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Immune monitoring with iTAg MHC Tetramers for prediction of recurrent or persistent cytomegalovirus infection or disease in allogeneic hematopoietic stem cell transplant recipients: a prospective multicenter study

Jan W. Gratama,[1] Michael Boeckh,[2] Ryotaro Nakamura,[3] Jan J. Cornelissen,[4] Rik A. Brooimans,[1] John A. Zaia,[5] Stephen J. Forman,[3] Karl Gaal,[6] Kurtis R. Bray,[7] Gail H. Gasior,[7] Christopher S. Boyce,[7] Linda A. Sullivan,[7] and Paula C. Southwick[7]

[1]Department of Clinical and Tumor Immunology, Erasmus Medical Center/Daniel den Hoed Cancer Center, Rotterdam, The Netherlands; [2]Program in Infectious Diseases, Fred Hutchinson Cancer Research Center, Seattle, WA; [3]Division of Hematology and Hematopoietic Cell Transplantation, City of Hope National Medical Center, Duarte, CA; [4]Department of Hematology, Erasmus Medical Center/Daniel den Hoed Cancer Center, Rotterdam, The Netherlands; [5]Department of Virology, City of Hope National Medical Center, Duarte, CA; [6]Division of Pathology, City of Hope National Medical Center, Duarte, CA; and [7]Department of Clinical Research, Beckman Coulter, Carlsbad, CA

Cytomegalovirus (CMV) infection is an important cause of morbidity and mortality in hematopoietic stem cell transplant recipients despite the introduction of posttransplantation viral monitoring and preemptive antiviral therapy. We evaluated the use of HLA class I tetramers in monitoring CMV-specific T-cell recovery to predict patients at risk for CMV-related complications. This prospective multicenter clinical trial obtained nearly 1400 tetramer/allele results in more than 800 biweekly blood samples from 83 patients monitored for 1 year after transplantation. Major HLA types were included (A*0101, A*0201, B*0702, B*0801, B*3501). iTAg MHC Tetramers (Beckman Coulter) were used to enumerate CMV-specific CD8$^+$ T cells by flow cytometry using a single-platform absolute counting method. Assay variability was 8% or less and results were available within 3 hours. Delayed recovery of CMV-specific T cells (< 7 cells/μL in all blood samples during the first 65 days after transplantation) was found to be a significant risk factor for CMV-related complications; these patients were more likely to develop recurrent or persistent CMV infection (relative risk 2.6, CI 1.2-5.8, $P = .01$) than patients showing rapid recovery, which was associated with protection from CMV-related complications ($P = .004$). CMV tetramer–based immune monitoring, in conjunction with virologic monitoring, can be an important new tool to assess risk of CMV-related complications and to guide preemptive therapeutic choices. (*Blood*. 2010;116(10):1655-1662)

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Introduction

Human cytomegalovirus (CMV) infects 60% to 90% of the population worldwide and remains latent in the infected host. CMV-specific CD8$^+$ cytotoxic T lymphocytes play a critical role in suppressing CMV reactivation. In healthy immunocompetent persons, CMV infection is asymptomatic when an equilibrium is achieved and CMV-specific T cells control the persisting virus. In immunosuppressed hematopoietic stem cell transplant (HSCT) recipients, CMV infection is an important cause of morbidity and mortality despite the introduction of routine posttransplantation viral monitoring and the use of preemptive antiviral therapy.[1]

Direct measurement of frequencies of antigen-specific CD8$^+$ T cells is now possible using major histocompatibility complex (MHC) tetramers. MHC tetramers are complexes between human leukocyte antigen (HLA) class I or class II molecules and specific antigenic peptides conjugated to fluorochromes.[2] The antigen-specific T lymphocytes may be enumerated by flow cytometry without the requirement of in vitro stimulation, allowing for rapid and sensitive quantitative measurement of an individual patient's T-cell response to a specific virus. Because development of a virus-specific response is crucial in suppressing CMV reactivation and preventing symptomatic CMV infection, and MHC tetramers allow measurement of this response, this study was undertaken to determine whether tetramers may be useful in assessing the CMV risk in HSCT recipients. Immune surveillance may provide additional information regarding the patient's risk of CMV reactivation, prolonged reactivation, recurrence of infection, or progression to CMV disease (CMVD).

The objective of this prospective multicenter study was to evaluate the use of iTAg MHC Tetramers (Beckman Coulter) in enumerating CMV-specific CD8$^+$ T-cell reconstitution in allogeneic HSCT recipients to predict patients at risk for recurrent or persistent CMV infection, CMVD, or transplant-related mortality (TRM).

Methods

Patients and study design

This multicenter prospective longitudinal clinical trial evaluated the use of tetramers in monitoring CMV-specific CD8$^+$ T-cell recovery after allogeneic HSCT in 83 CMV-seropositive recipients. Patients were tested every 2 weeks from day 28 to day 100, every 2 to 4 weeks from day 101 to day 270, and were monitored for 1 year for viral results, lymphocyte counts, antiviral and immunosuppressive drug regimen, and clinical status. The protocol and informed consent forms were reviewed and approved by

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the Institutional Review Board or Independent Ethics Committee at each site, and informed consent was obtained from each subject in accordance with the Declaration of Helsinki.

CMV surveillance and preemptive therapy

All patients underwent weekly surveillance by pp65 antigenemia, DNAemia, or shell vial culture, with preemptive antiviral therapy as per institutional guidelines. All sites initiated preemptive therapy when CMV reactivation was detected and continued it until clearance of viremia. Ganciclovir was used for preemptive therapy unless the patients had concomitant neutropenia, which prompted the use of foscarnet.

Definitions

CMV infection was defined as assay positivity for each of the methods as follows: quantitative CMV DNA polymerase chain reaction (PCR), viremia reaching the threshold used at each institution for implementation of antiviral therapy; and pp65 antigenemia, 2 or more positive cells per 200 000 cells (polymorphonuclear leukocytes). Shell vial culture assay or tissue culture was positive if replicating virus was detected or isolated. Recurrent CMV infection was defined as 2 or more episodes (separated by negative CMV results). Persistent CMV infection was defined as positive results for 4 or more weeks. CMVD was defined as biopsy-proven organ-involvement (tissue-invasive), or CMV in bronchoalveolar lavage fluid.

Enumeration of CMV-specific CD8$^+$ T cells

iTAg MHC Tetramers (Beckman Coulter) were used to enumerate CMV-specific CD8$^+$ T cells in ethylenediaminetetraacetic acid whole blood by flow cytometry using a single-platform absolute counting method. A panel of 5 CMV-derived peptides and corresponding MHC class I tetramers were used: pp50: A*0101 VTEHDTLLY; pp65: A*0201 NLVPMVATV, B*0702 TPRVTGGGAM, B*3501 IPSINVHHY; IE-1: B*0801 ELRRKMMYM. Donor and recipient were required to have one or more of the HLA types (A*0101, A*0201, B*0702, B*0801, B*3501) corresponding to available tetramers. These 5 HLA types are among the most common in many ethnic groups, with coverage of 65% to 77% in white populations, 39% to 48% in black populations, and 41% to 42% in Asian populations.[3-5]

iTAg MHC Tetramers are complexes of 4 MHC molecules, which are associated with a specific peptide and bound to a fluorochrome. These complexes bind to a distinct set of T-cell receptors on a subset of CD8$^+$ T cells. By mixing tetramers with whole blood and using flow cytometry as a detection system, a count of all T cells that are specific for one peptide and its matched allele is provided, regardless of functionality. Tetramers allow direct measurement of disease-specific T-cell responses. The iTAg MHC Tetramers, which have been constructed from mutated HLA molecules to minimize CD8-mediated binding of HLA to cell-surface CD8, show a greatly diminished binding to the general population of CD8 cells, but retain peptide-specific binding,[2] thus facilitating accurate discrimination of rare, specific T cells.

In this study, streptavidin-phycoerythrin (PE)–conjugated iTAg MHC Tetramers were used to enumerate CMV-specific CD8$^+$ T cells by flow cytometry using a single-platform absolute counting method. This is a 2-Panel assay. Panel 1 determines absolute CD4$^+$ and CD8$^+$ T-cell counts. Panel 2 determines the percentage of CD8$^+$ T cells that are CMV-specific. Absolute counts of CMV-specific T-cell subsets were calculated by multiplying the absolute CD8$^+$ T-cell count (determined in Panel 1) by the results from Panel 2. Tetramer values in this study were expressed in terms of percentage of the total CD8$^+$ cells that were CMV-specific (shown as CMV tetramer-positive percentage), as well as CMV tetramer-positive absolute counts (shown as cells per microliter).

For each specimen, a minimum of 3 tubes were required, as previously described in detail.[6] Panel 1 (Lyse/No Wash): CD8-fluorescein isothiocyanate (FITC)/CD4-PE/CD3-PC5/Flow-Count Fluorospheres (to obtain CD4$^+$ and CD8$^+$ absolute cell counts). Panel 2 (Lyse/Wash): CD8-FITC/Neg-iTAg-PE/CD3-PC5 (negative tetramer control). Panel 2 (Lyse/Wash): CD8-FITC/CMV-iTAg-PE/CD3-PC5 (to obtain percentage of CMV-specific CD8$^+$ cells). An additional tube identical to the last tube was added for each additional allele tested, based on the HLA type of the patient and the corresponding tetramers available.

The following reagents were provided by Beckman Coulter: iTAg MHC Tetramers, Negative Tetramer, Tetramer Lyse Reagent, Tetramer Fixative Reagent, anti–CD3-PC5 clone UCHT1, anti–CD4-PE clone SFCI12T4D11 (T4), anti–CD8-FITC clone SFCI21Thy2D3 (T8), and Flow-Count Fluorospheres. This assay was optimized for use with the Beckman Coulter EPICS XL using System II software, the Beckman Coulter FC500 using CXP software, and BD Biosciences FACSCalibur using CellQuest software. Recommended stop count for Panel 1 was 3000 singlet Flow-Count Fluorospheres. For Panel 2, a minimum of 100 iTAg MHC Tetramer-positive events or 20 000 to 30 000 CD3$^+$CD8$^+$ events were collected.

The lower limit of detection in whole blood is 0.2% of CD8$^+$ T cells (frequency) or 1 cell/μL (absolute cell count). Moderate-to-bright staining and clearly detectable proportions of tetramer-binding cells were observed for all 5 tetramers. Interassay variability was 8% or less for mid to high tetramer values (4-30 cells/μL), and 15% or less at low tetramer values (2 cells/μL). From the time the sample is available in the laboratory, sample staining and acquisition on the flow cytometer takes approximately 3 hours.

Healthy control subjects

Observed tetramer ranges were established for healthy CMV-seronegative donors and healthy CMV-seropositive donors. Testing was performed using all 5 CMV tetramers.

End points and statistical analysis

The objective of the study was to evaluate the use of tetramers in enumerating CMV-specific CD8$^+$ T cells to determine whether tetramers can be used to assess CMV-specific immune status and risk of recurrent or persistent CMV infection, CMVD, or TRM after allogeneic HSCT. The analysis also determined whether CMV-specific CD8$^+$ T-cell counts were an independent risk factor that can be used in conjunction with other known factors in identifying patients at risk of CMV infection and CMV-related complications.

The study end points were recurrent or persistent CMV infection as measured by each institution's standard method for virologic monitoring (quantitative CMV DNA PCR, pp65 antigenemia, shell vial culture), CMVD or TRM. End points were analyzed individually and as a composite.

To determine whether CMV-specific CD8$^+$ T cells were protective against CMV infection, the analysis evaluated recurrent or persistent CMV infection or CMVD occurring after cellular repopulation, that is, to evaluate if the presence or absence of these CMV-specific T cells would predict patients at risk for future episodes of recurrent or persistent infection or CMVD. The maximum response of any individual allele/tetramer (A1, A2, B7, B8, B35) within an individual patient was used in the analysis. A total of 83 subjects met study entry criteria with 3 or more blood samples (or early death with CMV complications) and were included in the analysis.

A χ^2 analysis compared CMV tetramer recovery (rapid/delayed) with recurrent or persistent CMV infection or CMVD (present/absent). Based on the χ^2 2 $\times$ 2 tables, the relative risk (95% confidence interval) of recurrent or persistent CMV infection, CMVD, or TRM was calculated. Univariate and bivariate logistic models assessed the predictive power of possible risk factors. Kaplan-Meier survival curves and cumulative incidence curves were used to assess time of event onset.

Results

Patient demographics

Data were analyzed for 83 allogeneic HSCT recipients. Demographic and clinical characteristics are shown in Table 1. Median follow-up was 9 months (range 2-12 months). Shorter follow-up times were primarily because of early deaths. The study protocol required blood draws and tetramer testing every 2 weeks from

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Table 1. Characteristics of allogeneic stem cell transplantation patients

Parameter	No. of patients (N = 83)
Sex, male/female	39/44
Median age, y (range)	50 (18-66)
Disease	
AML, ALL, CML (leukemias)	51
Myelodysplasia	5
Multiple myeloma	5
Non-Hodgkin lymphoma	11
Aplastic anemia	3
Other	8
CMV serology	
Recipient$^+$donor$^+$	50
Recipient$^+$donor$^-$	33
Donor-recipient relationship	
Related HLA-matched	32
Unrelated or HLA-mismatched	51
Stem cell source	
Marrow	10
Peripheral blood	73
Conditioning regimen	
Myeloablative	47
Nonmyeloablative	36
HLA types	
A*0101	31
A*0201	52
B*0702	21
B*0801	27
B*3501	11

AML indicates acute myeloid leukemia; ALL, acute lymphocytic/lymphoblastic leukemia; and CML, chronic myelocytic leukemia.

day 28 to day 100 (days 28, 42, 56, 70, 84, 98) after transplantation. Compliance rates with this schedule were 90% or higher at all sites.

Tetramer recovery and risk of CMV infection and CMVD

To compare CMV tetramer recovery (rapid/delayed) with recurrent or persistent CMV infection or CMVD (absent/present), a series of 2×2 χ^2 analyses were performed. Only those subjects with at least 5 months of viral test monitoring results (n = 72) were included in these analyses (median 9 months), allowing sufficient time to measure potential episodes of recurrent or persistent viral reactivation. Inclusion of patients with limited follow-up would introduce bias because the viral reactivation status was indeterminate due to insufficient data.

The first step in this analysis was to assess recovery kinetics (ie, to define and evaluate "rapid" vs "delayed" recovery of CMV-specific immunity). A range of cutoff days after transplantation (60 to 100 days) was analyzed. Table 2 presents relative risk results for a tetramer threshold of 7 cells/μL; comparable tables were generated for tetramer thresholds between 2 and 10 cells/μL (data not shown). Similar patterns of results were observed for each tetramer threshold, with day 65 yielding the highest relative risk and the fewest "low risk" patients above the tetramer threshold with CMV complications. By day 65, the majority of patients had at least 3 blood samples with tetramer results (86% compliance rate with the biweekly sampling schedule at days 28, 42, 56). Use of a cutoff day before day 65 yielded an insufficient number of blood draws and tetramer results per patient to be certain of the predictive validity. Use of a cutoff day after day 65 showed increasing difficulty in distinguishing between the "rapid" and "delayed" recovery groups as progressively more patients showed reconstitution of immunity approaching day 100, and was therefore less predictive. Use of day 65 as a cutoff day clearly grouped patients into "rapid" and "delayed" recovery groups and was strongly associated with patient outcome.

Table 2. Relative risk for tetramer thresholds and days after transplantation

	Relative risk (95% CI)	Subjects above cutoff, %*	*P*
Days after transplantation, using a tetramer threshold of 7 cells/μL			
60	2.46 (1.1-5.5)	19	.014
65	2.83 (1.3-6.3)	18	.004
70	2.13 (1.1-4.3)	22	.023
100	1.98 (1.0-3.8)	24	.031
Tetramer thresholds, cells/μL, on day 65			
2	1.74 (0.9-3.2)	28	.072
3	1.81 (1.0-3.4)	27	.056
4	2.22 (1.1-4.3)	23	.014
5	2.51 (1.2-5.1)	21	.006
6	2.51 (1.2-5.1)	21	.006
7	2.83 (1.3-6.3)	18	.004
8	2.26 (1.0-5.0)	20	.023
9	2.14 (1.0-4.7)	21	.034
10	2.02 (0.9-4.4)	21	.049

CI indicates confidence interval.
*Percentage of subjects above cutoff with CMV infection.

The second step was to define "protective tetramer recovery," that is, to determine the tetramer threshold associated with protection from recurrent or persistent CMV infection or CMVD. A range of CMV tetramer cell counts was evaluated (2 to 10 cells/μL). Table 2 presents relative risk results for day 65; comparable tables were generated for days 60 to 100 (data not shown). Similar patterns of results were observed for each cutoff day tested, with a threshold of 7 cells/μL yielding the highest relative risk (2.83), the greatest statistical significance ($P = .004$), and the fewest "low risk" patients with CMV complications (18%, range 18%-28%).

Table 3 shows that rapid recovery (≥ 7 cells/μL in any blood sample during the first 65 days after transplantation) was associated with protection from CMV-related complications ($P = .004$). In this group, 6 of 34 (18%) developed recurrent or persistent CMV infection or CMVD, whereas half (19 of 38, 50%) developed CMV complications in the delayed recovery group.

Table 4 shows the relative risk with 95% confidence interval for individual end points and composite end points. Results showed that delayed recovery of CMV-specific CD8$^+$ T cells (< 7 cells/μL in all blood samples during the first 65 days after transplantation) predisposes patients to CMV-related complications. These patients are more likely to develop recurrent or persistent CMV infection, CMVD, and fatal complications than patients showing rapid recovery.

Table 3. Protection from CMV infection with optimal cutoff of 7 cells/μL within 65 days

	Rcurrent or persistent CMV infection or CMVD (n, %)	
Recovery	**Absent**	**Present**
Rapid	28 (82)	6 (18)
Delayed	19 (50)	19 (50)

Rapid recovery of CMV-specific CD8$^+$ T cells (≥ 7 tetramer-positive cells/μL in any blood sample during the first 65 days after transplantation) was associated with protection from CMV-related complications ($P = .004$).

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Table 4. Relative risk for individual and composite end points

	Relative risk (95% CI)	*P*
Recurrent or persistent CMV infection	2.6 (1.2-5.8)	.01
CMVD	6.4 (0.8-49.2)	.03
TRM	2.4 (0.8-6.9)	.07
Composite		
Recurrent or persistent CMV infection or CMVD	2.8 (1.3-6.3)	.004
Recurrent or persistent CMV infection, CMVD, or TRM	2.4 (1.3-4.5)	.002

Delayed recovery of CMV-specific $CD8^+$ T cells (< 7 cells/μL in all blood samples during the first 65 days after transplantation) is a significant risk factor for CMV-related complications. CI indicates confidence interval.

Probability of recurrent or persistent CMV infection or CMVD

A total of 25 patients developed recurrent (n = 16) or persistent (n = 17) CMV infection or CMVD (n = 9). The cumulative incidence of recurrent or persistent CMV infection or CMVD is presented in Figure 1. Patients with delayed recovery of CMV-specific $CD8^+$ T cells were significantly more likely to develop CMV infection or CMVD than those patients with rapid recovery ($P = .003$).

Survival

Of the 83 patients in this study, 16 died of transplant-related complications. The Kaplan-Meier survival curve is shown in Figure 2. Patients with delayed recovery of CMV-specific $CD8^+$ T cells were less likely to survive than those patients with rapid recovery, although this was not statistically significant ($P = .08$) because of the relatively small number of deaths during the study period.

Examples of immune reconstitution patterns

Figure 3 shows examples of individual patient graphs with tetramer absolute cell counts and viral results over time (days posttransplantation). The first patient showed delayed recovery (< 7 cells/μL in all blood samples during the first 65 days); recurrent/persistent viremia was observed. The second patient (monitored with multiple tetramers) showed rapid recovery (at least 1 allele/tetramer ≥ 7 cells/μL in at least one blood sample before day 65); no viremia was observed. Tetramer results are shown for A*0101, A*0201, and B*0801. Rapid recovery was observed for 2 of the 3 alleles.

Individuals may respond with one or more alleles, and the responses may differ between alleles (Figure 3). The maximum response of any allele/tetramer (A1, A2, B7, B8, B35) within an individual patient was used to determine whether the patient recovered CMV immunity. Of the 83 subjects, 44 were A1, A2, B7, B8, or B35 alone; 21 subjects had some combination of 2 of the relevant alleles; 16 subjects had 3 alleles; and 2 subjects had 4 alleles.

Correlation between tetramer absolute count and tetramer percentage

Tetramer percentage (percentage shown) and absolute cell counts (as cells per microliter) are moderately correlated (r = 0.66, n = 1387 tetramer/allele results in 804 blood samples in 83 patients), but not strong enough to assume they provide the same information. Cell count is a more accurate reflection of CMV immune status than percentage, and a more reliable predictor of patient outcome. It is possible to have the same tetramer percentage in 2 different patients, but very different absolute cell counts depending upon the total $CD8^+$ cell counts.

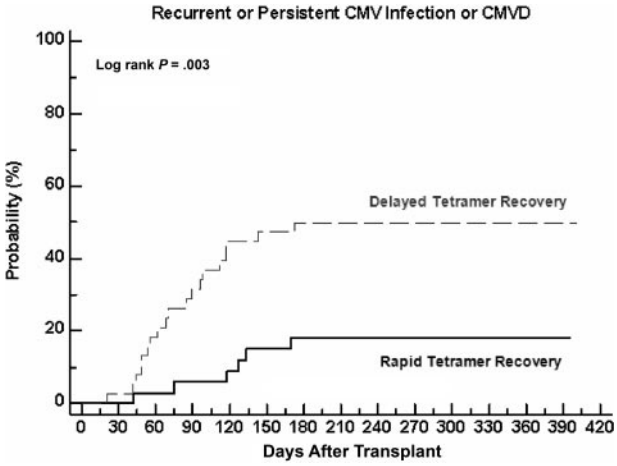

Figure 1. Cumulative incidence curves of CMV risks. Cumulative incidence of recurrent or persistent CMV infection or CMVD in patients with delayed (dashed histogram) versus rapid (solid histogram) recovery of CMV-specific $CD8^+$ T cells.

Allogeneic HSCT recipients and observed tetramer ranges

The observed range of tetramer values for allogeneic HSCT recipients in this study was 0 to 440 cells/μL (median 3.33, mean $\pm$ SD 25.45 $\pm$ 55.42, upper 95th percentile 149.12), or 0% to 42% (median 1.53, mean $\pm$ SD 4.29 $\pm$ 6.54, upper 95th percentile 18.80).

Of the 72 patients in the relative risk analysis, 34 (47%) had at least one sample with a tetramer value equal to or greater than 7 cells/μL at any time during the first 65 days after transplantation.

Healthy control subjects and observed tetramer ranges

Whole blood from 72 healthy control subjects was tested, consisting of 36 donors each that were CMV seronegative and CMV seropositive. A total of 99 distinct tetramer/allele results were obtained, consisting of 57 seronegative results and 42 seropositive results using the appropriate allele-matched tetramers for each donor.

The observed range based on CMV-seropositive donors was 0 to 47 cells/μL (median 4.03, mean $\pm$ SD 6.96 $\pm$ 9.49, upper 95th percentile 24.50), or 0% to 9% (median 0.85, mean $\pm$ SD

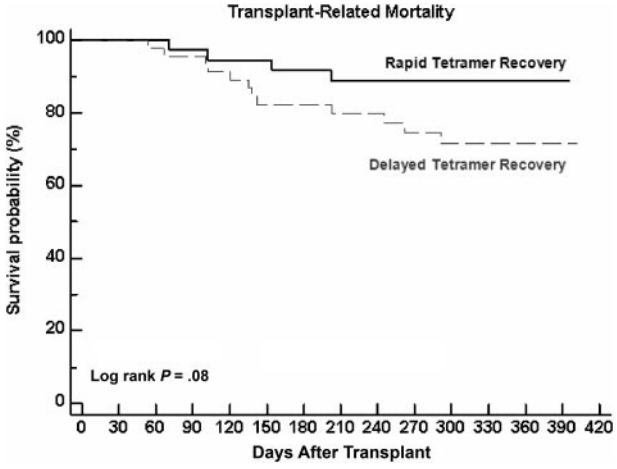

Figure 2. Kaplan-Meier survival curve of TRM. TRM in patients with delayed (dashed histogram) versus rapid (solid histogram) recovery of CMV-specific $CD8^+$ T cells.

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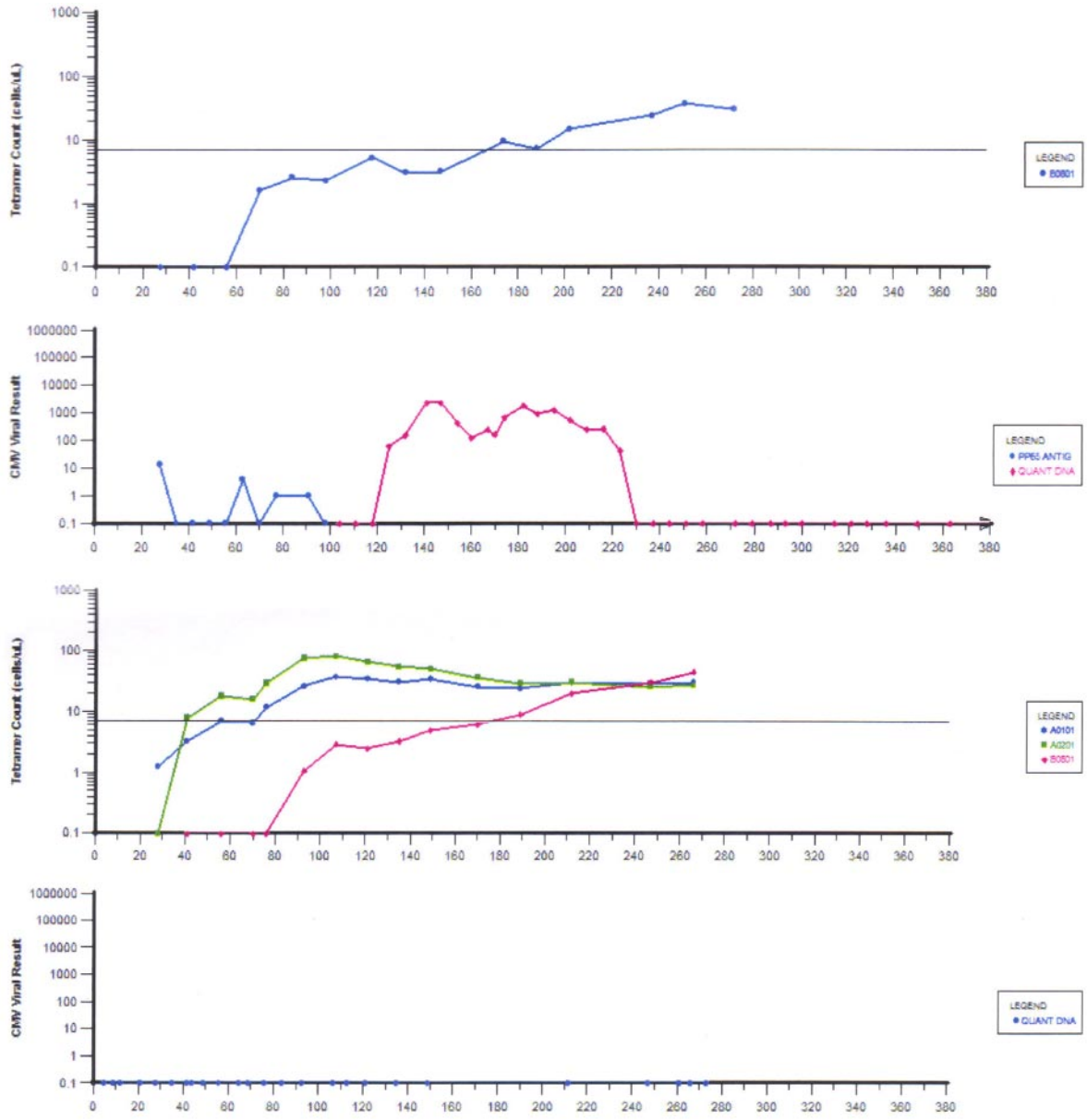

Figure 3. Individual patient graphs of CMV-related cell counts and viral results. Examples of CMV-specific CD8$^+$ T-cell absolute counts (shown as tetramer-positive cells per microliter) and viral results over time. The first patient (top 2 graphs) showed delayed recovery (< 7 cells/μL in all blood samples during the first 65 days); recurrent/persistent CMV viremia was observed. The second patient (bottom 2 graphs; monitored with multiple tetramers) showed rapid recovery (at least 1 allele/tetramer ≥ 7 cells/μL in at least 1 blood sample before day 65); no viremia was observed.

1.51 ± 1.79, upper 95th percentile 5.40). All of the CMV-seronegative donors tested had values below the limit of detection (1.0 cell/μL, 0.2%).

A greater proportion of transplant patients (47%, 34/72) had elevated tetramer values (≥ 7 cells/μL) compared with the healthy control subjects (29%, 12/42). The impaired immune system in transplant patients leads to frequent CMV reactivation and generation of CMV-specific T cells. In healthy controls, an equilibrium between T cells and virus has been achieved, and high values in either viral load or T-cell response are rarely observed. Tetramer values obtained in the healthy control subjects are not applicable to transplant patient management and are provided for reference only.

Comparison of tetramer and CD8$^+$ and CD4$^+$ absolute counts

CMV-specific CD8$^+$ (shown as tetramer-positive) absolute counts (shown in cells per microliter) and total CD8$^+$ absolute counts (in cells per microliter) showed a moderate correlation ($r = 0.57$, $y = 0.07x - 5.20$) because tetramer-positive cells are a subset of total CD8$^+$ cells. CMV-specific CD8$^+$ (tetramer-positive) absolute counts (in cells per microliter) and total CD4$^+$ absolute counts (in cells per microliter) showed a negligible correlation ($r = 0.14$, $y = 0.03x + 12.71$).

Univariate and bivariate logistic regression analysis

Possible risk factors were examined in a univariate analysis for their association with recurrent or persistent CMV infection or CMVD. The 13 factors examined included conditioning regimens (myeloablative/nonmyeloablative), use of antithymocyte globulin, HLA mismatch, unrelated donor status, donor CMV serology (negative), source of stem cells, age, sex, acute graft-versus-host disease (aGVHD, maximum grade ≥ 2), high-dose steroids, and delayed CD4$^+$, CD8$^+$, and tetramer counts (< 7 cells/μL).

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Table 5. Univariate and bivariate analysis of significant risk factors for recurrent or persistent CMV infection or CMVD

Covariate	Relative risk (95% CI)	
	Univariate*	Bivariate†
Donor CMV serology (negative)	2.0 (1.0-3.8)	1.6 (0.9-3.1)
Acute GVHD (grade 2 or higher)	3.2 (1.4-7.0)	2.6 (1.2-5.7)
Immune system recovery (absolute counts)		
$CD4^+$	2.2 (1.2-3.9)	2.0 (1.2-3.4)
$CD8^+$	2.6 (1.5-4.4)	1.9 (1.1-3.3)
Tetramer-positive	2.8 (1.3-6.3)	2.3-2.7‡

CI indicates confidence interval.

*All *P* values are less than .05.

†Individual bivariate models were run to control for sample size. Tetramer-positive absolute counts remained a significant predictor when paired with each covariate. All *P* values were less than or equal to .05.

‡Tetramer relative risk range compared individually with each of the other covariates in the bivariate models.

Only 5 of the 13 variables (donor CMV serology, aGVHD, $CD4^+$, $CD8^+$, tetramer-positive counts) showed a significant relationship ($P < .05$) with recurrent or persistent CMV infection or CMVD in univariate analysis. The study sample size did not allow for simultaneous inclusion of all possible risk factors in a multivariate analysis. Therefore, individual bivariate logistic regression models were run to control for sample size. Tetramer recovery was paired with each of the other covariates in bivariate models. Results showed that delayed tetramer recovery by day 65 after HSCT was independently associated with recurrent or persistent CMV infection or CMVD (Table 5) as a significant risk factor and is not a surrogate for other known risk factors.

Examination of general T-cell reconstitution data showed that 39% of patients (28/72) had rapid recovery of total $CD4^+$ cells ($\geq$ 100 cells/μL within the first 65 days) but delayed recovery of CMV-specific $CD8^+$ T cells; 44% (11/25) of patients with poor outcomes were in this group. In addition, 36% of patients (26/72) had rapid recovery of total $CD8^+$ cells ($\geq$ 100 cells/μL within the first 65 days) but delayed recovery of CMV-specific $CD8^+$ T cells; 40% (10/25) of patients with poor outcomes were in this group.

Discussion

The measurement of T-cell responses to pathogens, autoantigens, and tumor-derived antigens by MHC tetramers has become an established technique in preclinical research, and is becoming increasingly important for clinical trial monitoring of responses to vaccines and correlating natural T-cell responses with clinical outcomes. The current study demonstrates the effectiveness of tetramer-based immune monitoring as a promising new tool in patient management.

This study is the largest prospective multicenter clinical trial to date evaluating the use of tetramers in allogeneic stem cell transplant recipients, with nearly 1400 tetramer/allele results in more than 800 biweekly blood samples from 83 patients monitored for up to 1 year after transplantation. The major HLA types were included (A*0101, A*0201, B*0702, B*0801, B*3501). All sites followed the same standardized and optimized tetramer flow cytometric protocol using a single-platform absolute counting method that produced both frequency (shown as a percentage) and absolute cell count (in cells per microliter) results. A recent study demonstrated this iTAg MHC Tetramer–CMV assay to be simple, rapid, reproducible, and useful for assessing CMV-specific T cells across multiple centers at clinically relevant ranges.[6]

Results from the current study demonstrate utility for this tetramer assay in monitoring CMV-specific $CD8^+$ T cells to assess immune status and risk of recurrent or persistent CMV infection or CMVD in immunosuppressed stem cell transplant recipients, allowing clinicians to further refine preemptive therapeutic strategies in appropriate high-risk populations.

Rapid recovery ($\geq$ 7 cells/μL in any blood sample during the first 65 days after transplantation) of CMV-specific $CD8^+$ T cells (tetramer-positive cells) was associated with protection from recurrent or persistent CMV infection or CMVD. Delayed recovery ($<$ 7 cells/μL in all blood samples during the first 65 days after transplantation) of CMV-specific $CD8^+$ T cells predisposes patients to CMV-related complications. These patients are 2.6 times more likely to develop recurrent or persistent CMV infection, 6.4 times more likely to develop CMVD, and 2.4 times more likely to develop fatal complications than patients showing rapid recovery.

Other possible risk factors were examined for their association with recurrent or persistent CMV infection or CMVD. Donor CMV serology, moderate to severe aGVHD, and delayed $CD4^+$, $CD8^+$, and CMV-specific $CD8^+$ T-cell counts were shown to have a significant relationship with CMV infection. CMV-specific $CD8^+$ T-cell counts were found to have independent predictive power as a significant risk factor and are not a surrogate for other known risk factors.

Examination of cellular reconstitution data showed that 44% of patients with poor outcomes had rapid recovery of total $CD4^+$ cells but delayed recovery of CMV-specific $CD8^+$ T cells. In addition, 40% of patients with poor outcomes had rapid recovery of total $CD8^+$ cells but delayed recovery of CMV-specific $CD8^+$ T cells. For these patients, monitoring of total $CD4^+$ cells or total $CD8^+$ cells after transplantation was not sufficient to detect patients at risk of CMV infection and CMVD. Tetramer-based monitoring alone allowed identification of these high-risk patients.

CMV-specific $CD8^+$ (tetramer-positive) absolute counts showed a moderate correlation with total $CD8^+$ absolute counts ($r = 0.57$) and a negligible correlation with total $CD4^+$ absolute counts ($r = 0.14$). Tetramers provide unique information not available with these existing monitoring tests.

The observed range of tetramer values for allogeneic stem cell transplant recipients in this study was 0 to 440 cells/μL, or 0% to 42%. Some 47% of patients had at least one sample with a tetramer value equal or greater than 7 cells/μL at any time during the first 65 days after transplantation.

Monitoring individual patients over time using multiple tetramers showed that patients may respond with one or more alleles, and the responses may differ between alleles. The maximum response of any allele/tetramer (A1, A2, B7, B8, B35) within an individual patient was used to determine whether the patient recovered CMV immunity. It was observed that A1 and B7 tended to generate a stronger response than B8 and A2, but not in every case. Despite this diversity of response, a majority of patients (82%) capable of mounting a CMV-specific immune response (regardless of allele) appeared to be protected against recurrent or persistent infection or CMVD. All 5 alleles/tetramers in this study showed a strong response in at least one patient, and correlated with protection against recurrent or persistent CMV infection or CMVD in at least one patient. The results were consistent across alleles, that is, rapid recovery of CMV immunity ($\geq$ 7 cells/μL by any allele) appears to be associated with protection against CMV infection or CMVD.

Not all patients and alleles are covered with the tetramer panel used in our study. Research into other alleles and epitopes and the availability of additional tetramers in the future may improve

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results further. Rapid CMV-specific T-cell recovery indicates an immune system capable of mounting a response. However, an absence of detectable CMV-specific T cells (delayed recovery) may be because of an impaired immune system or the unavailability of all relevant tetramers for a specific patient. We cannot assess if another unmeasured allele is controlling the viremia. A "negative" tetramer finding may actually mean we are not measuring the more responsive allele. Despite this limitation, the panel of 5 tetramers in this study was highly effective in identifying high- and low-risk patients using only the available alleles/tetramers representing the most common HLA types.

Earlier studies in which tetramers were used to monitor the reconstitution of CMV-specific $CD8^+$ T lymphocytes also showed a relationship between the number of cells and the occurrence of CMV reactivation and CMVD.[7-10] These studies primarily focused on enumeration of lymphocytes restricted by HLA-A2 and HLA-B7 molecules because these alleles are among the most common HLA subtypes in many ethnic groups, with a frequency of up to 40% and 13%, respectively, in white populations, 19% and 10%, respectively, in black populations, and 25% and 4%, respectively, in Asian populations.[3-5] In the current study, the panel of 5 MHC tetramers expanded coverage up to 77% in white populations, 48% in black populations, and 42% in Asian populations.[3-5]

Previously, we used tetrameric complexes to quantify CMV-specific T cells in partially T cell–depleted grafts of 18 CMV-seropositive HSCT recipients, and monitored recovery of these T cells during the first 12 months after HSCT.[7] We found that the number of CMV-specific cells in the grafts correlated inversely with the number of preemptive ganciclovir courses administered. Thirteen of 14 patients who did not develop CMV disease regenerated CMV-specific T cells. Four patients developed CMVD despite preemptive ganciclovir treatment and all had failed to regenerate CMV-specific cells. These and current results showed that regeneration correlated with protection against progressive CMV infection and CMVD, suggesting that enumeration of CMV-specific T cells in the grafts and monitoring of these cells after HSCT may provide a new method to identify patients at risk of developing CMVD.

Cwynarski et al[8] used HLA tetramers to prospectively monitor the recovery of CMV-specific T cells in 24 recipients of allogeneic stem cell transplants. They found that recovery of CMV-specific cells was rapid and reached up to 21% of all $CD8^+$ T cells. Recovery of CMV-specific T cells to levels greater than 10 cells/μL was associated with protection from CMVD. It was concluded that use of HLA tetramers is valuable for monitoring T-cell responses and may assist in the development of adoptive CMV T-cell immunotherapy.

In contrast, Morita-Hoshi and colleagues[11] reported that CMVD could occur after HSCT even in patients with greater than 10 cells/μL. Possible explanations for these discrepant findings may be important differences in how the CMV-specific $CD8^+$ T lymphocytes were enumerated and CMV reactivation was monitored. Timing of tetramer testing is also critical; results of our current study indicate that a single sample at 30 days is not sufficient to assess risk of CMV reactivation.

Aubert et al[9] correlated CMV-specific T cells in 11 HSCT recipients with viral replication and clinical status, and showed that the level of tetramer-positive T cells provides an assessment of CMV immune reconstitution. Most patients with seropositive donors reconstituted long-term immunity, unless prolonged immunosuppression to control GVHD was required. They concluded that the use of tetramers in conjunction with quantitative CMV DNA PCR testing provide potential measures that can in the future be a guide to clinical management.

Ozdemir et al[10] did not find a relationship between the ability to control CMV reactivation and the recovery of sufficient numbers of CMV-specific T cells. However, this study did not test for rate of CMV-specific T-cell recovery soon after HSCT; a single tetramer result was obtained from a blood sample drawn at 3 months after HSCT. Our study findings indicate that tetramer testing every 2 weeks from day 28 to day 65 (3 to 4 test results) identifies patients with delayed recovery at risk of viral reactivation. A single tetramer result at 3 months cannot distinguish between the "delayed" (high risk) and "rapid" (low risk) recovery groups. In contrast, biweekly tetramer results in the second and third months were strongly associated with patient outcome.

Several studies have assessed intracellular cytokine production after CMV-specific T-cell stimulation, and found that the inability to control CMV reactivation may also be related to impaired function of antigen-specific $CD4^+$ and $CD8^+$ T cells.[10-13] Future studies may show an additive ability to predict high risk patients by combining both functional analysis and cellular count rate of recovery.

Conclusions

CMV tetramer-based immune monitoring, in conjunction with virologic monitoring, can be an important new tool that permits clinicians to assess the risk of CMV-related complications and to guide preemptive therapeutic choices. CMV tetramer testing performed every other week starting on day 28 after transplantation allows patients to be divided into 2 risk groups: rapid recovery, low risk and delayed recovery, high risk.

Rapid recovery, low-risk patients. If any tetramer result for any allele in any blood sample between day 28 and day 65 is greater than or equal to the threshold of 7 cells/μL, then the patient has shown rapid recovery of CMV-specific immunity and is at low risk of developing recurrent or persistent CMV infection or CMVD. For these low-risk patients, virologic monitoring should be continued following current practice. Studies are needed to determine how long virologic monitoring should be continued, and whether preemptive therapy may be reduced or introduced at a higher viral load threshold.

Delayed recovery, high-risk patients. If all tetramer results from all blood samples between day 28 and day 65 are below the threshold of 7 cells/μL, then the patient has shown delayed recovery of CMV-specific immunity and is at high risk of developing recurrent or persistent CMV infection or CMVD. For these high-risk patients, we suggest that virologic monitoring and preemptive strategies should be continued beyond 100 days after transplantation; immunologic monitoring with tetramers may prove useful in determining when virologic surveillance can be discontinued.

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Authorship

Contribution: J.W.G. contributed to the conception and design of the clinical trial, enrollment of patients, optimization of the tetramer flow cytometric assay, analysis and interpretation of results, and drafting of the manuscript; M.B. contributed to trial design, enrollment of patients, interpretation of results, and critical review of the manuscript with substantive suggestions for revision; R.N. contributed to trial design, enrollment of patients, and critical review of the manuscript; J.J.C., J.A.Z., and S.J.F. contributed to trial design and enrollment of patients; R.A.B. contributed to the optimization of the tetramer flow cytometric assay, design of experiments and analysis and interpretation of data, and drafting of the manuscript; K.G. performed CMV-specific tetramer flow cytometric assays and analyzed and interpreted data; K.R.B. provided important intellectual contributions to the study design and manuscript; G.H.G. made substantial contributions to acquisition and analysis of data; C.S.B. contributed to the optimization of the tetramer flow cytometric assay, design of experiments, and analysis and interpretation of data; L.A.S. performed statistical analysis and interpretation of results, and critical review and revision of the manuscript; P.C.S. contributed to trial design, analysis and interpretation of results, and drafting of the manuscript; and all authors reviewed and approved the final manuscript.

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Correspondence: Paula C. Southwick, PhD, Group Manager, Clinical Research, Beckman Coulter Inc, 2470 Faraday Ave, Carlsbad, CA 92010; e-mail: pcsouthwick@beckman.com.

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