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Identifying Cytomegalovirus Complications Using the Quantiferon-CMV Assay After Allogeneic Hematopoietic Stem Cell Transplantation

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Background. A simple test to identify recovery of CMV-specific T-cell immunity following hematopoietic stem cell transplantation (HSCT) could assist clinicians in managing CMV-related complications.

Methods. In an observational, multicenter, prospective study of 94 HSCT recipients we evaluated CMV-specific T-cell immunity at baseline, 3, 6, 9, and 12 months after transplant using the Quantiferon-CMV, an enzyme-linked immunosorbent spot assay (ELISpot), and intracellular cytokine staining.

Results. At 3 months after HSCT, participants who developed CMV disease (n = 8) compared with CMV reactivation (n = 26) or spontaneous viral control (n = 25) had significantly lower $CD8^+$ T-cell production of interferon-γ (IFN-γ) in response to CMV antigens measured by Quantiferon-CMV ($P = .0008$). An indeterminate Quantiferon-CMV result had a positive predictive value of 83% and a negative predictive value of 98% for identifying participants at risk of further CMV reactivation. Participants experiencing CMV reactivation compared with patients without CMV reactivation had a reduced proportion of polyfunctional ($IFN\text{-}\gamma^+$/tumor necrosis factor α–positive) $CD4^+$ and $CD8^+$ T cells and a higher proportion of interleukin 2–secreting cells ($P = .01$ and $P = .002$, respectively).

Conclusions. Quantifying CMV-specific T-cell immunity after HSCT can identify participants at increased risk of clinically relevant CMV-related outcomes.

Keywords. cytomegalovirus; viral immunity; stem cell transplantation; T-cell immunity; immunocompromised host.

Human cytomegalovirus (CMV) has long been regarded as an important opportunistic pathogen in recipients of hematopoietic stem cell transplantation (HSCT) [1, 2]. Individuals who lack CMV immunity and/or endure prolonged periods of immunosuppression are particularly susceptible [1]. Cytomegalovirus complications can range from asymptomatic spontaneously resolving low-level CMV viremia to CMV-related death. The associated morbidity and mortality of CMV reactivation and disease in HSCT are highly significant, with recipients at an increased risk of early death [3]. Furthermore, CMV recurrences frequently occur, and it can be difficult to predict which individuals will need prolonged surveillance [4].

Current preemptive and prophylactic treatment strategies for CMV have been successful in reducing overall rates of CMV disease [5, 6]. However, both strategies have clinical limitations and primarily rely on detection of CMV DNA or virus. Based on regular monitoring of CMV DNA to detect early replicating virus before anti-CMV treatment, the preemptive strategy is often limited by a delay in obtaining results and commencing treatment [7]. Furthermore, not all end-organ disease is preceded by CMV DNA in plasma [8]. Alternatively, CMV disease can be prevented by initiating universal prophylaxis, whereby all individuals at risk of disease are given anti-CMV drugs from the time of engraftment [9]. However, current anti-CMV drugs are associated with significant bone marrow toxicity [10], universal prophylaxis has not been shown to improve survival[5], and many participants are treated unnecessarily [11]. Overall, neither strategy assesses an individual's immune response to better identify individuals at highest risk.

A simple test to identify recovery of CMV-specific T-cell immunity after HSCT could assist clinicians in managing CMV-related complications such as reactivation or disease [12]. Control of CMV viral replication is highly dependent on an

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intact cellular immune response, in particular on CMV-specific cytotoxic $CD8^+$ T lymphocytes [13, 14]. Current assays used to measure CMV-specific T-cell immunity include enzyme-linked immunosorbent spot assay (ELISpot), flow cytometry with intracellular cytokine staining (ICS), tetramer staining, and more recently, enzyme-linked immunosorbent assays (ELISAs) such as Quantiferon-CMV testing[12]. With the exception of Quantiferon-CMV, the other techniques require specialized personnel and equipment, are time consuming, and are often not available in a routine diagnostic laboratory [15].

Here we assessed CMV-specific $CD8^+$ T-cell immunity using the rapid high-throughput Quantiferon-CMV assay and compared these results with the traditional CMV ELISpot to characterize the kinetics of CMV-specific immunity after HSCT to determine the relationship of CMV-specific $CD8^+$ T-cell immunity to clinical outcome. We further assessed polyfunctional T-cell profiles using ICS in a subset of participants. We found that the Quantiferon-CMV assay identified individuals after HSCT at high risk of CMV-related complications and therefore could potentially be incorporated into clinical care.

METHODS

Study Participants

Eligible participants who were at risk of CMV disease and undergoing allogeneic HSCT were recruited from the Alfred Hospital and the Royal Melbourne Hospital, Melbourne, Australia, between February 2011 and May 2013. The study was approved by the human research ethics committees of the Alfred (339/10), Melbourne Health (MH2010.290), and Monash University (CF11/0238-2011000078). Written informed consent was obtained from all participants. Low-risk participants (CMV donor-negative [D−]/recipient-negative [R−]) were excluded. Blood was collected before transplantation (baseline) and at 3, 6, 9, and 12 months after HSCT.

Two different CMV prophylactic strategies were used whereby at 1 site all participants were managed with a preemptive CMV approach, undergoing weekly plasma CMV DNA polymerase chain reaction (PCR) testing for 100 days or longer if graft-versus-host disease was present. Anti-CMV drugs were commenced once a clinically significant viral threshold (usually >1000 IU/mL) was reached. Participants at the other site were either given universal prophylaxis (ganciclovir 5mg/kg intravenously 3×/week until day 100) or preemptive CMV prophylaxis at the discretion of the treating clinician.

Cytomegalovirus Clinical Outcomes

Cytomegalovirus disease was confirmed by biopsy with histological evidence of viral cytopathic changes or positive immunohistochemistry [16]. Cytomegalovirus reactivation was defined as detection of CMV DNA in plasma by PCR ≥546 IU/mL (the lower limit of detection was 136 IU/mL; COBAS AmpliPrep/COBAS Taqman CMV test, Roche), and treated CMV reactivation included participants who received anti-CMV therapy. Spontaneous viral control was defined as the resolution of any level of CMV DNA in plasma without antiviral therapy.

QuantiFERON-CMV Assay

The QuantiFERON-CMV assay (Qiagen) is an in vitro diagnostic test providing both qualitative (reactive, nonreactive, indeterminate) and quantitative (concentration of interferon γ [IFN-γ] in international units per milliliter) results using human leucocyte antigen-restricted CMV epitopes and therefore only quantifies $CD8^+$ CMV-specific T cells [17].

As described previously [17], this assay consists of 3 1-mL QuantiFERON-CMV blood tubes, each containing 1 of CMV peptide antigens, a positive mitogen control of phytohemagglutinin, or no antigens (negative control). The CMV peptide pool contains 22 peptides derived from epitopes of the major CMV proteins (pp65 and IE-1) and other less-common CMV proteins (pp50, gB, pp28, and IE-2) [17].

The assay was processed in accordance with the manufacturer's instructions. In brief, after whole-blood collection, the tubes were incubated at 37°C for 16–24 hours before the supernatants were harvested and analyzed for IFN-γ production by ELISA. A positive result was determined after subtracting the IFN-γ production from the negative control and was defined as an IFN-γ level ≥ 0.2 IU/mL.

Cytomegalovirus Enzyme-Linked Immunosorbent Spot Assay

The synthetic CMV peptides used in the assay were of 15 amino-acid length (overlapping by 10 amino acids) spanning the pp65 and IE-1 human CMV proteins [18]. The 110 pp65 peptides were clustered in 11 pools, and the 96 IE-1 peptides were clustered in 10 pools [18]. All of the pp65 and IE-1 peptides used in the Quantiferon-CMV assay were also included in the CMV peptides used for the ELISpot assay. The ELISpot testing was performed using cryopreserved peripheral blood mononuclear cells (PBMCs) as previously described [18]. In brief, 96-well plates were coated with 100 μL of human monoclonal anti–IFN-γ immunoglobulin (Eurobio). Dispensed into each well were 1×10^5 cells together with the CMV overlapping synthetic peptide pools, plus a negative and positive control (phytohemagglutinin). Spot-forming cells (SFCs) were read using an ELISpot reader (Autoimmun Diagnostika GmbH). The ELISpot results therefore include both $CD4^+$ and $CD8^+$ CMV-specific T cells.

Intracellular Cytokine Staining and Flow Cytometry

One million PBMCs were stimulated with the same synthetic CMV overlapping peptides used in the ELISpot assay as previously described [20], a negative control, or *Staphylococcal* enterotoxin B positive control. The pp65 and IE-1 CMV peptides covered in the ICS study were inclusive of the CMV peptides

used in the Quantiferon-CMV assay. Florescence-labeled conjugated antibodies for CD3-pacific blue, CD8-peridinin chlorophyll protein, and CD4-fluorescein isothiocyanate were used, as well as anti-IFN-γ–Alexa700, interleukin 2 (IL-2)–allophycocyanin, tumor necrosis factor α (TNF-α)–phycoerythrin Cy7, and CD40 ligand (CD40L)–phycoerythrin with surface and intracellular expression quantified using a FACS Canto II (Becton Dickinson) [20]. Data analysis was performed using Flow Jo Version 9.7.5 USA and SPICE version 5.3, downloaded from http://exon.niaid.nih.gov [21]. Both CMV-specific $CD4^+$ and $CD8^+$ T cells were quantified.

Statistical Analysis

Categorical variables between 2 groups were compared using a χ^2 test or Fisher's exact test as appropriate. A Wilcoxon rank-sum test or Kruskal-Wallis test was used where ≥3 or more groups of nonparametric continuous data were compared. A P value < .05 was considered to be statistically significant. The change in IFN-γ over time in each participant was modeled using generalized estimating equations (GEEs). Baseline was taken as the production of IFN-γ in response to CMV peptides at 3 months (after transplant), and this model considered both variation at baseline as well as change over time (3–12 months) as an interaction with each CMV group. Standard errors were calculated using the robust Huber-White sandwich estimator. Statistical analyses were performed using Stata 14.1 (StataCorp LP) and GraphPad Prism v6.

RESULTS

Participant Characteristics

In total, 96 individuals were enrolled with 94 participants completing the study (Figure 1). Baseline characteristics are shown in Table 1.

Cytomegalovirus Clinical Outcomes

Sixty-three (67%) study participants experienced post-HSCT detectable CMV with only baseline CMV D−/R+ serostatus compared with CMV D+/R+ being an independent protective risk factor for viremia (multivariate odds ratio [OR] = 0.04; 95% CI = .01–.18; P < .0001) (Table 1). Acute graft-versus-host disease was not a risk factor in either univariate or multivariate analysis for any CMV reactivation in this study. Of the participants with detectable viremia, 26 experienced CMV reactivation, 25 had spontaneous CMV viral clearance, and 8 had CMV disease (n = 7 gastrointestinal, n = 1 pneumonitis) (Figure 1). The median times to first CMV reactivation and CMV disease were 48 (interquartile range [IQR] =38–62) days and 65 (IQR = 64–176) days after HSCT, respectively. A further 7 (7.4%) participants had a second defined episode of CMV reactivation with a minimum of 3 weeks of undetectable CMV by PCR and a median time to second reactivation of 185 (IQR = 97–269) days. Eighty-eight percent of all CMV clinical outcomes occurred in 42 study participants and before the first study time point. At baseline, before the transplant, no participant had detectable plasma CMV DNA. No participant who

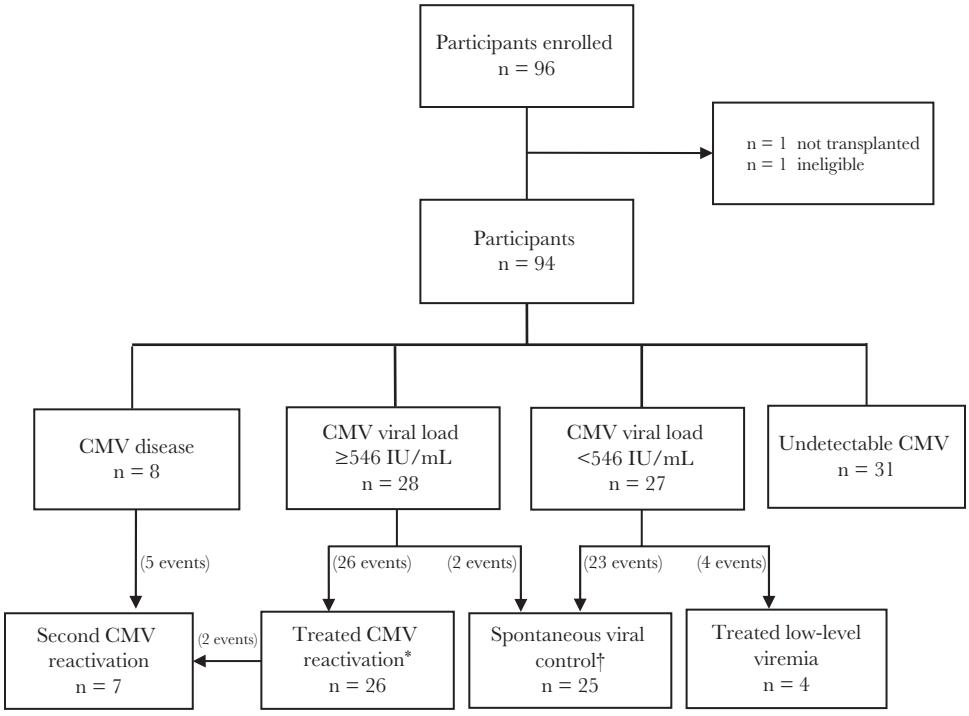

Figure 1. Study participant flowchart. *Defined as cytomegalovirus (CMV) DNA viral load > 546 IU/mL plus use of CMV-directed antivirals. †Defined as the resolution of any level of CMV DNA without use of CMV-directed antivirals. Abbreviation: CMV, cytomegalovirus.

Table 1. Baseline Demographics of Study Participants

Characteristic	All participants, n = 94 (%)	CMV disease, n = 8 (%)	Any CMV reactivation, n = 63 (%)	No CMV reactivation, n = 31 (%)	Univariate *P* value	Multivariate analysis
Age, y, median (range)	50 (18–68)	52 (36–56)	48 (31–56)	51 (41–56)	1.00	…
Sex, no., male/female	44/50	2/6	29/34	15/16	.80	…
Primary diagnosis						
Acute myeloid leukemia	33 (35)	5 (63)	21 (33)	12 (39)	.20	…
Acute lymphoblastic leukemia	10 (11)	1 (12)	7 (11)	3 (10)		
Multiple myeloma	14 (15)	0	10 (16)	4 (13)		
Myelodysplasia	7 (7)	1 (12)	5 (8)	2 (6)		
Chronic leukemia	7 (7)	1 (12)	5 (8)	2 (6)		
Non-Hodgkins lymphoma	10 (9)	0	9 (14)	1 (3)		
Hodgkins disease	6 (6)	0	5 (8)	1 (3)		
Other	7 (9)	0	1 (2)	6 (18)		
Type of transplant						
Myeloablative	59 (63)	7 (88)	40 (63)	19 (61)	.80	…
Reduced intensity conditioning	35 (37)	1 (12)	23 (37)	12 (39)		
Donor source						
Sibling related	43 (46)	1 (12)	27 (43)	16 (52)	.40	…
Volunteer unrelated	51 (53)	7 (88)	36 (57)	15 (48)		
Graft source						
Peripheral blood stem cells	79 (84)	4 (50)	51 (81)	28 (90)	.10	NS
Umbilical cord	8 (8)	4 (50)	8 (13)	0		
Bone marrow	7 (7)	0	4 (6)	3 (10)		
CMV donor/recipient serostatus						
Positive/positive	43 (46)	1 (12)	34 (54)	9 (29)	<.0001	D+/R–: OR = 0.04; 95% CI = .01–.18; <.0001
Negative/positive	31 (33)	7 (88)	26 (41)	5 (16)		
Positive/negative	20(21)	0	3 (5)	17 (55)		
CMV prophylaxis						
Preemptive monitoring	69 (74)	7 (88)	50 (79)	19 (61)	.06	…
Universal prophylaxis	25 (26)	1 (12)	13 (21)	12 (39)		
Conditioning						
Fludarabine/melphelan	25 (27)	0	18 (29)	7 (23)	.07	NS
Cyclophosphamide/TBI	16 (17)	1 (12)	9 (14)	7 (23)		
Busulphan/cyclophosphamide	14 (15)	1 (12)	9 (14)	5 (16)		
Fludarabine/TBI	14 (15)	1 (12)	9 (14)	5 (16)		
Etoposide/TBI	6 (6)	0	4 (6)	2 (6)		
Fludarabine/cyclophosphamide/TBI	5 (5)	2 (25)	5 (6)	0		
Fludarabine/cyclophosphamide	4 (4)	0	4 (6)	0		
Fludarabine/cyclophosphamide/TBI/ Thiotepa	4(4)	2 (25)	1 (2)	0		
Other	6 (6)	1 (12)	1 (2)	5 (16)		
T-cell depletion						
Antithymocyte globulin	26 (28)	1 (12)	16 (25)	10 (32)	.50	…
Alemtuzumab	9 (10)	0	5 (8)	4 (13)	.40	
Acute GVHD						
Nil	47 (50)	1 (12)	31 (49)	16 (52)	.60	NS
Grade I–II	33 (35)	4 (50)	24 (38)	9 (29)		
Grade III–IV	14 (15)	3 (38)	8 (13)	6 (19)		
Chronic GVHD[a]						
Nil	32 (39)	1 (14)	20 (34)	12 (48)	.50	…
Limited	17 (21)	1 (14)	13 (22)	4 (16)		
Extensive	34 (40)	5 (71)	25 (43)	9 (36)		
Death, 12-month mortality						
Total	23 (24)	4 (50)	14 (22)	9 (29)	.50	…
GVHD	7 (7)	2 (25)	4 (6)	3 (10)		
Relapse/progressive disease	6 (6)	0	3 (5)	3 (10)		

Table 1. Continued

Characteristic	All participants, n = 94 (%)	CMV disease, n = 8 (%)	Any CMV reactivation, n = 63 (%)	No CMV reactivation, n = 31 (%)	Univariate *P* value	Multivariate analysis
Infection[b]	5 (5)	1 (12)	4 (6)	1 (3)		
Multiorgan failure[c]	3 (3)	1 (12)	2 (3)	1 (3)		
Hemorrhage/bleeding	2 (2)	0	1 (2)	1 (3)		

All data are no. (%) unless otherwise stated.

Abbreviations: CI, confidence interval; CMV, cytomegalovirus; D, donor; GVHD, graft-versus-host disease; NS, not significant; OR, odds ratio; R, recipient; TBI, total body irradiation.

[a]n = 83 participants with data available

[b]n = 1 *Candida guilliermondii*, n = 1 *Eschericia coli*, n = 1 *Nocardia* spp, n = 1 *Prototheca* spp, n = 1 unknown organism.

[c]n = 1 kidney/lung failure, n = 1 renal/liver failure, n = 1 lung failure.

had spontaneously controlled virus experienced a second CMV reactivation, whereas 5 of 8 (62.5%) and 2 of 26 (7.7%) participants with CMV disease and CMV reactivation, respectively, had a second episode of CMV reactivation ($P < .0001$). Late CMV disease occurred in 3 participants, all with CMV colitis with a median time to diagnosis of 191 (range = 176–540) days.

The all-cause mortality rate at 12 months was 24.5%. There was a trend toward a lower 12-month survival in those with CMV disease compared with those without CMV disease (Kaplan-Meier survival = 41.7% vs 73.7%; $P = .09$). No difference was seen in 12-month survival between different baseline CMV sero-groups ($P = .50$).

Quantiferon-CMV and Cytomegalovirus Enzyme-Linked Immunosorbent Spot Assay

Three hundred two Quantiferon-CMV assays were assessed (median = 3; range = 1–5 per participant). The sensitivity and specificity of the Quantiferon-CMV assay relative to CMV serology before HSCT were 74% and 100%, respectively. Reducing the lower cut-off of IFN-γ in the Quantiferon-CMV assay to 0.1 IU/mL improved the sensitivity to 86% without affecting specificity (100%). Thus, all further analysis of Quantiferon-CMV assay results used this lower cut-off level.

Two hundred nineteen CMV ELISpot assays measuring total CMV-specific T cells were performed. The sensitivity and specificity of the CMV ELISpot relative to pre-HSCT CMV serology were 98% and 44%, respectively. The Pearson correlation between the Quantiferon-CMV and the CMV ELISpot was 0.66 ($P < .0001$). The sensitivity and specificity of the Quantiferon-CMV assay relative to the CMV ELISpot were 87% and 85%, respectively, with a positive predictive value of 99% and a negative predictive value of 66%.

Before HSCT, there was no difference in the magnitude of IFN-γ production measured by the Quantiferon-CMV test between participants who subsequently developed CMV disease, CMV reactivation, or spontaneous viral control ($P = .24$). Qualitative Quantiferon-CMV results before HSCT were not associated with subsequent CMV reactivation ($P = .16$) or CMV disease ($P = .30$). We also did not observe any association with pretransplant CMV ELISpot pp65-specific and IE-specific responses and subsequent CMV outcomes (Kruskal–Wallis $P = .90$ and $P = .20$, respectively).

At 3 months after HSCT, participants who developed CMV disease compared with those with CMV reactivation or spontaneous viral control had a significantly lower magnitude of IFN-γ production in the Quantiferon-CMV assay (median IFN-γ = 0.04 vs 0.23 vs 1.86 IU/mL, respectively; Kruskal–Wallis $P = .0008$) (Figure 2A). We also assessed IFN-γ responses in individuals with CMV disease, plasma CMV DNA ≥ 546 IU/mL or plasma CMV DNA ≥ 136 IU/mL as any reactivation and observed a median IFN-γ of 0.04 versus 0.38 versus 1.32 IU/mL, respectively (Kruskal-Wallis $P = .006$). Because CMV D+/R− recipients are at a lower risk of CMV reactivation, we also analyzed the data excluding this group (n = 20), with results of CMV disease versus CMV reactivation and spontaneous viral control being a median IFN-γ level of 0.04 versus 0.2 versus 1.5 IU/mL (Kruskal–Wallis $P = .003$).

Similar results were observed at 3 months using the CMV ELISpot assay, whereby participants with CMV disease compared with those with CMV reactivation or spontaneous viral control had significantly lower ELISpot IFN-γ (median = 198 vs 1670 vs 2513 SFC/10^6 cells; Krukal-Wallis test $P = .002$) (Figure 2B). When we analyzed the pp65-specific and IE-specific responses separately, the response to pp65 was associated with CMV outcome (Kruskal-Wallis $P = .01$) but not in response to IE (Kruskal-Wallis $P = .15$).

In a longitudinal assessment, the Quantiferon-CMV IFN-γ responses were significantly different among all 3 clinical groups (Figure 3A). Compared with participants with spontaneous viral control, participants with CMV reactivation and CMV disease had significantly higher rates of CMV immune recovery between 3 and 12 months as measured by Quantiferon-CMV (GEE estimated coefficient of 0.17 and. 0.30; $P = .04$ and $P = .03$, respectively) but not when using the ELISpot assay (GEE estimated coefficient of 0.02 and 0.07; $P = .5$ and $P = .3$, respectively) (Figure 3B). We also found differences in CMV immune reconstitution using the Quantiferon-CMV assay according to CMV baseline serogroup whereby CMV D−/R+ and CMV D+/R− recipients showed an increase and small decrease, respectively, in rates of recovery compared with CMV D+/R+

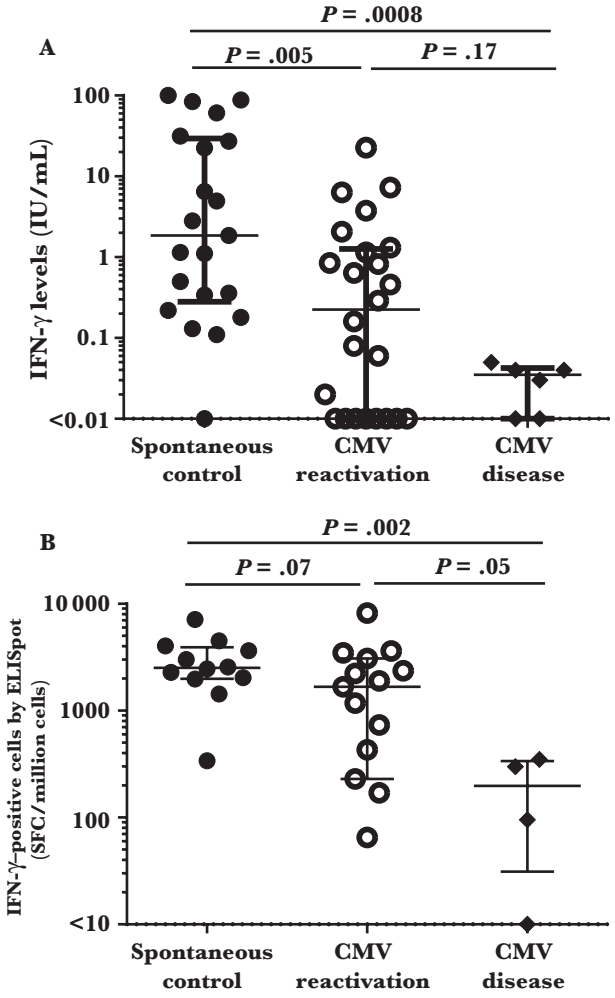

Figure 2. Cytomegalovirus (CMV)–specific responses measured 3 months after hematopoietic stem cell transplantation. Cytomegalovirus-specific responses were quantified using the Quantiferon-CMV assay to measure interferon γ (IFN-γ) production (IU/mL) (*A*) and CMV enzyme-linked immunosorbent spot assay to measure IFN-γ spot-forming cells (SFC/10^6 cells) (*B*) in individuals with spontaneous control (solid circles), CMV reactivation (open circles), and CMV disease (closed triangles). The horizontal line represents the median, and error bars represent the interquartile range. Abbreviations: CMV, cytomegalovirus; ELISpot, enzyme-linked immunosorbent spot assay; IFN-γ, interferon γ; SFC, spot-forming cell.

recipients (GEE estimated coefficient of 0.22 and −0.01; $P = .03$ and $P = .006$, respectively) (Figure 3C). There was no significant difference in immune responses by either Quantiferon-CMV or CMV ELIspot between participants who received preemptive monitoring and those who received universal prophylaxis. ($P = .20$ and $P = .90$, respectively).

The qualitative results of the Quantiferon-CMV assay at 3 months were found to be significantly associated with CMV clinical outcomes (Figure 4A), and the transplant factors associated with test outcomes included baseline CMV donor/recipient serostatus ($P < .0001$), graft source ($P = .02$), and unrelated grafts ($P = .04$) (Supplementary Table 1). In participants with a reactive, nonreactive, or indeterminate Quantiferon-CMV result, spontaneous viral clearance was observed in 49%, 0%, and 10% of participants, respectively ($P < .001$). Cytomegalovirus disease was only observed in participants with an indeterminate result at 3 months ($P < .0001$). An indeterminate Quantiferon-CMV result at 3 months had a positive predictive value of 83% and a negative predictive value of 98% for identifying participants at risk of further CMV reactivation. A positive Quantiferon-CMV response at 3 months was 89% predictive of future positive responses. Thirty-two of 36 participants who had positive responses at 3 months continued to have positive responses at 6, 9, and 12 months, where data were available. Of the remaining participants (n = 4), none had CMV-related complications at 6, 9, or 12 months.

We next evaluated the association between CMV DNA in plasma and the Quantiferon-CMV assay. Participants with a reactive compared with a nonreactive or indeterminate Quantiferon-CMV result at 3 months after HSCT had a significantly lower peak CMV DNA in plasma (median viral load = 546 vs 4543 vs 4076 IU/mL, respectively; $P = .003$) (Figure 4B). There was a significant delay in the time to development of CMV-specific immunity (defined as CMV IFN-γ ≥ 0.1 IU/mL) in participants with CMV disease compared with CMV reactivation and spontaneous control (median time = 276 vs 116 vs 97 days; cumulative incidence function with competing-risks regression $P < .0001$) (Figure 5).

Flow Cytometry and Intracellular Cytokine Staining

In a subset of participants (n = 10; demographic details in Supplementary Table 2) we assessed the breadth and specificity of the CMV-specific response using ICS in participants with CMV reactivation (n = 5) and without CMV reactivation (n = 5) (Figure 6). Before HSCT, the most frequently produced cytokine from CMV-specific $CD4^+$ and $CD8^+$ T cells was IFN-γ with no significant difference in the frequency of IFN-γ–positive cells between participants with and without CMV reactivation ($P = .18$). At 3 months after HSCT, the proportion of dual-functional (IFN-γ^+/TNFα$^+$) CMV-specific $CD4^+$ and $CD8^+$ T cells was reduced in those who developed CMV reactivation ($P = .02$) (Figure 6B). At 6 months after HSCT, the proportion of dual-functional (IFN-γ^+/TNFα$^+$) CMV-specific $CD4^+$ and $CD8^+$ T cells remained lower in the group with CMV reactivation, and the profile of cytokine production in cells that produced a single cytokine was significantly different ($P = .0008$) (Figure 6C). Participants experiencing CMV reactivation had a much higher proportion of $CD4^+$ and $CD8^+$ IL-2–secreting T cells compared with participants without CMV reactivation, who had a higher proportion of $CD4^+$ and CD8v TNFα+ or IFN-γ–secreting T cells ($P = .0008$). Similar findings were observed at month 6 after HSCT when CMV-specific $CD4^+$ T cells ($P = .02$) and CMV-specific $CD8^+$ T cells ($P = .05$) were analyzed separately (see Supplementary Figures 3*C* and 4*C*).

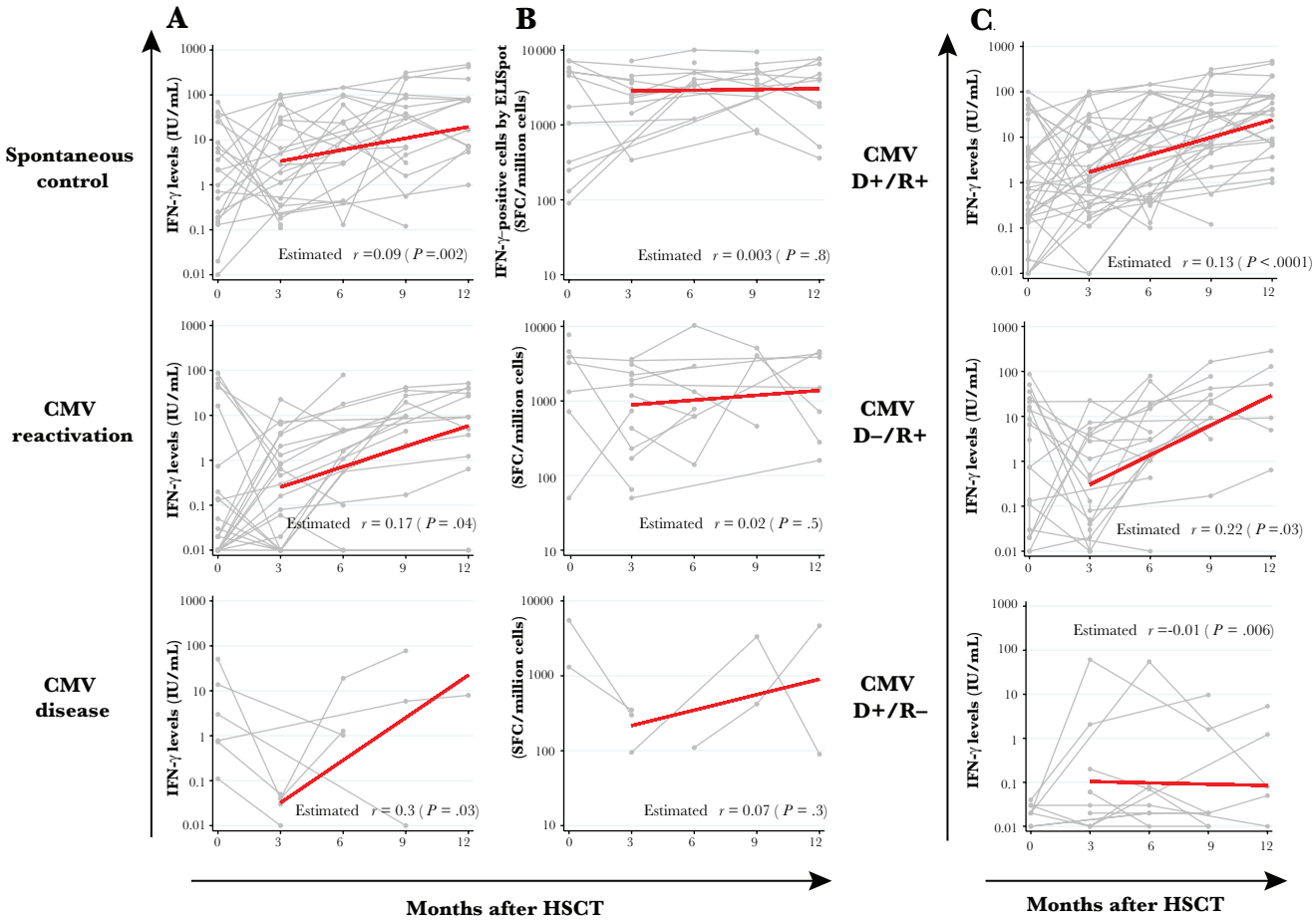

Figure 3. Cytomegalovirus (CMV)–specific responses measured over time for 12 months after hematopoietic stem cell transplantation (HSCT). Cytomegalovirus-specific responses were quantified using the Quantiferon-CMV assay to measure interferon γ (IFN-γ) production (IU/mL) (*A*) and CMV enzyme-linked immunosorbent spot assay to measure IFN-γ spot-forming cells (SFC/10^6 cells) (*B*) in individuals with spontaneous control (top row), CMV reactivation (middle row), and CMV disease (bottom row). *C*, Cytomegalovirus-specific responses measured over time for 12 months after HSCT using the Quantiferon-CMV assay in CMV D+/R+, CMV D–/R+, and CMV D+/R– recipients. Each dot and line in light gray represent data from a single participant. The red (if color) or gray (if grayscale) bold line represents the statistically modeled trend based on the generalized estimating equation. Abbreviations: CMV, cytomegalovirus; D, donor; ELISpot, enzyme-linked immunosorbent spot assay; HSCT, hematopoietic stem cell transplantation; IFN-γ, interferon γ; R, recipient; SFC, spot-forming cell.

DISCUSSION

In this prospective study of recipients of allogeneic HSCT, the Quantiferon-CMV assay, which measures CMV-specific $CD8^+$ T cells using a high-throughput format, was able to distinguish between CMV-related clinical outcomes, including CMV-related clinical recurrences. Cytomegalovirus-specific $CD8^+$ T cells, measured by the Quantiferon-CMV assay or by ELISpot measured at 3 months and longitudinally over time were able to clearly distinguish CMV disease, CMV reactivation, or spontaneous CMV viral control after HSCT. In addition, a reactive Quantiferon-CMV result was associated with spontaneous viral clearance and a lower peak CMV DNA level, whereas an indeterminate result identified participants at further risk of CMV reactivation. Finally, the quality of the CMV-specific $CD4^+$ and $CD8^+$ T-cell response differed between participants with and without CMV reactivation with regard to the number and cytokine profile detected. The Quantiferon-CMV assay has the potential to be incorporated into current clinical practice and could be of high clinical utility.

This is the first study to comprehensively evaluate the Quantiferon-CMV assay longitudinally in the largest cohort to date after HSCT. Tey et al studied 41 recipients of allogeneic HSCT in the early post-transplant period with much more frequent monitoring of the Quantiferon-CMV assay [22]. Consistent with our findings, the authors observed that a positive Quantiferon-CMV assay was associated with a significantly lower peak CMV DNA in blood [22]. This suggests that recovery of an adequate CMV-specific T-cell response is essential for controlling CMV virus [23]. However, the study also showed variable Quantiferon-CMV results when the test was performed early post-transplant, suggesting that frequent monitoring early after HSCT may not have much additive clinical utility [22]. Fleming et al found that the Quantiferon-CMV assay correlated well with ICS studies but were unable to identify a significant

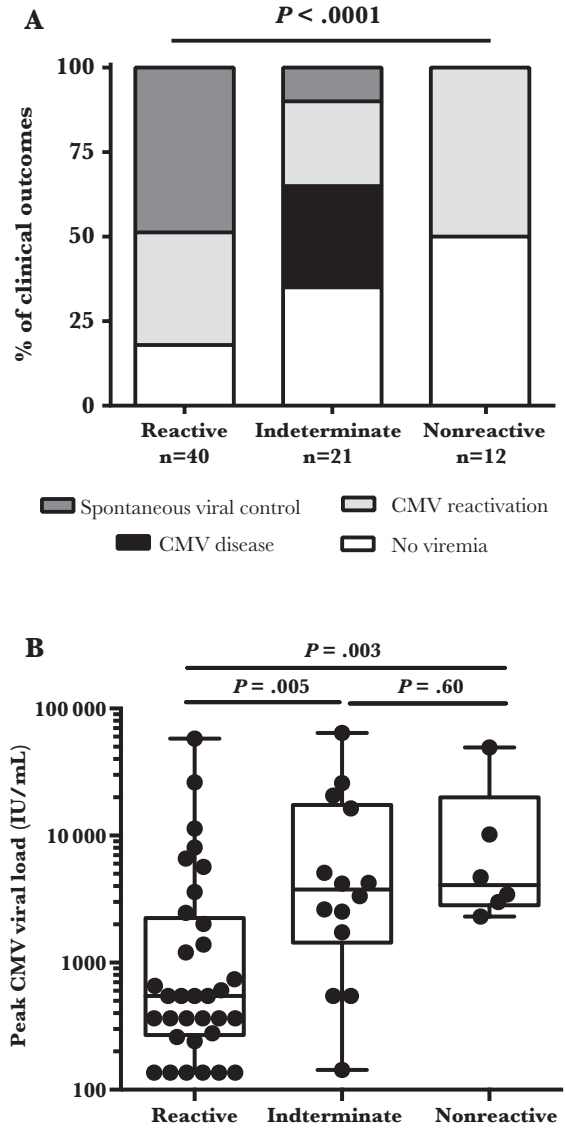

Figure 4. Comparison of CMV outcomes by the Quantiferon-CMV assay qualitative result. *A,* The relationship between the Quantiferon-CMV assay qualitative result at 3 months and clinical outcomes of spontaneous viral control, cytomegalovirus (CMV) reactivation, CMV disease and no viremia. *B,* Peak CMV DNA in blood in international units per milliliter. The box represents the interquartile range, with the middle horizontal bar representing the median. The whiskers represent the minimum and maximum range. Abbreviation: CMV, cytomegalovirus.

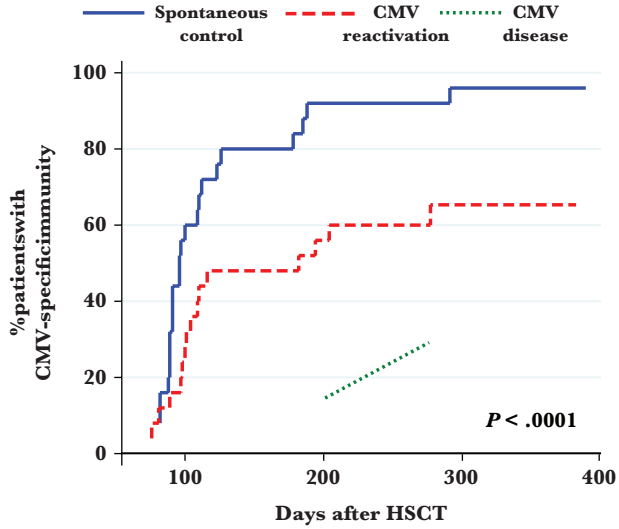

Figure 5. A cumulative incidence curve of time to achieve cytomegalovirus (CMV)–specific immunity (defined as CMV interferon γ [IFN-γ] level of > 0.1 IU/mL measured by the Quantiferon-CMV assay) in participants who had spontaneous control (solid line), CMV reactivation (dashed line), and CMV disease (dotted line). Abbreviations: CMV, cytomegalovirus; HSCT, hematopoietic stem cell transplantation.

association between these assays and specific CMV clinical outcomes [24]. Similarly Bono et al describe immune monitoring with the Quantiferon-CMV assay in 22 HSCT recipients but without clear association with clinical outcomes [25]. Other studies assessing the clinical use of the Quantiferon-CMV assay have largely been in the solid-organ transplant setting [26–29]. We note that 9% of all participants in this cohort developed CMV disease, likely driven by a rate of CMV disease of 50% seen in individuals who received umbilical cord transplants.

In addition to immune monitoring with a single cytokine IFN-γ, we studied the breadth and cytokine profile of the CMV-specific $CD4^+$ and $CD8^+$ T-cell response in participants with and without CMV reactivation. Participants who did not experience CMV reactivation were more likely to have dual functional T cells (IFN-γ^+/TNF-α^+), which is consistent with other studies showing that polyfunctional antigen-specific T cells are better able to control virus and prevent disease progression [30–33]. The specificity of these polyfunctional CMV-specific T cells can vary, with Krol et al finding that IFN-γ^+/$IL2^+$ $CD8^+$ T cells were associated with better CMV viral control after HSCT in children [34]. However, in healthy control subjects, cells stimulated with CMV antigen predominantly expressed dual IFN-γ^+/TNFα^+ cytokines [35]. It is thought that cytokines other than IFN-γ are important to enable and enhance effective cytotoxic effect on virus [31]. Although the Quantiferon-CMV assay only measured a single cytokine IFN-γ, our results were concordant with the estimates of polyfunctional CMV-specific T-cell responses when determined by ICS [36].

This study contributes to the growing body of literature in support of CMV immune monitoring [4, 22, 37, 38]. Most recently, Navarro et al determined that use of immunological monitoring shortened the length of antiviral treatment and reduced the incidence of recurrent CMV viremia [4]. Avetisyan et al also demonstrated in small numbers that participants with CMV-specific immune recovery could have antiviral therapy delayed without development of CMV disease [38]. These studies measured CMV-specific immunity using intracellular IFN-γ production by flow cytometry and/or CMV ELISpot [4, 38]. Both these techniques, as well as tetramer staining, require isolation of PBMCs using specialized

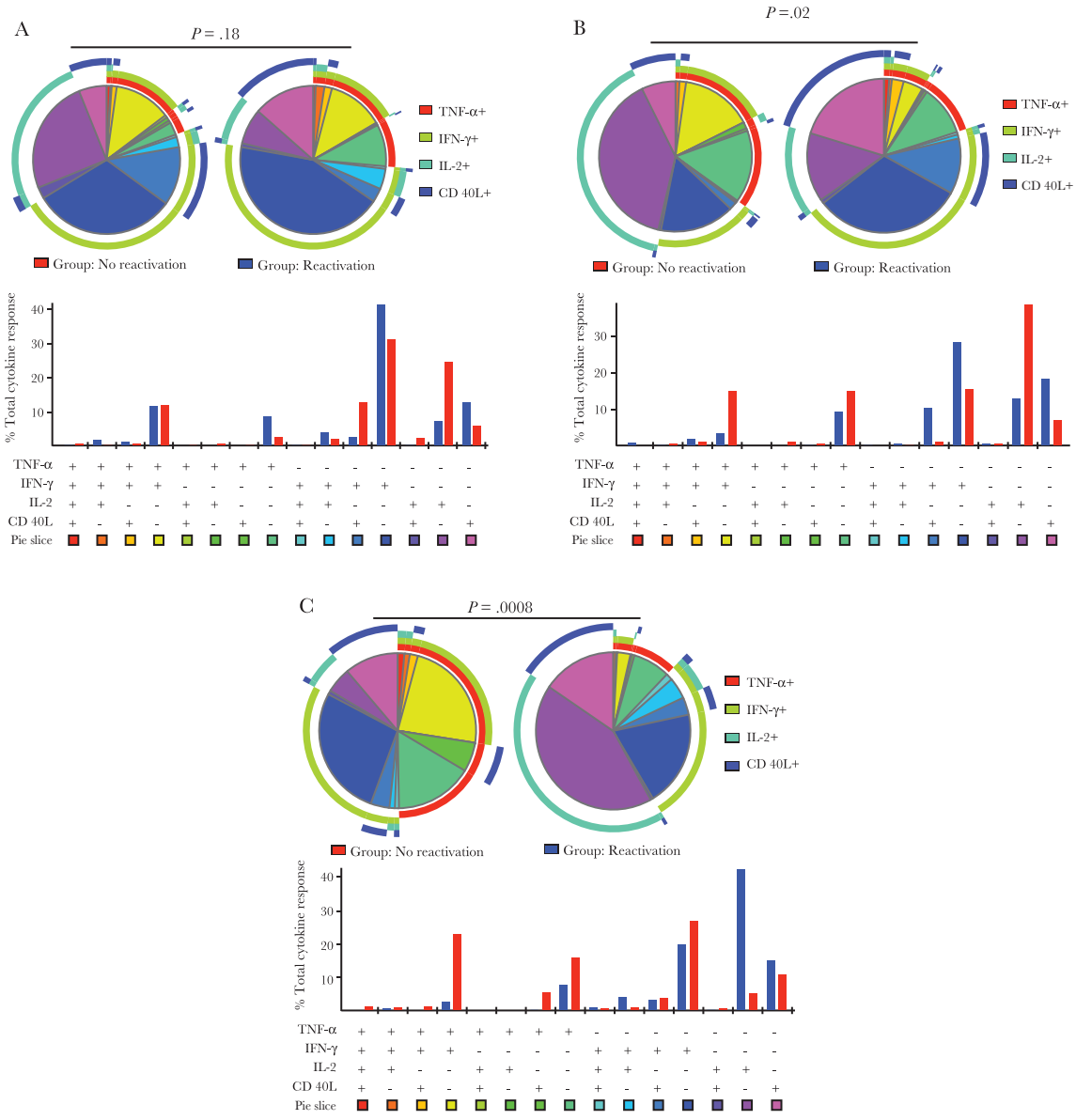

Figure 6. Assessment of cytomegalovirus (CMV)–specific CD4+ and CD8+ T-cells by intracellular cytokine staining in participants with no CMV reactivation (n = 5) and CMV reactivation (n = 5) before 3 months (*A*), at 3 months (*B*), and at 6 months after hematopoietic stem cell transplantation (*C*). The pie charts represent the 2 patient groups, with no CMV reactivation displayed on the left side and CMV reactivation on the right side and the cytokine profile of CMV-specific CD4+ and CD8+ T cells are combined. Each of the pie slices represent the proportion of cells that produce 1, 2, 3, or 4 cytokines either alone or in combination. The same data are also represented as a bar graph, where each column shows the percentage of cells that produce each cytokine in participants with (blue) or without (red) CMV reactivation. The arcs represent the proportion of total cells producing a particular cytokine and, where the arcs overlap, indicate cells producing >1 cytokine. Abbreviations: CD40L, cluster differentiation 40 ligand; IFN-γ, interferon γ; IL-2, interleukin 2; TNFα, tumor necrosis factor α.

equipment, are time consuming to perform, and are not standardized [12]. In contrast, the Quantiferon-CMV assay that we used in this study provides an in vitro functional assessment of CMV-specific CD8+ T cells, which can be performed in most diagnostic pathology laboratories [39]. Use of whole blood in this assay also has the advantage of more accurately reflecting physiological cell conditions in vivo, particularly with regard to the presence of functional dendritic cells required in antigen presentation. The process of cryopreserving PBMCs for assays such as ELISpot significantly reduces dendritic cell function, potentially affecting results.

Although this was a prospective longitudinal study, there were several limitations in this study. First, we elected to collect blood at 3 months after transplantation by which time most participants had already experienced viremia. Second, the Quantiferon-CMV assay includes peptides that are MHC class I restricted and thus does not stimulate CD4+ T cells, which have also been found to be important in CMV viral control [40, 41].

Finally, the study also did not measure $CD4^+/CD8^+$ T-cell subsets, which would have provided additional data on immune reconstitution.

In conclusion, we demonstrate that the frequency of CMV-specific $CD8^+$ T cells as measured by the Quantiferon-CMV assay after an allogeneic HSCT is highly associated with CMV clinical outcomes. We evaluated several methods for detecting CMV-specific T cells with the Quantiferon-CMV assay having the additional benefit of being a simple and high-throughput assay that can be performed in most routine diagnostic laboratories. Further large randomized controlled trials of monitoring CMV-specific T cells in addition to CMV viremia to determine the optimal CMV prophylaxis strategies are indicated.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

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