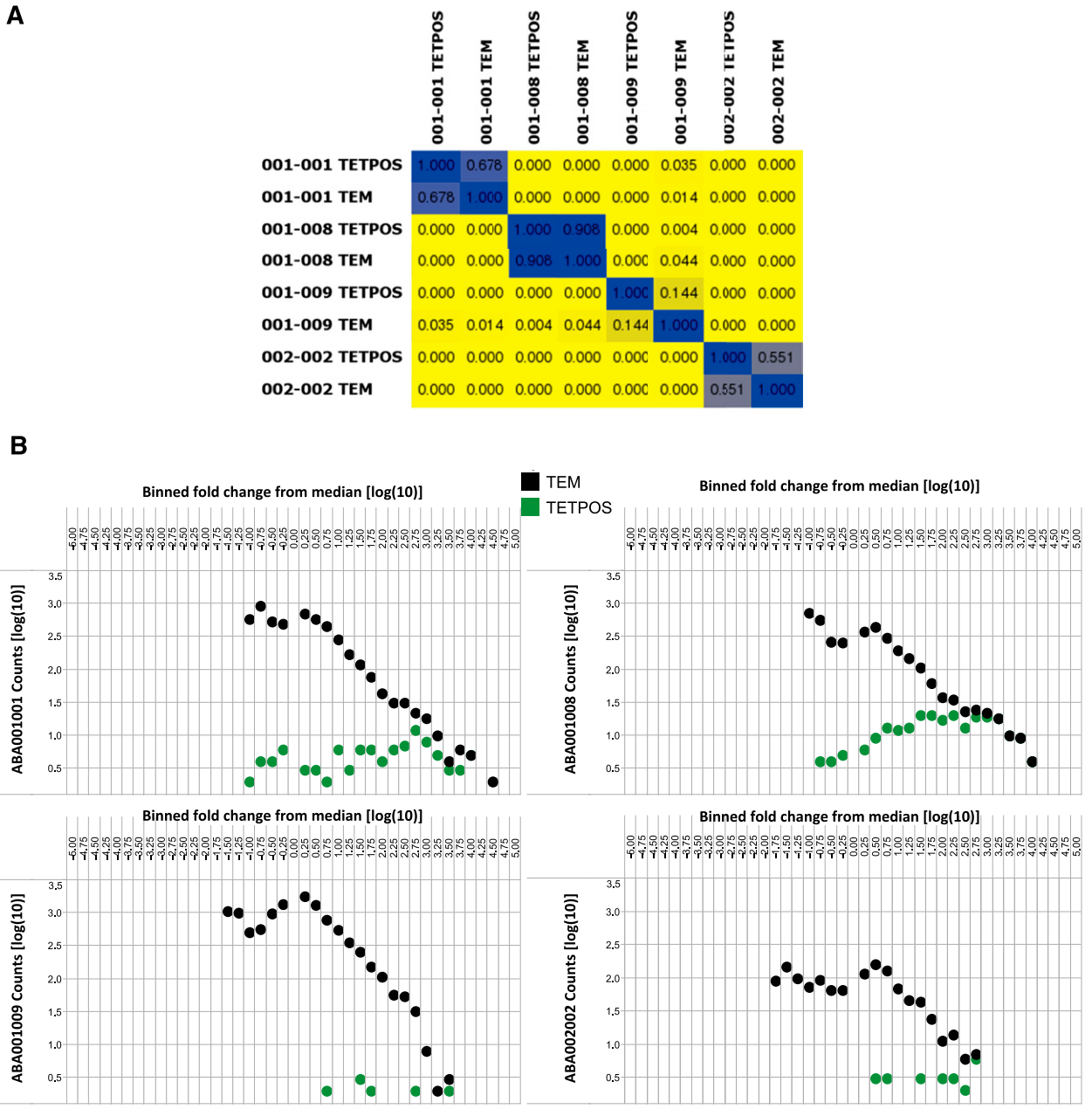

Figure 5. Inter- and intrapatient evaluation of shared TCRβ clones between purified Tem and tetramer$^+$ cells. (A) Color-coded pairwise TCRβ clone sharing between samples. Colors range from yellow (indicates no sharing) to blue (indicates 100% sequence sharing). This matrix depicts the degree of clone sharing between all identified TCRβ sequences in the sorted Tem and tetramer$^+$ cells. (B) Enrichment of Tetramer$^+$ cells in the clonally expanded Tem. Shown are dot plots for 4 +CMV patients which depict the binned frequencies for each of the TCR clones in the sorted CD8$^+$ Tem ("TEM"; black) and sorted tetramer$^+$ cells ("TETPOS"; green). These graphs depict the change in frequencies of productive clones, each compared with a sample-specific median frequency value. Bins represent 0.25 log(10) intervals, with binned data reported using a $\log_{10}$ scale. Tem clones are shown as black circles and the Tetramer$^+$ clones are shown as green circles.

relevant. As shown in Figure 6A-D, CMV reactivation was associated with a compromised CD8 Tem TCR repertoire, in which the TCR repertoire of +CMV patients demonstrated significantly more holes than were present in −CMV patients.

To investigate the potential confounding contribution of GVHD to holes in the repertoire, 2 additional analyses were performed. First, as shown in Figure 6E, −CMV patients were divided into those that did not develop significant aGVHD or cGVHD (patients 001-002, 002-001, 003-001, 004-001) and those that did develop significant GVHD (patients 001-005, 001-006, 003-003). As shown in the figure, this analysis documented no difference in the number of holes in −CMV patients based on GVHD status. Second, as shown in Figure 6F, we compared the number of holes in the subset of patients who all developed significant aGVHD or cGVHD, based on their CMV status, comparing +GVHD/+CMV patients (001-001, 001-008, 001-009, 002-002, 003-002) to +GVHD/−CMV patients (001-005, 001-006, 003-003). As shown in the figure, this subset analysis recapitulated the results in Figure 6C-D, documenting more holes in the +CMV/+GVHD patients compared with the −CMV/+GVHD patients. This constitutes the first exhaustive analysis of the underlying TCR repertoire after CMV reactivation, and provides the strongest evidence to date for significant molecular immune compromise in these patients.

Table 3. Rank order list of highly clonal TCR sequences from CD8$^+$ Tem and CMV tetramer$^+$CD8$^+$ T cells after CMV reactivation

UPN	Rank Order	Clone Frequency (%) Tem	Tem TCR V Gene Sequence	Tem TCR J Gene Sequence	Clone Frequency (%) Tetramer+	CMV-Specific Tetramer+ V gene sequence	CMV-Specific Tetramer+ J gene sequence	Rank in Tem	Color Code
001-001	1	11.673	TGTGCCAGCAGCTTAG	TTT	53.272	TGCGCCAGCAGCTTGG	ACTGAAGCTTTCTTT	#1	
001-001	2	11.277	TGTGCCAGCAGC	ACAATGAGCAGTTCTTC	31.844	TGTGCCAGCAGCTTGG	TGAACACTGAAGCTTTCTTT	#2	
001-001	3	6.844	TGTGCCAGCAGCTTGG	TGAACACTGAAGCTTTCTTT	12.006	TGTGCCAGCAG	CTCCTACAATGAGCAGTTCTTC	#3	
001-001	4	5.114	TGCGCCAGCAGCCAAGA	ACAATGAGCAGTTCTTC	0.278	TGTGCCAGCAGTG	CGGGGAGCTGTTTTTT	#4	
001-001	5	4.164	TGTGCCAGCAG	AAGAGACCCAGTACTTC	0.159	TGTGCCAGCAGCTTGG	ACTGAAGCTTTCTTT	#5	
001-001	6	3.437	TGTGCCAGCAGCTTAGG	CGAGCAGTACTTC	0.133	TGTGCCAGCAGCC	CGAGCAGTACTTC	#6	
001-001	7	3.291	TGTGCCAGCAG	CTCCTACAATGAGCAGTTCTTC	0.118	TGTGCAAGTCGCTTAGTAG	AGAGACCCAGTACTTC	#7	
001-001	8	2.984	TGTGCCAGCAGC	ACTATGGCTACACCTTC	0.113	TGTGCCAGCAGCTTAGG	CGAGCAGTACTTC	#8	
001-001	9	2.823	TGTGCCAGCAGCGTAG	TGGCTACACCTTC	0.112	TGTGCCAGCAGC	CACAGATACGCAGTATTTT	#9	
001-001	10	1.98	TGTGCCAGCAGTG	GAACACTGAAGCTTTCTTT	0.091	TGCGCCAGCAGCCAAGA	ACAATGAGCAGTTCTTC	#10	
001-008	1	9.844	TGTGCCAGCAGTT	CTCCTACGAGCAGTACTTC	12.237	TGTGCCATCAGTGA	CTCCTACGAGCAGTACTTC		
001-008	2	7.476	TGTGCCAGTAGTAT	CCGGGGAGCTGTTTTTT	8.043	TGTGCCAGTAGTAT	CCGGGGAGCTGTTTTTT		
001-008	3	3.942	TGTGCCAG	GAGACCCAGTACTTC	3.477	TGCAGTGCTACGGGGGAA	CTACGAGCAGTACTTC		
001-008	4	3.738	TGTGCCACCAG	TACAATGAGCAGTTCTTC	2.548	TGCGCCAGCAG	ATGAGCAGTTCTTC		
001-008	5	3.57	TGTGCCAGCAGCTTG	CCAAGAGACCCAGTACTTC	2.515	TGTGCCAGCAGCGTAGGA	CCTACGAGCAGTACTTC		
001-008	6	3.311	TGCAGTGCTACGGGGGAA	CTACGAGCAGTACTTC	2.474	TGTGCCAGCAGCTTGG	TCACCCCTCCACTTT		
001-008	7	2.593	TGTGCCATCAGTGA	CTCCTACGAGCAGTACTTC	2.313	TGTGCCAGCAGTT	CTCCTACGAGCAGTACTTC		
001-008	8	2.353	TGTGCCAGCAGCGTAGGA	CCTACGAGCAGTACTTC	2.301	TGTGCCAGCAGCGTAG	TAATTCACCCCTCCACTTT		
001-008	9	2.312	TGTGCCAGCAGCTTGG	TCACCCCTCCACTTT	2.134	TGTGCCAGTAGT	CTCTGGAAACACCATATATTTT		
001-008	10	2.132	TGTGCCAGC	TGAACACTGAAGCTTTCTTT	2.032	TGTGCCAGC	TGAACACTGAAGCTTTCTTT		
001-009	1	2.923	TGTGCCAGCAGCTTGG	CTCCTACAATGAGCAGTTCTTC	51.613	TGTGCCAGCAGTTA	CGAGCAGTACTTC		
001-009	2	2.15	TGCGCCAGCAGC	ACACTGAAGCTTTCTTT	31.85	TGTGCCAGCAGT	CTATGGCTACACCTTC		
001-009	3	2.092	TGTGCCAGCAGTGA	CCTACGAGCAGTACTTC	7.563	TGTGCCAGCAGTTAC	TATGGCTACACCTTC		
001-009	4	1.859	TGTGCCAGCAGTTTA	CGCAGTATTTT	2.421	TGCAGCGCGGGGGGTATT	ACACTGAAGCTTTCTTT		
001-009	5	1.363	TGTGCCAGCAGTTACTC	TAACTATGGCTACACCTTC	2.064	TGTGCCAGCAG	CACCGGGGAGCTGTTTTTT		
001-009	6	1.113	TGTGCCAGCAGCTTAG	CCGGGGAGCTGTTTTTT	1.224	TGTGCCAGCAGT	CTATGGCTACACCTTC		
001-009	7	1.056	TGTGCTAG	CTATGGCTACACCTTC	0.914	TGTGCCAGCAGT	CCGGGGAGCTGTTTTTT		
001-009	8	0.93	TGTGCCAGCAGTGACGGG	AACACCGGGGAGCTGTTTTTT	0.811	TGTGCCAGCAGCG	ACCGGGGAGCTGTTTTTT		
001-009	9	0.873	TGTGCCAGCAGTGAGGGG	CACTGAAGCTTTCTTT	0.34	TGTGCCAGCAGTTTA	CGCAGTATTTT		
001-009	10	0.863	TGTGCCAGCAGTTACGGGA	TACGAGCAGTACTTC	0.243	TGTGCCAGCAGC	CACCGGGGAGCTGTTTTTT		
002-002	1	10.544	TGTGCCTGGA	CTATGGCTACACCTTC	82.046	TGTGCCAGCAGC	CCTACGAGCAGTACTTC		
002-002	2	3.579	TGTGCCAGCAGT	ACTAATGAAAAACTGTTTTTT	7.513	TGTGCCAGCAGT	TCCTACGAGCAGTACTTC		
002-002	3	3.445	TGTGCCAGCAGTT	ACGAGCAGTACTTC	2.868	TGTGCCAGCAGT	ACTAATGAAAAACTGTTTTTT		
002-002	4	2.754	TGTGCCAGCAGCCAAGA	CTGAAGCTTTCTTT	1.388	TGTGCCAGCAGTGAAT	ATGGCTACACCTTC		
002-002	5	2.752	TGCGCCAGCAGCCAAGA	TGAACACTGAAGCTTTCTTT	0.824	TGTGCCAGCAGTTGGGACA	TATGGCTACACCTTC		
002-002	6	2.7	CGTGCCAGCAGC	GCCCCAGCATTTT	0.579	CGTGCCAGCAGC	GCCCCAGCATTTT		
002-002	7	2.676	TGTGCCAGCAGT	GGGGCCAACGTCCTGACTTTC	0.557	TGCGCCAGCAGCCAAGA	TGAACACTGAAGCTTTCTTT		
002-002	8	2.637	TGTGCCAGCAGTGAAT	ATGGCTACACCTTC	0.499	TGTGCCAGCAGCC	TTTTT		
002-002	9	2.399	TGTGCCAGTA	ACACTGAAGCTTTCTTT	0.33	TGTGCCAGCAGTTTAGGGT	TCAGCCCCAGCATTTT		
002-002	10	2.041	TGTGCCAGCAGTTGGGACA	TATGGCTACACCTTC	0.322	TGTGCCAGCAGC	AGCACAGATACGCAGTATTTT		

Color coding schema: Each of the top 10 TCRβ sequences within the sorted CD8$^+$ Tem from each patient was ranked and color coded. If a top 10 Tem clone was shared with a tetramer$^+$ clone from the same patient, the tetramer$^+$ clone was color coded accordingly.

CMV reactivation is associated with reduced Tnaive and thymic output after transplant

Given the observed defects in the TCR repertoire in the setting of CMV reactivation, we queried our data set to investigate whether a connection existed between reactivation and naive T-cell reconstitution as well as thymic output. We found that although patients reactivating CMV demonstrated specific expansion of CD8 Tem (Figure 2B-C), they demonstrated a global contraction of both CD4 and CD8 Tnaive (Figures 2B and 7A). In addition, as shown in Figure 7B, these patients demonstrated a progressive loss of CD31$^+$CD4$^+$ Tnaive, a subpopulation that has been previously shown to be enriched in new thymic emigrants.[40,41] These results are consistent with thymic compromise in CMV-reactivating patients, an observation that has previously been shown in humanized murine models.[42,43] Although Figures 2B and 7A-B document the impact of CMV reactivation on Tnaive reconstitution, when patients were dichotomized by the presence or absence of GVHD, no such correlation with Tnaive reconstitution was observed (Figure 7C-E).

Discussion

We have performed an analysis of the impact of CMV reactivation on posttransplant immune reconstitution at an unprecedented level of cellular and molecular detail. Given that immune reconstitution must occur de novo in HSCT patients, and that these patients are at high risk for CMV reactivation, studying this patient population with the tools of multiparameter flow cytometry, cell sorting, tetramer-based cell purification, and TCRβ deep sequencing provides a rigorous time-collapsed experiment on the impact of CMV on global immune phenotype and function. Moreover, the ability to correlate changes in T-cell composition and reconstitution with the inflection point of viral reactivation in these patients further strengthens the mechanistic links that can be made between CMV reactivation and immune homeostasis. Although other reactivating viruses (eg, Epstein-Barr virus, herpes simplex virus) were not studied, and may also impact immune reconstitution, these experiments provided quantitative molecular evidence for CMV-specific T-cell clonal expansion and, importantly, for the development of holes in in the underlying T-cell repertoire in the setting of CMV reactivation.

In this study, 17 patients underwent detailed longitudinal immune analysis, which included both phenotypic and functional assays, as well as exhaustive TCRβ deep sequencing of sorted Tem, Tnaive, and Tetramer$^+$ cells. In addition, 10 healthy controls were used as a comparator group for the longitudinal flow cytometric analysis and an additional 7 healthy controls were used to create the reference TCRβ repertoire. Although the number of transplanted patients that were exhaustively analyzed in this study is still relatively small, several factors strengthen the conclusions that we have been able to draw: first, each of the patients underwent detailed longitudinal analysis, with 10 time points analyzed per patient (Figures 1-2). This provided

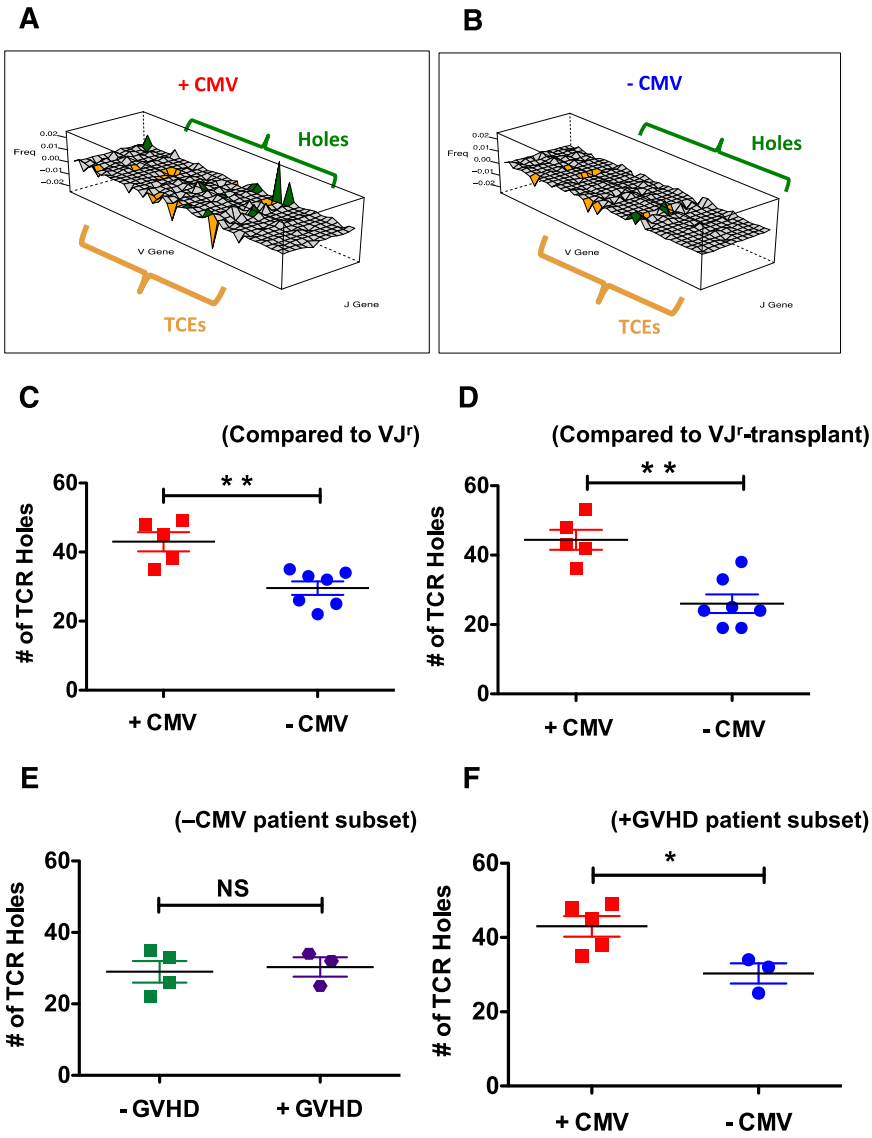

Figure 6. Impact of CMV reactivation on CD8 Tem TCR repertoire holes. (A-B) For panels A-B, V, and J genes are represented along the x-y axes, whereas the difference in the V-J–specific proportions of unique clones are represented along the z-axis and labeled "Freq." TCE and holes analysis was performed as described in "Methods." Panels A and B depict wire-frame 3-dimensional graphs (created using the Lattice graphics package in R) for 2 representative patients (001-008, panel A and 001-005, panel B). Both TCEs and holes in the TCR repertoire are depicted in these graphs, with V-J families in orange depicting TCEs and those in green depicting holes. Those families shown in light gray are already sparse in the VJ^r and thus were not evaluated for holes. (C) Summary analysis showing the mean number of holes in the CD8 Tem from the +CMV (red squares) and −CMV (blue circles) patients using the VJ^r. $^{**}P < .01$. (D) Summary analysis showing the mean number of holes in the CD8 Tem from the +CMV (red) and −CMV (blue) patients using the VJ^r-transplant. $^{**}P < .01$. (E) Analysis of the number of holes in −CMV patients that either did not develop GVHD (green squares, left) or did develop GVHD (purple hexagons, right) compared with the VJ^r. $P = 1.0$. (F) Analysis of the number of holes in +GVHD patients that either did reactivate CMV (red squares) or did not reactivate CMV (blue circles) compared with the VJ^r. $^{*}P < .05$.

significant intrapatient controls for the longitudinal evaluation, and allowed a detailed correlation of changes in T-cell reconstitution with the inflection point of CMV reactivation. Second, a larger cohort of 46 patients, who underwent standard day 100 CD4 and CD8 counts at our institution, were also evaluated for CD4:CD8 ratio (Figure 1F), and confirmed the results that were obtained with the 17 exhaustively analyzed patients. Given the pivotal importance of the expansion of CD8 T cells to the results in this study, this external validation was of critical importance. Finally, the depth of the analysis that we performed on the patients enrolled provided a great deal of statistical power: high statistical significance was obtained for the flow cytometric as well as the deep-sequencing results where a mean of >3 million reads per sample was obtained. Together, these support the high fidelity of the data set used in the current analysis.

Our results support a model wherein CMV reactivation can drive posttransplant T-cell reconstitution. Indeed, several of the traditional hallmarks of T-cell reconstitution, often attributed to post-HSCT immune reconstitution per se,[44-51] occurred exclusively in those patients that reactivated CMV. These included the inversion of the CD4:CD8 T-cell ratio, as well as the enhanced expression of the terminal differentiation marker CD57[52-55] on $CD8^+$ T cells in the +CMV cohort.

TCRβ deep sequencing enabled measurement of the impact of posttransplant CMV reactivation on immune reconstitution at a level of detail unavailable with previous technologies. This included the identification of T-cell clonal expansions in the setting of CMV reactivation, as has been previously documented.[32,56] Importantly and unique to the current study, the linkage of CD8 Tem deep sequencing with CMV $Tetramer^+$ deep sequencing provided incontrovertible evidence that the (often extreme) expansion of CD8 Tem (Figure 2B-C) in the setting of CMV reactivation was due to patient-specific CMV-directed clones (Figure 5A-B), rather than to nonspecific global expansion of Tem.

Although the analysis of the TCEs provided several novel insights into the impact of CMV on immune reconstitution posttransplant, perhaps the most important insights concerned the TCR repertoire that lied "underneath" the clonal expansions. Thus, detailed molecular analysis of this repertoire in toto has been previously unattainable, given technical limitations of TCR spectratyping or targeted sequencing. However, the deep-sequencing techniques applied in this study have allowed us to evaluate in detail the structure of the entire TCR repertoire and the impact of CMV reactivation on this structure. Given the documented existence of CD8 Tem TCEs in the setting of CMV reactivation, 2 mutually

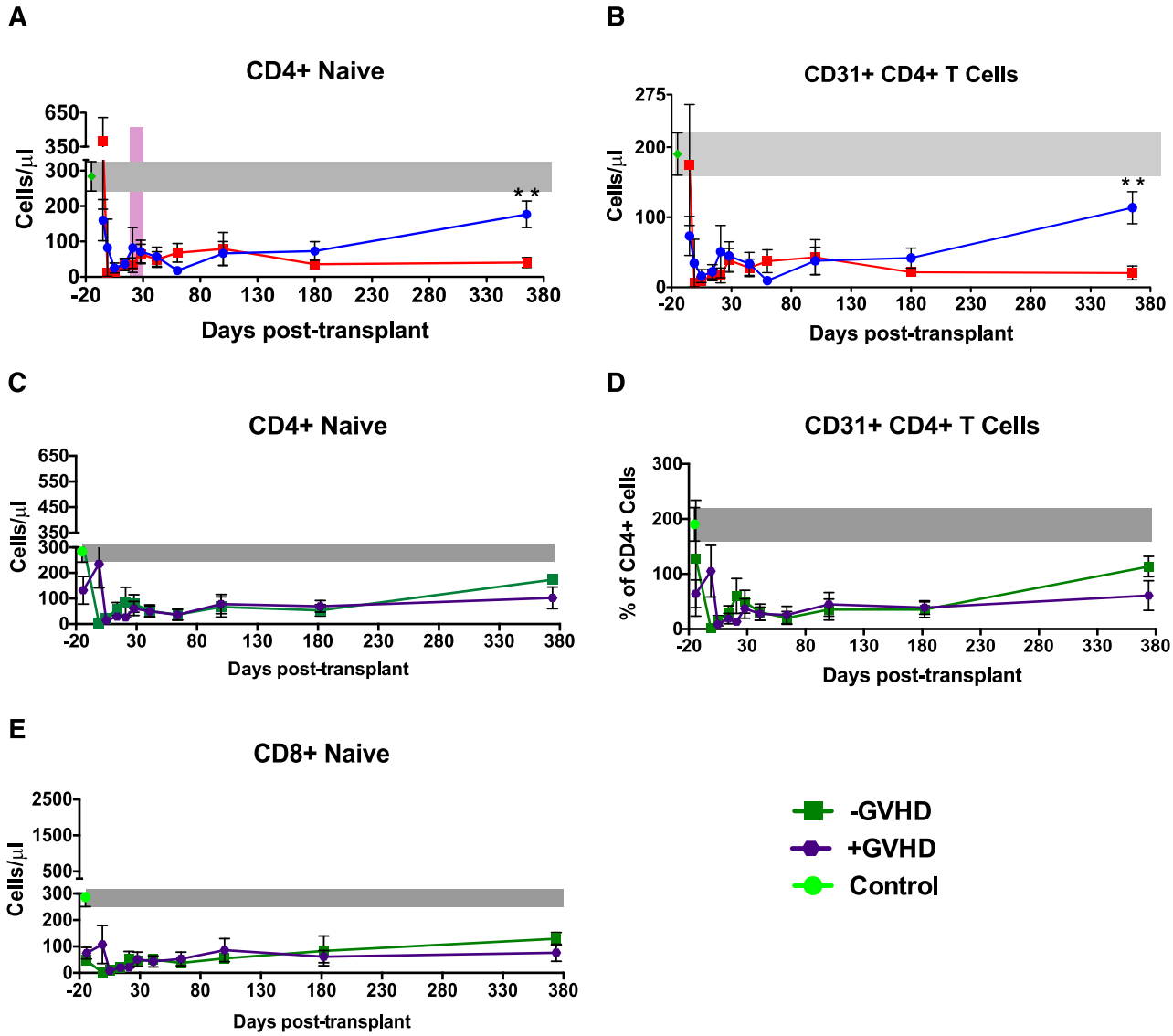

Figure 7. Impact of CMV reactivation and GVHD on the reconstitution of CD4$^+$ Tnaive and CD31$^+$ recent thymic emigrants. (A) Longitudinal analysis of the reconstitution of CD4$^+$ Tnaive. Data are mean ± SEM in +CMV patients (n = 7), −CMV patients (n = 10), and healthy controls (n = 10). Also shown is the mean day of CMV reactivation (±SEM), depicted as a purple bar. (B) Longitudinal analysis of the reconstitution of CD31$^+$/CD4$^+$ recent thymic emigrants. Data are mean ± SEM in +CMV (n = 7), −CMV patients (n = 10), and healthy controls (n = 10). *$P \leq .05$; **$P \leq .01$; NS, nonsignificant; Wilcoxon rank-sum test. (C) Longitudinal analysis of the reconstitution of CD4$^+$ Tnaive dichotomized based on GVHD status. Data are mean ± SEM in +GVHD patients (purple circles, n = 10), −GVHD patients (green squares, n = 7), and healthy controls (green circle and gray bar, n = 10). (D) Longitudinal analysis of the reconstitution of CD31$^+$/CD4$^+$ recent thymic emigrants dichotomized based on GVHD status. Data are mean ± SEM in +GVHD patients (purple circles, n = 10), −GVHD patients (green squares n = 7), and healthy controls (green circle and gray bar, n = 7). (E) Longitudinal analysis of the reconstitution of naive CD8$^+$ T cells dichotomized based on GVHD status. Data are mean ± SEM in +GVHD patients (purple circles, n = 10), −GVHD patients (green squares, n = 7), and healthy controls (green circle and gray bar, n = 10).

exclusive scenarios with respect to the remainder of the TCR repertoire were possible: In the first scenario, TCEs exist, but do not alter the topology of the underlying repertoire, implying intact T-cell immunity despite clonal expansions. In the second, TCEs exist concomitant with defects in the underlying reservoir: this scenario predicts defects in T-cell immunity. As shown in Figure 6, in the current study, we were able to measure, for the first time, the impact of CMV-specific TCEs on the underlying CD8 Tem immune reservoir and find that there were significantly more holes in the Tem reservoir of CMV-reactivating patients than in those that did not reactivate virus. This provides the first molecular link between CMV reactivation and a detrimental impact on subsequent protective immunity in transplant patients.

The underlying cause of the defects in the TCR repertoire in +CMV HSCT patients remains unknown, but 2 possible hypotheses have been considered. In the first, the TCEs caused by CMV reactivation result in competition for T-cell "space" in a scenario in which there is a zero-sum game for the number of T-cell clones that can exist in any individual.[57] Although this scenario is possible, 3 lines of evidence suggest that it is unlikely. First, mouse models have shown that the number of CD8 Tem in the mammalian host adapts according to immunologic experience.[58] Second, holes in the repertoire were not observed in aging CMV-seropositive individuals (R.M., Paul Lindau, C.D., Jeanne DaGloria, Heidi Utsugi, Edus H. Warren, Stanley R. Riddell, Karen W. Makar, Cameron J. Turtle, H.S.R., manuscript submitted April 2015), providing evidence that TCEs alone do not necessitate defects in the repertoire. Finally, CMV-reactivating transplant patients demonstrated significant growth in their total CD8$^+$ and CD8 Tem compartment (Figures 1D and 2C), suggesting a large capacity for CD8 expansion in these patients.

The second hypothesis concerns the health of the thymus in CMV-reactivating patients, and suggests that thymic damage may result in defects in T-cell immunity. Indeed, one of the unexpected observations made in the present study was that, although CMV reactivation drove a specific expansion of $CD8^+$ Tem cells, it was associated with a global contraction of both CD4 and CD8 Tnaive, accompanied by significantly fewer $CD31^+CD4^+$ putative recent thymic emigrants.[40,41] Although the addition of T-cell receptor excision circles (TREC) analysis[59,60] (which was not feasible given sample constraints in the current study) would strengthen this data set, the flow cytometric observations suggest that a defect in thymopoiesis may have been linked to CMV reactivation and to the CMV-driven expansion of $CD8^+$ Tem. Indeed, there is supportive precedent in the literature for this, as previous studies have documented that CMV can infect thymic epithelium and that activated and effector T cells can directly infiltrate and damage the thymus.[43,61-63] Moreover, a previous study of young adults, thymectomized as children, showed that the combination of the lack of thymic function and CMV infection lead to dramatic changes in T-cell homeostasis.[55] These data suggest the novel hypothesis that CMV-associated thymic damage may be one of the contributory mechanisms for the immune compromise that accompanies CMV reactivation after transplant.

In determining the impact of CMV reactivation on posttransplant immune reconstitution, it is critical to also consider the potential impact that GVHD can make on thymic and immune dysfunction, given the fact that GVHD and CMV reactivation are often linked. Indeed, previous studies often[59,60] but not always[64,65] have documented decreased TRECs in patients that have developed cGVHD. In the current study, we have documented that (1) +CMV patients had more CD8 Tem repertoire holes than −CMV patients (Figure 6A-D); (2) when a subanalysis of only patients that developed GVHD was performed, there were more holes in the CD8 Tem in the +CMV/+GVHD patients compared with the −CMV/+GVHD patients (Figure 6F); (3) in contrast, there was no increase in holes when −CMV/−GVHD patients were compared with −CMV/+GVHD patients (Figure 6E); and (4) when flow cytometric analysis of Tnaive reconstitution was dichotomized on the basis of CMV reactivation, defective reconstitution in CMV-reactivating patients was apparent (Figures 2B-C and 7A-B), but these differences were not observed when patients were dichotomized based on GVHD (Figure 7C-E). It is important to note, however, that our study was not sufficiently powered to perform the final subanalysis, to specifically determine whether patients that experienced both CMV reactivation and GVHD (often linked together) displayed more repertoire defects than patients who had reactivated CMV but had not developed GVHD. Given the evidence in the current study for CMV-induced TCEs and holes in the CD8 Tem reservoir, and previous data supporting the impact of GVHD on thymic dysfunction,[59,60] we believe that it is possible that there could be a combinatorial effect of both GVHD and CMV reactivation, such that patients that experience both complications would demonstrate the most severe defects in protective immunity. This represents a critical area for future analysis with a larger patient cohort.

This study has provided convincing molecular evidence that CMV is a major driver of $CD8^+$ Tem expansion posttransplant, and for a linked appearance of defects in the CD8 Tem TCRβ repertoire amid contraction of the Tnaive compartment. The implications of this study are provocative, given newly available agents being tested for primary CMV prophylaxis during HSCT.[15] Our results predict that preventing CMV reactivation will profoundly impact immune reconstitution after transplant, and that although quantitative CD8 reconstitution may be slower with CMV prophylaxis, qualitative T-cell reconstitution should be significantly improved.

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Authorship

Contribution: Y.S. performed experiments, analyzed data, and wrote the paper; R.M., A.D.K., B.R.B., A.K.M., and H.S.R. analyzed data and wrote the paper; B.W., K.F., C.D., and J.R. analyzed data; D.T.K. performed the clinical trial and performed experiments; L.S., J. A. Cheeseman, J. A. Conger, and A. Garrett performed experiments; A. Grizzle performed the clinical trial; J.T.H., A.L., M.Q., H.J.K., and E.K.W. performed the clinical trial and wrote the paper; and L.S.K. conceived the study, performed the clinical trial, analyzed data, and wrote the paper.

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