Simultaneous Reconstitution of Multiple Cytomegalovirus-Specific CD8+ Cell Populations with Divergent Functionality in Hematopoietic Stem-Cell Transplant Recipients

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A panel of 7 human cytomegalovirus (CMV) epitope peptides and corresponding major histocompatibility class 1 tetramers was used to evaluate cellular immunity in healthy seropositive donors and in hematopoietic stem-cell transplant recipients. Broad CMV-specific T cell responses to epitopes were found within several CMV polypeptides and were restricted by multiple human leukocyte antigen alleles. Their cytotoxic functionality was evaluated by use of an assay that measures transient surface levels of lysosomal membrane proteins LAMP-1 (CD107a) and LAMP-2 (CD107b) after peptide stimulation. This assay can be combined with tetramer staining of antigen-specific CD8+ T lymphocytes and has potential as a surrogate marker for cytotoxic function. CD8+ T lymphocytes specific for epitopes within the pp65 or pp50 gene products exhibited significantly higher functionality, compared with populations recognizing CMV major immediate early–1 epitopes. These functional differences between T lymphocyte populations within the same individual may have implications for protection against CMV.

Several studies have established the central importance of cytotoxic T lymphocytes (CTLs) and T helper lymphocytes in providing protection against cytomegalovirus (CMV) disease during the reconstitution of immunity after hematopoietic stem-cell transplant (HSCT) [1–5]. However, there is still an incomplete understanding of which CMV antigens and epitopes are protective as part of these cellular immune responses. The majority of studies of cellular immunity against CMV have focused on the pp65 tegument polypeptide [6–14], with a smaller number of reports describing immune responses to the major immediate early (IE)–1 [15–20] and pp150 [16, 21–23] polypeptides. Recently, Elkington et al. [24] described the use of enzyme-linked immunospot assays with multiple peptides that were based on computer algorithms predicting binding to major histocompatibility class (MHC) type 1 molecules, to demonstrate that broadly directed immune responses to multiple epitopes in several polypeptides exist in healthy CMV-seropositive individuals. We used flow-based intracellular cytokine (ICC) assays and MHC 1 tetramer staining to investigate the frequency of CD8+ lymphocytes specific for newly reported CMV peptide epitopes in samples from healthy CMV-seropositive donors and from HSCT recipients.

Betts et al. [25–27] recently described a novel flow-based degranulation assay that measures the mobilization of the lysosome-associated membrane proteins LAMP-1 (CD107a) and LAMP-2 (CD107b) in response...
to antigen-specific stimulation. As the cytolytic granules fuse with the cell membrane of antigen-specific CD8+ T lymphocytes, these proteins are exposed on the cell surface and can be quantitated by flow cytometry. Degranulation has been directly correlated with cytolytic activity [25, 28], and this interesting new assay is potentially useful for the assessment of the functionality of viral antigen-specific CD8+ cells. We describe the use of this assay to investigate diversity in the cytolytic potential of CMV-specific CD8+ T lymphocyte populations in samples from HSCT recipients and healthy volunteers.

SUBJECTS, MATERIALS, AND METHODS

Subjects. Peripheral blood mononuclear cells (PBMCs) were obtained from 6 healthy donors and from 7 HSCT recipients who were part of a large prospective study of the reconstitution of cellular immunity to CMV after transplant. The 7 patients were included because of their samples having been studied with the full panel of 8 peptides and tetramers listed below. The 6 healthy volunteers were from a study that required that the subjects have no recent clinical symptoms of CMV disease. The subject demographics are shown in table 1. The study protocols were approved by the City of Hope institutional review board, and specimens and data were obtained prospectively after informed consent was obtained from the enrollees. HLA typing was performed by polymerase chain reaction (PCR), as described elsewhere [29].


Preparation and use of MHC I tetramers. The HLA-A*02 CMVpp65495–503 tetramer (A2 pp65 N9V-Tet), the HLA-A*02 CMV IE-1199–207 tetramer (A2 IE-1 V9L-Tet), the HLA-B*0702 CMV pp65417–426 tetramer (B7 pp65 T10M-Tet), the HLA-B*0702 CMV pp65265–275 tetramer (B7 pp65 R11L-Tet), the HLA-B*08 CMV IE-1188–96 tetramer (B8 IE-1 Q9V-Tet), the HLA-B*08 CMV IE-1199–207 tetramer (B8 IE-1 E9M-Tet), and the HLA-A*01 CMV pp50245–253 tetramer (A1 pp50 V9Y-Tet) were refolded, purified, and conjugated to fluorochromes as described elsewhere [32]. The HLA-B*08 CMV IE-1199–207 tetramer (B8 IE-1 E9M-Tet) was refolded with both sequence variants described above. Both tetramers were functional; the higher of the 2 values obtained with these variant peptides/tetramers are reported. Staining and labeling were performed as described elsewhere [32].

Detection of interferon (IFN-γ) production by lymphocytes on peptide stimulation. The detection of IFN-γ production was performed as described elsewhere [33, 34]. Briefly, thawed aliquots of PBMCs were washed and in some cases labeled with Tet for 20 min. The cells were then washed, resuspended in culture medium and incubated overnight with 10 μg/mL viral epitope peptide or control peptide at 37°C. Brefeldin A was added to 1 μmol/L after the first hour, and this was maintained until the end of incubation. The stimulated cells were then washed and labeled with fluorescein isothiocyanate (FITC)–conjugated antibody to CD8 (Phar-mingen) for 20 min at 4°C in 50 μL of buffer before washing, fixation, permeabilization, and labeling with allophycocyanin (APC)–conjugated antibody to IFN-γ (Phar-mingen) for 20 min at 4°C, followed by a final wash and flow analysis.

CD107 mobilization/degranulation assay. The CD107 mobilization/degranulation assay was performed essentially as described elsewhere [25]. Cryopreserved PBMCs were washed once with RPMI 10 medium. Aliquots of 10^6 cells were labeled with CMV-specific tetramers in 100 μL of medium for 30 min at room temperature. Then, 1 mL of RPMI 10 medium and FITC-conjugated antibodies to CD107a and CD107b (Phar-mingen) were added to each aliquot, followed by costimulatory antibodies to CD28 and CD49d (Phar-mingen) to give a concentration of 1 μg/mL each. CMV antigenic peptides incorporated in the tetramers were then added to a final concentration of 5 μmol/L. The tubes were incubated at 37°C in a 5% CO2 gassed incubator for 1 h before the addition of 1 μL of monensin (Golgistop; Phar-mingen) to all cultures, followed by a further 4 h of incubation at 37°C. The cells were then washed with PBS/0.5% bovine serum albumin before they were labeled for 20 min at 4°C with phycoerythrin- or APC-conjugated antibody to CD8 (Phar-mingen), and cells were washed again before flow analysis. A primary gate was set on lymphocytes by use of forward and side scatter, and a secondary gate was set on CD8+ tetramer-binding cells. At least 100,000 events were collected per sample. The percentage of CD8+ tetramer-binding lymphocytes expressing elevated surface CD107a/b was determined by reference to controls incubated with costimulatory antibodies to CD28 and CD49d but no CMV peptides.

Statistical methods. The comparison of tetramer and degranulation results with respect to peptide and HLA restriction presents 2 major statistical issues. The first is that measurements of different cell populations from the same subject may be correlated, contrary to the assumption of independence that motivates most simple statistical comparisons. We dealt with this issue by using F statistics, with the interaction of subjects and cell populations providing the error mean square. Simple non-parametric (rank sum) comparisons were also computed, to verify the robustness of results. The second issue is that peptides and HLA restrictions are substantially confounded because of
Table 1. Demographics and clinical characteristics of normal donors and hematopoietic stem-cell transplant (HSCT) recipients.

<table>
<thead>
<tr>
<th>UPN</th>
<th>Diagnosis</th>
<th>Age at time of study entry, years</th>
<th>CMV serostatus</th>
<th>HLA type</th>
<th>Transplant type</th>
<th>Transplant regimen, days after HSCT</th>
<th>CMV reactivation after HSCT</th>
<th>Note on GVHD</th>
<th>Date of onset of GVHD</th>
<th>Survival status, day 360</th>
<th>Medication</th>
<th>Survival status, day 360</th>
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<td>1</td>
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<td>...</td>
<td>49</td>
<td>CMV+</td>
<td>A01, A02, B08, B44</td>
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<td>...</td>
<td>51</td>
<td>CMV+</td>
<td>A020, B3501, B4402</td>
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<td>57</td>
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<td>A0201, A1101, B0702, B4402</td>
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<td>35</td>
<td>CMV+</td>
<td>A0201, B3512</td>
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<td>HSCT recipients</td>
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<td>54</td>
<td>AML</td>
<td>34</td>
<td>D’/R*</td>
<td>A0101, A0201, B1501</td>
<td>MUD/ BMT</td>
<td>FTBI/Cy</td>
<td>Day 69 (37)</td>
<td>Day 26, GVHD; day 310, GVHD; mouth/eyes</td>
<td>PSE, MMF</td>
<td>Day 765, alive</td>
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<td>HD, MDS</td>
<td>36</td>
<td>D’/R*</td>
<td>A0201, A0301, B0702, B3503</td>
<td>MUD/ PBSC</td>
<td>Fludarabine/Melphalan</td>
<td>Day 52 (Negative)</td>
<td>Day 58, GVHD; mouth; day 51 GVHD liver</td>
<td>CSA, MMF</td>
<td>Day 394, alive</td>
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<td>42</td>
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<td>A0101, A0301, B0702</td>
<td>MUD/Allo</td>
<td>FTBI/Cy</td>
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<td>No GVHD</td>
<td>None</td>
<td>Day 672, alive</td>
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<td>AML</td>
<td>51</td>
<td>D’/R*</td>
<td>A0101, A0201, B0801, B4402</td>
<td>MUD/ PBSC</td>
<td>Fludarabine/Melphalan</td>
<td>Day 43 (36)</td>
<td>No GVHD</td>
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<td>D’/R*</td>
<td>A0101, A0301, B0801, B1402, A5201</td>
<td>Allo/PBSC</td>
<td>FTBI/VP16</td>
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<td>AML</td>
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<td>D’/R*</td>
<td>A0101, A0201, B0801, B5201</td>
<td>Allo/PBSC</td>
<td>Fludarabine/Melphalan</td>
<td>None</td>
<td>No GVHD</td>
<td>None</td>
<td>Day 99, died</td>
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<tr>
<td>171</td>
<td>preB-ALL</td>
<td>28</td>
<td>D’/R*</td>
<td>A0201, A0301, B0702, B4402</td>
<td>MUD/Allo</td>
<td>Fludarabine/Melphalan</td>
<td>None</td>
<td>Day 123, GVHD gut</td>
<td>None</td>
<td>Day 239, alive</td>
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**NOTE.** ALL, acute lymphoblastic leukemia; AML, acute myelogenous leukemia; BMT, bone marrow transplant; CML, chronic myelocytic leukemia; CMV, cytomegalovirus; CSA, cyclosporin; D, donor; FK506, tacrolimus; GCV, ganciclovir; GVHD, graft-vs-host disease; HD, Hodgkin disease; MDS, myelodysplastic syndrome; MMF or cellcept, mycophenolate mofetil; MUD, matched unrelated donor; PBSC, peripheral blood stem cells; pre-B-ALL, precursor B cell acute lymphoblastic leukemia; PSE, prednisone; R, recipient.

* Positive by polymerase chain reaction (PCR) or shell-vial plasma CMV culture assay. If 2 values are given, the first refers to shell-vial culture and the second to PCR.
the limited number of known immunodominant epitopes within individual CMV polypeptides; we dealt with this by testing each factor both with and without adjustment for the other factor.

RESULTS

Simultaneous reconstitution of immune responses to multiple CMV epitopes after HSCT. Samples from 7 HSCT recipients possessing combinations of the A2, A1, B7, and B8 alleles were evaluated by use of ICC assays. Data from 3 representative individuals are shown in figure 1. The individual designated UPN54 (figure 1A) had a ganciclovir-treated CMV reactivation episode during days 37–100 after transplant, as measured by serial plasma quantitative PCR (qPCR) and positive shell vial blood-culture assays (table 1). This patient’s PBMCs harbored significant (>2 × 10^6 cells/L of peripheral blood) populations of CD8^+ T lymphocytes specific for the A1 pp50_{245–253}, A2 pp65_{495–503}, and B8 IE-1_{88–96} epitopes that exhibited similar kinetics, simultaneously increasing from days 120 to 150 and declining somewhat by day 180 after transplant. This is, to our knowledge, the first report of immune responses to the A1 pp50_{245–253} and the B8 IE-1_{199–207} and IE-1_{88–96} CMV epitopes in HSCT recipients.

A second individual, UPN108, possessed populations of CD8^+ T lymphocytes specific for the A1 pp50_{245–253} and B8 IE-1_{199–207} epitopes (figure 1B). The kinetics of these cells were similar to those seen in patient UPN54, with a peak at day 150 after transplant and a decline thereafter. No CMV reactivation was detected in this individual’s samples, as assessed by serial qPCR or blood cultures. The third individual, patient UPN130, shown in figure 1C, had an immune response dominated by a very high level (day 150 after transplant, 6.5 × 10^7 cells/L; day 180 after transplant, 8 × 10^7 cells/L) of cells specific for the A2 pp65_{495–503} epitope, but, at day 90 after transplant, we also detected cells specific for the A1 pp50_{245–253} epitope, which were joined at days 150–180 by expanding populations of cells specific for both of the B^*08-restricted IE-1 epitopes. Thus, all 4 populations expanded 90–180 days after transplant. At this time point, when measured by ICC assay, >20% of this individual’s CD8^+ T lymphocytes were CMV specific. Chimerism analysis indicated that this individual was a full chimera at day 104 after transplant (data not shown). This patient had a prolonged CMV reactivation episode, as detected by both serial qPCR and blood cultures, at days 36 and 92. This reactivation, which was treated by ganciclovir, may have driven the expansion of these CMV-specific CD8^+ T lymphocytes to such remarkable levels. These results illustrate that immunocompromised HSCT recipients can simultaneously expand CD8^+ T lymphocyte populations that are specific for epitopes within multiple CMV antigens and that are restricted by several HLA alleles.

Higher functionality of CD8^+ T cells recognizing pp65 and pp50 epitopes, compared with CD8^+ T cells specific for IE-1, in CD107a/b mobilization/degranulation assays. The ICC assays described above indicated no obvious differences between the CMV-specific lymphocyte populations with respect to cytokine production, but we and others have observed a lack of concordance between cytotoxicity and cytokine production by CD8^+ T lymphocytes specific for the A2 pp65_{495–503} epitope [33, 35, 36]. We tested the hypothesis, which is based on linkage of degranulation and cytotoxic function [25, 28], that the CD107a mobilization assay might be able to detect functional and protective CMV-specific CD8^+ cell populations. We used a recently described degranulation assay [25] that measures the
transient expression of LAMP-1 (CD107a) and LAMP-2 (CD107b) on the surface of the CD8+ lymphocyte in response to stimulation with CMV antigenic peptides to test a panel of samples from 12 individuals: 6 healthy CMV-seropositive donors and 6 HSCT recipients for whom we had documented CMV-tetramer binding cells that made up at least 1% of CD8+ T lymphocytes. Figure 2A shows a representative example of the degranulation assay flow plots for donor UPN01. This donor had no clinical symptoms of CMV infection, and PCR data on CMV reactivation were not available. This donor’s PBMCs contained significant populations of cells specific for 4 CMV epitopes in 3 antigens: pp65, pp50, and IE-1 (figure 2B). As can be seen in figure 2C, in samples from this individual, >30% of the CD8+ T lymphocytes bound by the pp65 and pp50 tetramers degranulated on stimulation with the cognate peptide, but <9% and 12% of the cells bound by the IE-1 restricted epitopes mobilized CD107a/b in response to specific stimulation. Analogous results were found in the other individuals, and these are summarized in figure 2C and 2D.

The degranulation data for both healthy and immunocompromised subjects were combined and subjected to statistical analysis. The median percentage of the CD8+ tetramer-binding cells degranulating on specific stimulation was 26.0% for pp65-specific cells and 19.8% for pp50-specific cells. By contrast, only 5.6% of the IE-1-specific cells degranulated on stimulation with the cognate peptides. Degranulation of the IE-1-specific T cells was significantly lower than degranulation of combined pp65- and pp50-specific T cells (P = .003, F test adjusted for HLA effects because of restriction). Degranulation percentages of IE-1-specific T cells were markedly lower than those of pp65- and pp50-specific T cells in 8 of 10 subjects in whose samples IE-1-specific T cells were measured. In summary, the data from this degranulation assay indicate that, in both healthy individuals and HSCT recipients, significantly larger proportions of CD8+ T lymphocyte populations specific for pp65 or pp50 had lytic functionality, compared with cells in the same individuals that recognize the IE-1 polypeptide. A similar conclusion concerning differences in the functionality of pp65 or pp50 versus IE-1 T cells was found for multiple antigenic epitopes and HLA alleles and within both immunocompetent and immunocompromised individuals.

**Surface marker phenotypes of CMV-specific CD8+ T lymphocyte populations.** PBMCs from the subjects were stained with tetramer and anti-CD8 antibody and costained with a panel of antibodies to surface markers. The results (data not shown) indicated that the majority of the CD8+ T lymphocytes bound by the CMV IE, pp65, and pp50 tetramers that we used were CD45RA+, CD27+, CD62L+, and CCR7+, which indicates that the majority of the CMV-specific cells are differentiated effector memory cells, consistent with data from previous reports [37, 38]. We observed no consistent pattern among the cell-surface markers differentiating IE-1-specific from pp65- and pp50-specific CD8+ T lymphocytes, in agreement with the results of Khan et al. [18].

**DISCUSSION**

Our studies using ICC assays and MHC 1 tetramers confirm and extend the findings of Elkington et al. [24] to HSCT recipients. It is clear from these studies that CD8+ T lymphocyte-mediated immune responses to CMV in both healthy and immunocompromised individuals are directed toward a much wider range of antigens than has previously been appreciated. In a recent report, Manley et al. [39] described the use of a CMV deletion mutant lacking 4 immunoevasins to infect fibroblasts for use in ICC assays of PBMCs from healthy CMV-seropositive donors. Their data support the concept of broad immune responses to CMV antigens other than pp65 and IE-1 in healthy individuals, which we have extended to HSCT recipients. To guide vaccine development, it will be important to determine which of these antigenic responses are involved in control or protection against viral reactivation and disease.

We addressed this issue by examining the degranulation of CMV-specific T cells in response to specific stimulation. Degranulation is an initial event in the process of target cell lysis and is thus a measure of cytotoxic potential. The researchers who developed this assay presented data from flow cytometry–based killing assays to show that antigen-specific CD8+ T lymphocytes that degranulate directly mediate cytotoxicity [25]. Our results with the degranulation assay suggest that, irrespective of the specific epitope within the pp65 or pp50 polypeptide (4 examined) or the restricting HLA allele (A*01, A*02, or B*07), a significant percentage (12%–32%) of CD8+ T lymphocytes recognizing the CMV pp65 and pp50 antigens are potentially functional cytotoxic effectors. By contrast, a smaller proportion (0.3%–11%) of CD8+ T lymphocytes recognizing antigenic epitopes within the IE-1 polypeptide were able to degranulate on specific stimulation. These apparently noncytotoxic IE-1-specific T cells make up a significant proportion of the detected CMV-specific cells in many individuals, and, in some people, they match or outnumber the combined pp50- and pp65-specific cells. Our finding of large numbers of IE-1-specific CD8+ T cells with reduced functional potential is in accord with the observation by Cobbold et al. in P.A. Moss’s laboratory [40] of large expansions of IE-1 CTLs after HSCT that were unable to lyse CMV-infected fibroblasts in vitro. Rubio et al. [28], using the CD107a/b degranulation assay to characterize melanoma-specific T cells, reported that, in samples from peptide-vaccinated patients, only 10%–20% of melanoma-specific tetramer-binding cells degranulated in response to melanoma antigen—a much lower proportion than that of cells labeled with CMV pp65res-50 tetramer and stimulated with the cognate peptide.
Figure 2. Impaired degranulation of immediate early (IE)-1–specific CD8+ T lymphocytes vs. pp65- and pp50-specific CD8+ T lymphocytes in response to specific peptide stimulation. Degranulation and tetramer binding data are shown for 6 healthy cytomegalovirus (CMV)–seropositive donors and 6 hematopoietic stem-cell transplant recipients tested with 7 CMV-specific tetramers. A, Representative flow data obtained with cryopreserved peripheral blood mononuclear cells from a healthy seropositive donor (UPN01). The cells were labeled separately with 2 CMV-specific tetramers and then stimulated in the presence of costimulatory peptides for 5 h, including monensin for the last 4 h. Fluorescein isothiocyanate–conjugated antibodies to CD107a and to CD107b detected transient exposure of these degranulation markers on the cell surface. At the end of incubation, cells were washed and labeled with fluorescently conjugated antibodies to CD8 before flow analysis. B, Levels of CD8+ T lymphocytes for each subject, bound by these tetramers as percentages of all CD8+ T lymphocytes. C, Percentages of these tetramer-binding cells for each subject that displayed increased CD107a/b on the surface after cognate peptide stimulation, compared with control samples. Black symbols, CMV IE-1–specific cells; white symbols, populations recognizing pp50/pp65. The individual HLA alleles restricting the epitopes are represented by different geometric shapes: squares, HLA-A*01; circles, HLA-A*02; triangles, HLA-B*07; and diamonds, HLA-B*08. APC, allophycocyanin; NA, no CMV cellular epitopes restricted by the corresponding HLA allele were available for study; PE, phycoerythrin.

High levels of IE-1–specific T cells indicate that the antigenic presentation of IE-1 peptides has occurred efficiently enough to drive a vigorous expansion of populations to levels that match or exceed those of populations specific for pp65 and pp50. However, the lower levels and/or slower expansion of IE-1–specific CD8+ T lymphocytes, compared with those of lymphocytes specific for pp65 and pp50—which were seen in all 3 of the HSCT recipients shown in figure 1—combined with the observed less-functional phenotype of IE-1–specific cells, suggests partial impairment of IE-1–specific responses. This IE-1–specific difference could be explained by the effect of different in vivo host cells on the effectiveness of viral immunoevasion.
strategies [41, 42]. How this might happen has been shown by in vitro experiments that have reported the escape from inhibition of IE-1 peptide presentation by macrophages [43], compared with the tight control of IE-1 antigen presentation in fibroblasts [44]. In this scenario, historical CMV replication in a cellular environment where IE presentation was able to escape the activity of viral immunoevasins could have led to the expansion of CTL precursors specific for IE, as well as for other CMV antigens, that were not fully functional for degradation and cytotoxicity. Subsequent reactivation of virus in tissues where, as is the case with fibroblasts [44], immunoevasins prevented efficient presentation of IE-1 peptides could lead to preferential in vivo restimulation of pp65 and pp50, rather than IE-specific CTLs, and maturation to a functional cytotoxic phenotype. It is even possible that the IE-specific effect in our patients could have been an effect of graft-versus-host disease or its treatment or of some aspect of the conditioning regimens, but this does not explain the occurrence of the effect in the healthy donors.

These findings underline the caution required when the clinical impact of the presence of large numbers of CMV-specific T cells with different levels of cytotoxic functionality in patients is interpreted. If protection against CMV reactivation and/or disease is associated with these functional subsets of CMV-specific T cells, then the implication of our data is that IE-1–specific T cells may be less valuable than pp65- and pp50-specific populations. The CD107 mobilization/degranulation assay, in combination with tetramer staining, thus offers the potential for identifying and monitoring the levels of the functional and, hence, possibly protective subsets of CMV antigen–specific T cells in patients. We are currently engaged in a longitudinal study of HSCT and solid-organ transplant recipients, to evaluate the functional phenotypes and frequencies of T cell populations specific for epitopes from within multiple CMV antigens.

Acknowledgments

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References


