Functional Comparison of T Cells Recognizing Cytomegalovirus pp65 and Intermediate-Early Antigen Polypeptides in Hematopoietic Stem-Cell Transplant and Solid Organ Transplant Recipients


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The functional status of cytotoxic T lymphocyte (CTL) populations recognizing cytomegalovirus intermediate-early antigen (IE1) and pp65 polypeptides was investigated in peripheral blood mononuclear cells from hematopoietic stem-cell transplant (HSCT) and solid organ transplant recipients. Combined flow-based CD107a/b degranulation/mobilization and intracellular cytokine (ICC) assays using peptide libraries as antigens indicated that a significantly higher proportion of pp65-specific CTLs were in a more mature functional state, compared with IE1-specific CTLs. Degranulation/multiple cytokine ICC assays also indicated that a significantly higher proportion of pp65-specific than IE1-specific CTLs secreted both interferon-γ and tumor necrosis factor-α and possessed greater cytotoxic potential. These results support our earlier findings of functional differences between CTLs recognizing individual epitopes within the IE1 and pp65 antigens in healthy donors and HSCT recipients and extend them to a broader array of human leukocyte antigen–restricted responses to those antigens. We also provide evidence of a relationship between cytotoxic function and the ability of cytomegalovirus-specific CTLs to secrete multiple cytokines.

Reconstitution of adaptive T cell responses to human cytomegalovirus (CMV) is critical to protection from CMV disease after hematopoietic stem-cell transplantation (HSCT) [1–5]. Similarly, studies of immunity in solid organ transplant (SOT) recipients have shown an association between CMV immune responses and risk of CMV disease [6–8]. However, there is an incomplete understanding of which CMV antigens and epitopes are most crucial to providing protective responses. Two well-studied CMV antigens—the pp65 tegument polypeptide and the major immediate-early antigen (IE1)—are immunodominant and, thus, are recognized by >50% of CMV-positive adults, with similar levels of CD8+ T cell memory responses for both [9]. Prior work has suggested that pp65-specific [2] and IE1-specific [10] CTLs have a protective function against viremia in transplant recipients, although the precise mechanism is unknown.

Using a panel of major histocompatibility complex (MHC) class I tetramers recognizing epitopes within the CMV IE1, pp65, and pp50 polypeptides, we previously reported that CD8+ T cells specific for IE1 in peripheral blood mononuclear cells (PBMCs) from
healthy donors and HSCT recipients exhibited reduced degranulation of cytotoxic granules, compared with T cells specific for the pp65 and pp50 polypeptides [11]. CD8+ T cells stained by these tetramers were evaluated for their ability to mobilize the lysosome-associated membrane proteins LAMP-1 (CD107a) and LAMP-2 (CD107b) found within cytotoxic granules in response to antigenic peptide stimulation. This CD107 degranulation/mobilization assay was described by Betts et al. [12, 13] and Wolint et al. [14] and has been used by other researchers to evaluate the functionality of NK cells [15] and of T cells specific for poxvirus [16], CMV [11–13], BK virus [17], lymphocytic choriomeningitis virus (LCMV) [14], and melanoma [18].

Our previous finding of differing levels of degranulation between CMV IE1- and pp65/pp50-specific T cells [11] was complicated by the possibility that differences were epitope and/or HLA specific. We generalized the approach by substituting tetramers with intracellular cytokine (ICC) assays using pools of overlapping peptides spanning the full-length pp65 and IE1 open reading frames as stimulatory antigens [19]. The advantages of this approach include no required knowledge of specific T cell epitopes and no HLA typing of research subjects. This modified assay was used to investigate the comparative degranulation levels of IE1- and pp65-specific CD8+ T cells in 2 cohorts of immunocompromised subjects—HSCT and SOT recipients—and to shed light on the controversy about the function, role, and relative importance of CTLs specific for either antigen. Apart from cytotoxicity, a second function of CTLs is the secretion of cytokines, chemokines, and other soluble factors that contribute to the control of viral replication [20]. The ability to secrete multiple cytokines is associated with differentiated effector memory CTLs rather than naive T cells, which implies an association with a more-robust protective function [21, 22]. In this context, a report [23] that a subset of HIV-1–specific CTLs capable of producing both interferon (IFN)–γ and tumor necrosis factor (TNF)–α was associated with improved cytotoxic activity prompted us to investigate whether degranulation, a functional correlate of cytotoxicity, was positively associated with dual cytokine production and whether degranulation, a functional correlate of cytotoxicity, with improved cytotoxic activity prompted us to investigate the comparative degranulation/mobilization levels of IE1- and pp65-specific CD8+ T cells in 2 cohorts of immunocompromised subjects—HSCT and SOT recipients—and to shed light on the controversy about the function, role, and relative importance of CTLs specific for either antigen. Apart from cytotoxicity, a second function of CTLs is the secretion of cytokines, chemokines, and other soluble factors that contribute to the control of viral replication [20]. The ability to secrete multiple cytokines is associated with differentiated effector memory CTLs rather than naive T cells, which implies an association with a more-robust protective function [21, 22]. In this context, a report [23] that a subset of HIV-1–specific CTLs capable of producing both interferon (IFN)–γ and tumor necrosis factor (TNF)–α was associated with improved cytotoxic activity prompted us to investigate whether degranulation, a functional correlate of cytotoxicity, was positively associated with dual cytokine production and predicted differences between IE1 and pp65-specific CD8+ T cells.

SUBJECTS, MATERIALS, AND METHODS

HSCT transplant recipients. PBMCs were collected at intervals (40, 90, 120, 150, 180, and 360 days after transplant) from 17 HSCT recipients at the City of Hope (COH) Comprehensive Cancer Center. Patient demographics for the HSCT cohort are shown in table 1. The study protocols were approved by the institutional review board at COH, and specimens and data were obtained prospectively after informed consent was obtained from subjects. HLA typing was performed by polymerase chain reaction, as described elsewhere [24]. None of these subjects developed clinical CMV disease during the 360 days after transplant.

Liver-transplant recipients. SOT recipients in the present study (table 2) were from a cohort of 26 patients enrolled between January 2004 and March 2005 in a longitudinal collaborative study between University of Washington Medical Center (UWMC) and COH. The study protocols were approved by institutional review boards at COH and UWMC, and specimens and data were obtained prospectively after informed consent was obtained from subjects. Multiple blood specimens were collected 3–12 months after orthotopic liver transplant (OLT) for unique patient numbers (UPNs) 1–20 and at a single time point for UPNs 21–26. Briefly, all CMV-negative recipients (R−) of an OLT from a CMV-positive donor (D+) received a 3-month CMV prophylaxis regimen with valganciclovir (Valcyte; Roche) and were treated with intravenous ganciclovir (Cytovene; Roche) or valganciclovir for CMV disease [25].

Immunological evaluations of HSCT and SOT recipients. The inclusion criterion for this analysis was that each of the 10 SOT and 17 HSCT recipients had, at 1 time point, a measurable CD8+ IFN-γ response to pp65 and/or IE PepMix libraries (JPT Peptide Technologies), which we defined as a level of ≥0.2% after background subtraction. CMV-specific CD107 mobilization/degranulation [11] and CD4+ IFN-γ–positive activity [26] were evaluated using pp65 and IE1 PepMix libraries (or diluent as the background control) in 12-h stimulations. There were no other selection or exclusion criteria other than sample availability.

All peptide stimulations were initiated in the absence of protein transport inhibitors and with fluorescein isothiocyanate–conjugated antibodies to CD107a and CD107b present. After 1 h in culture, monensin (GolgiStop; Becton Dickinson Biosciences) was added, and incubation continued for an additional 11 h. For the experiments that measured TNF-α secretion in addition to IFN-γ secretion and CD107a/b mobilization, both monensin and brefeldin A (GolgiPlug; Becton Dickinson Biosciences) were added. After the stimulation step, the cells were washed, labeled with phycoerythrin (PE)–conjugated anti-CD8 antibody, fixed, and permeabilized (Cytofix-Cytoperm; Becton Dickinson Biosciences) before they were labeled with allophycocyanin (APC)–conjugated antibody to IFN-γ and, in some experiments, PE-Cy7–conjugated antibody to TNF-α. All antibodies were from Pharmingen. The stained cells were analyzed on a FACSCanto flow cytometer (Becton Dickinson Biosciences), and data were analyzed using FCS Express (version 3.0; DeNovo Software).

Statistical analysis. IFN-γ and degranulation percentages were compared using the Wilcoxon signed-rank test. For SOT recipients, repeated measurements were reduced to a single
### Table 1. Demographics of cohort 1 (17 hematopoietic stem-cell transplant [HSCT] recipients).

<table>
<thead>
<tr>
<th>UPN</th>
<th>Diagnosis</th>
<th>Age, years</th>
<th>CMV serostatus</th>
<th>Transplant type</th>
<th>Transplant regimen</th>
<th>CMV reactivation after HSCT, day</th>
<th>Date of onset of GVHD grade &gt;2, day</th>
<th>Medication with dosages &gt;1 mg/kg</th>
<th>Survival status at day 360</th>
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<tbody>
<tr>
<td>99</td>
<td>AML</td>
<td>38</td>
<td>D+/R⁺</td>
<td>Allogeneic</td>
<td>Busulfan/FTBI/VP-16</td>
<td>21</td>
<td>13</td>
<td>PSE, MMF, CSA</td>
<td>Alive</td>
</tr>
<tr>
<td>125</td>
<td>AML</td>
<td>60</td>
<td>D+/R⁺</td>
<td>MUD</td>
<td>Fludarabine/melphalan</td>
<td>48</td>
<td>24</td>
<td>PSE, MMF, CSA, daclizumab</td>
<td>Alive</td>
</tr>
<tr>
<td>136</td>
<td>ALL</td>
<td>50</td>
<td>D+/R⁺</td>
<td>Allogeneic</td>
<td>FTBI/VP-16</td>
<td>55</td>
<td>None</td>
<td>None</td>
<td>Alive; relapsed day 205 after HSCT</td>
</tr>
<tr>
<td>139</td>
<td>CML</td>
<td>45</td>
<td>D+/R⁺</td>
<td>Allogeneic</td>
<td>Busulfan/cytoxan</td>
<td>36</td>
<td>23</td>
<td>PSE</td>
<td>Alive</td>
</tr>
<tr>
<td>146</td>
<td>AML</td>
<td>63</td>
<td>D+/R⁺</td>
<td>Allogeneic</td>
<td>Fludarabine/melphalan</td>
<td>37</td>
<td>105</td>
<td>CSA</td>
<td>Alive</td>
</tr>
<tr>
<td>161</td>
<td>ALL</td>
<td>43</td>
<td>D+/R⁺</td>
<td>Allogeneic</td>
<td>FTBI/VP-16</td>
<td>51</td>
<td>148</td>
<td>CSA</td>
<td>Alive; relapsed day 337 after HSCT</td>
</tr>
<tr>
<td>165</td>
<td>MDS</td>
<td>45</td>
<td>D+/R⁺</td>
<td>Allogeneic</td>
<td>Busulfan/cytoxan</td>
<td>61</td>
<td>None</td>
<td>None</td>
<td>Alive</td>
</tr>
<tr>
<td>187</td>
<td>MDS/AML</td>
<td>37</td>
<td>D+/R⁺</td>
<td>MUD</td>
<td>Fludarabine/melphalan</td>
<td>197</td>
<td>148</td>
<td>MMF, CSA, PSE</td>
<td>Alive</td>
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<tr>
<td>207</td>
<td>ALL (PH+)</td>
<td>57</td>
<td>D+/R⁺</td>
<td>Allogeneic</td>
<td>Fludarabine/melphalan</td>
<td>None</td>
<td>322</td>
<td>CSA</td>
<td>Alive</td>
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<tr>
<td>212</td>
<td>AA</td>
<td>26</td>
<td>D+/R⁺</td>
<td>Allogeneic</td>
<td>Cytoxan</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>Alive</td>
</tr>
<tr>
<td>220</td>
<td>NHL</td>
<td>43</td>
<td>D+/R⁺</td>
<td>MUD</td>
<td>Fludarabine/melphalan</td>
<td>44</td>
<td>83</td>
<td>MMF</td>
<td>Alive; relapsed day 182 after HSCT</td>
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<tr>
<td>234</td>
<td>AA</td>
<td>40</td>
<td>D+/R⁺</td>
<td>Allogeneic</td>
<td>Cytoxan/ATG</td>
<td>37</td>
<td>38</td>
<td>PSE, CSA</td>
<td>Alive</td>
</tr>
<tr>
<td>251</td>
<td>CML</td>
<td>63</td>
<td>D+/R⁺</td>
<td>Allogeneic</td>
<td>Fludarabine/melphalan</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>Alive</td>
</tr>
<tr>
<td>252</td>
<td>NHL</td>
<td>33</td>
<td>D+/R⁺</td>
<td>Allogeneic</td>
<td>Fludarabine/melphalan</td>
<td>123</td>
<td>311</td>
<td>MMF, PSE</td>
<td>Alive; relapsed day 237 after HSCT; secondary lymphoma day 246 after HSCT</td>
</tr>
<tr>
<td>259</td>
<td>AML</td>
<td>59</td>
<td>D+/R⁺</td>
<td>Allogeneic</td>
<td>Fludarabine/melphalan</td>
<td>None</td>
<td>117</td>
<td>MMF</td>
<td>Alive at day 194 after HSCT</td>
</tr>
<tr>
<td>260</td>
<td>NHL</td>
<td>36</td>
<td>D+/R⁺</td>
<td>Allogeneic</td>
<td>FTBI/cytoxan</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>Alive</td>
</tr>
<tr>
<td>272</td>
<td>AML</td>
<td>26</td>
<td>D+/R⁺</td>
<td>MUD</td>
<td>Fludarabine/cytoxan</td>
<td>44</td>
<td>None</td>
<td>PSE, MMF</td>
<td>Alive; renal insufficiency day 261 after HSCT; congestive heart failure day 267 after HSCT</td>
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</table>

**NOTE.** +, positive; −, negative; AA, severe aplastic anemia; ALL, acute lymphocytic leukemia; ALL (PH+), ALL and Philadelphia chromosome positive; AML, acute myelogenous leukemia; ATG, anti-thymocyte globulin; CML, chronic myelocytic leukemia; CMV, cytomegalovirus; CSA, cyclosporin; D, donor; GVHD, graft-vs.-host disease; FTBI, fractionated total body irradiation; MDS, myelodysplastic syndrome; MMF, mycophenolate mofetil; MUD, matched unrelated donor; NHL, non-Hodgkin lymphoma; PSE, prednisone; R, recipient; UPN, unique patient no.; VP-16, etoposide.
mean measurement for each individual before testing, so that all observations were stochastically independent.

RESULTS

**IFN-γ and CD107 monitoring of CMV-specific T cells in HSCT recipients.** We used a combined flow-based ICC/degranulation method with PepMix as the antigen to measure both IFN-γ expression and CD107 mobilization in T cells specific for pp65 and IE1 in frozen PBMC samples from 17 HSCT recipients (table 1). PepMixes are composed of a set of overlapping 15-mer peptides spanning the length of the antigen, and they are designed to be used together in a single tube assay for readout by flow-based analytical methods. We and others have successfully evaluated such cryopreserved PBMCs for their cytokine production and have found little difference from results with fresh material [11, 19, 27–29]. Figure 1A shows representative ICC/degranulation flow histograms from HSCT subject 136. The histograms in the middle row show PBMCs stimulated with a pp65 PepMix, and the bottom row shows an aliquot stimulated with an IE1 PepMix. The top row shows a mock stimulation with costimulatory antibodies but no peptides. This individual, like most of the other HSCT recipients (figure 2B), produced IFN-γ in response to both pp65 and IE1 antigens. Events in the upper right quadrants of the left column of plots in figure 1 show CD8+ T cells labeled with an APC-conjugated antibody to IFN-γ. The right-hand column of plots in figure 1, gated on the CD8+ and IFN-γ+ lymphocytes, illustrates the levels of CD107a/b on the surface of IE1- and pp65-specific CTLs. A higher proportion (~44%) of pp65-specific than IE1-specific (~5%) CD8+ T cells degranulated in response to peptide stimulation.

Figure 2 summarizes the ICC/degranulation data from samples collected between days 120 and 180 after transplant from all 17 HSCT recipients. This time period was chosen because IE-specific T cells are less frequently detected before day 120 (figure 3). The dot plots in figure 3A and 3B indicate the proportions of CD4+ and CD8+ T cells secreting IFN-γ in response to stimulation with IE1 and pp65 PepMixes. A significantly higher proportion of CD4+ T cells in samples from this cohort recognized pp65, compared with IE1 (P = .007734). The lower frequencies of IE1-specific CD4+ T cells is similar to data regarding both healthy adults [9] and SOT recipients [10], which suggests its generality among all adults, regardless of their degree of immunocompetence. This observation may reflect the 120–180 day posttransplant time period during which these samples were obtained. There was no significant difference between the percentages of CD8+ T cells recognizing pp65 and those recognizing IE1. As has been reported elsewhere [30] and illustrated in figure 3A in a much greater number of patients, CD8+ T cell immune responses to IE reconstitute more slowly after HSCT than CD8+ T cell immune responses to pp65.

Degranulation analyses were performed on samples containing populations that constituted >0.2% of either IE1- or pp65-specific CD8+ T cells. This cutoff was chosen to allow flow acquisition of sufficient antigen-specific cells for analysis. Figure 2C indicates the proportion of the CD8+ antigen-specific T cells that also mobilized CD107a/b. Fourteen of 17 subjects had a CD8+ T cell response to pp65, whereas 9 had a CD8+ T cell response to IE1; thus, 6 patients harbored CD8+ T cell populations specific for each of these 2 CMV antigens. The most notable feature of these results was the difference between the mean percentages of IE1-specific (20.88%) and pp65-specific

Table 2. Demographic information for the liver-transplant cohort.

| UPN | Age, years | HLA type | Reason for OLT | D/R CMV serostatus | CMV PCR
<table>
<thead>
<tr>
<th></th>
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</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10</td>
<td>A01, A02, B07</td>
<td>Liver cirrhosis</td>
<td>D+/R-</td>
<td>Negative</td>
</tr>
<tr>
<td>2</td>
<td>15</td>
<td>A02, A03</td>
<td>Liver cirrhosis</td>
<td>D+/R-</td>
<td>Negative</td>
</tr>
<tr>
<td>3</td>
<td>20</td>
<td>A03, A02, B07</td>
<td>Liver cirrhosis</td>
<td>D+/R-</td>
<td>Negative</td>
</tr>
<tr>
<td>4</td>
<td>25</td>
<td>A02, A03</td>
<td>Liver cirrhosis</td>
<td>D+/R-</td>
<td>Negative</td>
</tr>
<tr>
<td>5</td>
<td>30</td>
<td>A02, A03</td>
<td>Liver cirrhosis</td>
<td>D+/R-</td>
<td>Negative</td>
</tr>
<tr>
<td>6</td>
<td>35</td>
<td>A02, A03</td>
<td>Liver cirrhosis</td>
<td>D+/R-</td>
<td>Negative</td>
</tr>
<tr>
<td>7</td>
<td>40</td>
<td>A02, A03</td>
<td>Liver cirrhosis</td>
<td>D+/R-</td>
<td>Negative</td>
</tr>
<tr>
<td>8</td>
<td>45</td>
<td>A02, A03</td>
<td>Liver cirrhosis</td>
<td>D+/R-</td>
<td>Negative</td>
</tr>
<tr>
<td>9</td>
<td>50</td>
<td>A02, A03</td>
<td>Liver cirrhosis</td>
<td>D+/R-</td>
<td>Negative</td>
</tr>
<tr>
<td>10</td>
<td>55</td>
<td>A02, A03</td>
<td>Liver cirrhosis</td>
<td>D+/R-</td>
<td>Negative</td>
</tr>
<tr>
<td>11</td>
<td>60</td>
<td>A02, A03</td>
<td>Liver cirrhosis</td>
<td>D+/R-</td>
<td>Negative</td>
</tr>
<tr>
<td>12</td>
<td>65</td>
<td>A02, A03</td>
<td>Liver cirrhosis</td>
<td>D+/R-</td>
<td>Negative</td>
</tr>
</tbody>
</table>

NOTE. +, positive; −, negative; CMV, cytomegalovirus; D, donor; ETOH, alcoholic cirrhosis; HCV, hepatitis C; IC, idiopathic/cryptogenic autoimmune chronic active hepatitis; NASH, nonalcoholic steatohepatitis; NT, not tested; OLT, orthotopic liver transplant; PBC, primary biliary cirrhosis; PCR, polymerase chain reaction; R, recipient; UPN, unique patient no.

* Measurement of >10^6 copies/mL detected at least at 1 time point.

a Total of 5.5 months of prophylaxis received; the patient died at 11 months after OLT for transplant related lymphoma.
Figure 1. Intracellular cytokine (ICC) assay and degranulation plots. Representative flow plots show combined PepMix ICC/degranulation assays performed on peripheral blood mononuclear cell (PBMC) samples obtained from a hematopoietic stem-cell transplant recipient (unique patient no. [UPN] 136) at 180 days after transplant (A) and from solid organ transplant recipient UPN 15, 12 months after orthotopic liver transplant (B). All plots were primary-gated on lymphocytes by forward and side scatter, and the right column of plots was additionally gated on interferon (IFN)–γ–producing (+) CD8+ T cells (left, upper right quadrants). For both panels, the stimulation performed using pp65, immediate-early antigen (IE1) libraries, or diluent (mock, indicated at left). The values in the left column of plots indicate the IFN-γ T cells as percentage of the CD8+ lymphocytes. The values in the right column of plots indicate the CD107a/b-positive and -negative cells as the percentage of CD8+ IFN-γ T cells. Phycoerythrin-conjugated antibodies to CD8, allophycocyanin-conjugated antibodies to IFN-γ, and fluorescein isothiocyanate–conjugated antibodies to CD107a and CD107b were used in the flow-cytometric analyses.
Figure 2. Summary of intracellular cytokine (ICC) assay/degranulation data of the type shown in figure 1 for 17 hematopoietic stem-cell transplant (HSCT) recipients. Samples shown in this figure were collected at 120–180 days after transplant, as indicated below panel C. A, Levels of CD4+ T cells producing interferon (IFN)–γ on stimulation with pp65 PepMix (white circles) and immediate-early antigen (IE1) PepMix (black triangles). B, Levels of CD8+ T cells producing IFN-γ on stimulation with pp65 PepMix (white circles) and IE1 PepMix (black triangles). C, Proportion of these CD8+ IFN-γ–producing T cells mobilizing CD107a/b.

(43.86%) CD8+ T cells that degranulated in response to PepMix stimulation (P < .001, Wilcoxon rank sum test). This finding is consistent with our previous study that used a tetramer-based degranulation assay to investigate a panel of 6 healthy donors and 6 HSCT recipients [11]. In our earlier study, the median percentage of degranulation for pp65-specific T cells was 26%, and that of IE-specific T cells was 5.6% (P = .003, Wilcoxon signed-rank test). The present findings not only confirm our earlier results but also strongly indicate that these antigen-specific differences are not HLA specific or an epitope-specific artifact.

We were interested in determining whether these functional differences between T cells specific for pp65 and IE1 might decrease as the immune system of the recipients reconstituted. We used pp65 and IE1 PepMixes as antigens in ICC assays to track CMV-specific T cell immunity in a cohort of HSCT recipients between 40 and 360 days after transplant. Figure 3A shows the mean levels of IE1- and pp65-specific CD8+ T cells in PBMC samples obtained from 67 (IE1) and 81 (pp65) subjects at days 40 and 180 after transplant. Levels of pp65-specific CD8+ T cells were higher than those of IE1-specific cells at day 40 (P = .017), but IE-1 responses had been reconstituted significantly by day 180 (P = .0022). Figure 3B illustrates the tracking of IE1- and pp65-specific CD8+ T cell reconstitution in 1 subject from this cohort (UPN 187) during the first year after HSCT. Levels of IE1-specific T cells were low or undetectable between days 40 and 180 and then expanded by 1 year after transplant to a level roughly equal to that of pp65-specific CD8+ T cells, which showed a modest decrease between days 120 and 360 (figure 3B). We measured degranulation using the
Figure 3. Evaluation of the levels and functionality of pp65 and immediate-early antigen (IE1)–specific T cells in samples from hematopoietic stem-cell transplant (HSCT) recipients. A, Summary of mean levels of antigen-specific CD8+ T cells present in peripheral blood mononuclear cells (PBMCs) from a cohort of HSCT recipients at the City of Hope. Intracellular cytokine assays using pp65 and IE1 PepMixes as antigens were performed on 81 (pp65) and 67 (IE1) PBMC samples collected at 40 and 180 days after transplant. The significance of differences between the means of observations with different antigens and at different time points was calculated using Student’s t-test. B, Evaluation of the levels of pp65 (diamonds) and IE1 (squares)–specific CD8+ T cells in serial PBMC samples from HSCT recipient 187 obtained between 40 and 360 days after transplant. C, Functionality as measured by CD107 mobilization/degranulation assay of the pp65- and IE1-specific–CD8+ T cells from HSCT recipient 187 shown in panel B. CD107a/b mobilization/degranulation values for IE1-specific T cells were not obtained on days 40, 90, 120, and 150 because of the low no. of these cell populations, making the analysis infeasible to conduct. These points are accordingly marked unevaluable (UE). IFN, interferon.
CD107 assay of pp65- and IE-specific CD8+ T cells between days 40 and 360 after transplant in several such patients. Despite the increase in the frequency of IE1-specific T-cells, their percentage of degranulation at both day 180 and 360 was lower than that of pp65-specific T-cells (figure 3C). The degranulation of pp65-specific T cells increased from 23% to 42% between days 120 and 360 after stimulation with pp65 PepMix. There were minimal differences in the degranulation of IE1-specific CD8+ T cells between days 180 and 360, despite an increase in the frequency of IE1 T cells that expressed IFN-γ. These results suggest that the reconstitution of the immune response does not change the magnitude of difference in percentages of degranulation between both IE1 and pp65 antigen-specific populations.

**Immune function of CMV-specific CD4+ and CD8+ T cells in SOT recipients.** We tested whether differences in the degranulation of IE1- and pp65-specific T cells would also be found in D+R/H SOT recipients. Ten SOT recipients (table 2) were analyzed for IFN-γ production in CD4+ and CD8+ T cells in response to antigenic stimulation with pp65 and IE1 PepMixes between 3 and 12 months after OLT. Of these 10 SOT recipients, 2 (UPNs 3 and 14) had tissue-invasive CMV disease, 3 (UPNs 8, 9, and 15) had CMV syndrome with pyrexial debilitating illness [31], and the remaining 5 had no CMV disease. Analysis of IFN-γ production by CD4+ T cells (figure 4A) indicated percentages of IE1-specific T cells (range, 0%-0.16%) that were markedly lower than those of pp65-specific T cells (range, 0.05%-0.7%). This difference was significant (P = .002), which confirmed earlier findings [9, 32] and our results in HSCT recipients (figure 2). In all 10 patients, a brisk IFN-γ response to the pp65 PepMix was detected in CD8+ T cells (range, 0.2%-11.7%) at ≥1 time point. In UPNs 8, 15, and 20, levels of pp65-specific IFN-γ-producing CD8+ T cells remained constant throughout the 9-month observation period (figure 4B). However, in UPN 3, levels of these cells decreased at 1 year after transplant; and, in UPN 4, levels showed substantial variations during 4.5–5.5 months after transplant (figure 4B). One-half of the recipients had considerable recognition of the IE1 PepMix, as assessed by the percentages of CD8+ T cells producing IFN-γ (range, 0.9%-18.3%); whereas, for UPNs

![Figure 4](description of figure 4)
12, 22, and 23, low IFN-γ levels in IE1-specific T cells were detected, and UPNs 8 and 9 had no activity (figure 4B). No time-dependent decrease was observed for levels of IE1-specific CD8+ T cells after transplant. Differences in the IFN-γ response of CD8+ T cells to the pp65 and IE1 PepMix did not reach statistical significance.

We observed striking differences in the degree of mobilization of CD107 membrane proteins to the surface of IE1- and pp65-specific CD8+ T cells after stimulation with cognate PepMix reagents. A considerably higher proportion of pp65-specific than IE1-specific CD8+ T cells degranulated (P = .008) in response to stimulation with the corresponding PepMix (figures 1 and 4C). For recipients tested at multiple time points, no significant variation in degranulation levels was found between different time points after OLT (figure 4C). The results obtained with both SOT and HSCT recipients confirms the existence of profound differences in cytotoxic potential between IE1- and pp65-specific CD8+ T cell populations, which provides further support for the generalizability of this observation.

**Cytokine production profiles of CMV and IE1-specific CD8+ T cells in HSCT recipients.** It was reported elsewhere [23] that superior cytotoxic functionality, as measured in a flow-based assay of populations of CD8+ T cells specific for HIV-1, was associated with the ability of these T cells to secrete both TNF-α and IFN-γ. We hypothesized that differences in the cytotoxic potential of IE1- and pp65-specific CD8+ T cells might be associated with the ability to secrete both Th1 cytokines. We analyzed PBMCs collected at days 120–180 after transplant from 9 of the HSCT recipients listed in table 1, using a combined ICC/degranulation assay that simultaneously measured production of the cytokines IFN-γ and TNF-α and detected the mobilization of CD107. A multigate flow analysis was performed that examined all combinations of CD107 mobilization with production of these 2 cytokines. A primary gate was placed on lymphocytes as assessed by forward and side scatter. A secondary gate was placed on CD8+ T cells secreting IFN-γ in response to CMV IE1 or pp65 PepMix stimulation, and the cells in this secondary gate were assessed for TNF-α production and for mobilization of CD107. This gating strategy does not formally exclude CD8dim NK cells, but such cells were considered to be unlikely to respond to peptide stimulation.

The results for each group of subjects are summarized as the average percentages of CD8+ T cells exhibiting each combination of phenotypes in the bar charts on the right of figure 5A and B. Seven subjects had comparable CMV antigen-specific CD8+ T cell cytokine expression profiles (designated “cytokine profile 1”), with easily detected populations of T cells secreting both IFN-γ and TNF-α in response to stimulation with IE1 and/or pp65 PepMix and statistical significance in 2 important comparisons (figure 5A). A higher proportion of pp65-specific, compared with IE1-specific, T cells were present in the CD107+ subset that produces IFN-γ but not TNF-α (P = .015), the CD107− subset that produces IFN-γ but not TNF-α (P = .049), and most significantly, in the trifunctional population CD107+ subset that produces both IFN-γ and TNF-α (P = .012). The remaining 2 subjects had a distinctly different cytokine secretion profile (cytokine profile 2) and were analyzed separately (figure 5B). In these 2 subjects, pp65-specific CD8+ T cells produced either IFN-γ or TNF-α but not both. The IE1-specific cells in these 2 subjects included a large TNF-α–positive population that neither produced IFN-γ nor mobilized CD107, which had no counterpart in PBMCs from the group with cytokine profile 1. Because there were only 2 individuals exhibiting cytokine profile 2, it was not possible to ascribe statistical significance to observations about these samples, but it was observed that very few of the IE1-specific CD8+ T cells mobilized CD107, and the majority of the IE1-specific cells were either CD107− cells that produced IFN-γ but not TNF-α or CD107+ cells that produced TNF-α but not IFN-γ. In summary, 9 individuals represented in figure 5A and 5B had profiles consistent with a smaller proportion of degranulating IE1-specific, compared with pp65-specific, CD8+ T cells.

**DISCUSSION**

The present article both confirms and extends previous results regarding a panel of 8 MHC class I tetramers from healthy subjects and HSCT recipients [11]. The PepMix antigen ICC assay used in the present study examines a broader HLA-restricted response to pp65 and IE1, which minimizes the individual contribution of single epitopes. It is theoretically possible that our results may be explained by peptides corresponding to the immunodominant epitopes within the pp65 antigen having, as a group, a higher avidity than peptides representing the immunodominant epitopes within the IE1 antigen. However, we used a commercial preparation that frequently demonstrates higher levels of IE1-specific than pp65-specific CD8+ T cells (figure 4B). Our findings indicate that IE1-specific CD8+ T cells have a lower cytotoxic potential than pp65-specific cells, whether they are examined in the context of healthy immunocompetent subjects [11], in the reconstituting immune system of HSCT recipients (the present study) [11], or in immunocompromised SOT recipients (the present study). This may be related to the observation of others that expansions of IE1-specific CD8+ T cells inefficiently lyse CMV-infected fibroblasts in vitro [33, 34].

CMV encodes a number of polyepitopes that have functions in evading host immunological responses [35]. The interaction of the host immune system with viral antigens and immune evasion will differ according to the infected cell type, which is known to affect the presentation of different CMV antigens. For example, pp65, but not IE1, is presented by CMV-infected fibroblasts [36], but macrophages can present both murine...
Figure 5. Cytokine production profiles of populations of cytomegalovirus pp65 and immediate-early antigen (IE1)–specific CD8+ T cells correlated with their cytotoxic potential. A, Representative flow data from analyses of peripheral blood mononuclear cells from 1 of 7 hematopoietic stem-cell transplant (HSCT) recipients with both IE1- and pp65-specific CD8+ T cells detected by PepMix stimulation and exhibiting cytokine production profile 1 (see text). The top row of plots was gated on lymphocytes by forward and side scatter and then additionally gated on CD8+ T cells. The second row of plots was gated on interferon (IFN)–γ–producing (+) CD8+ T cells. The nos. in the quadrant of each plot indicate the cells in that quadrant as percentage of gated cells. The bar chart at the right summarizes the distribution of pp65 and IE1-specific T cells according to phenotype as averages for all 7 subjects. P values, calculated using Student’s t test by comparing the percentages of pp65- and IE1-specific CD8+ T cells with defined phenotypes, are shown. *** , IFN-γ+; **, IFN-γ+/TNF-α+; and *, IFN-γ+/CD107+. B, Corresponding flow plots from 1 of 2 additional individuals exhibiting cytokine production profile 2 (see text) and the distribution of pp65- and IE1-specific T cells as an average for these 2 subjects. All samples were collected at 180 days after transplant.

CMV pp65 and IE1 epitopes [37]. We speculate, on the basis of the present observations, that IE and pp65 antigens are differentially presented to CMV-specific memory T cells in different immunological compartments, which affects the functional phenotype of CD8+ T cells recognizing these 2 antigens. Studies in murine models of infection with LCMV [38, 39] and influenza virus [40] suggested that such IFN-γ and TNF-α–producing CD8+ T cells correlate with the established memory
T cell pool rather than with activated CD8+ T cells present during the acute phase of infection. Considering the differences in both cytokine profiles and cytotoxic potential that we observed, it seems probable that the pp65-specific CD8+ T cells represent a more mature effector population than do the IE1-specific CD8+ T cells.

Bunde et al. [10] associated IE1-specific, but not pp65-specific, CD8+ T cells with protection against CMV infection after SOT. Their findings are discordant with those of other researchers, who reported that pp65-specific T cells are associated with the control of CMV viremia after HSCT [2, 41–43]. It was acknowledged by Bunde et al. that factors other than the magnitude of the IE1-specific CD8+ T cell response were related to protection. Nonetheless, findings peculiar to a cohort of patients who were all seropositive for CMV before transplantation were considered to be universally applicable to any recipient, regardless of the CMV serostatus and the antiviral regimen [10]. In contrast to our prophylactically treated high-risk D+/R- SOT recipients [31], the lower risk D+ or D-/R+ cohort of heart- and lung-transplant recipients studied by Bunde et al. included only 2 individuals who were prophylactically treated for CMV, and immune monitoring started within days after transplant. In fact, the discriminating IE1-specific protective role of CD8+ T cells was only apparent during the first month after transplant, when IE1-specific T cells have been reported by others to be undetectable in D+/R- liver-transplant recipients receiving oral prophylaxis [44]. Moreover, the only functional assessment of CMV-specific T cells in common between the study of Bunde et al. and our study was IFN-γ production, whereas we also measured degranulation and multiple cytokine production. These aspects of experimental design may explain, in part, our divergent conclusions about the protective function of T cell responses to pp65 and IE1.

Our data support a correlation between cytotoxic potential as measured by CD107 mobilization/degranulation and the ability of CMV-specific CTLs to produce both IFN-γ and TNF-α. Prior studies have indicated that assessment of IFN-γ production alone does not accurately correlate with cytotoxicity [14, 45]. The evaluation of LCMV-specific mouse and CMV-specific human CTLs using the Lysispot assay showed widely varying ratios of cytokine secretion and target cell lysis [46]. Similarly, it has been shown that the proliferation [47] and degranulation [12] of HIV-1–specific T cells can occur independently of IFN-γ secretion. Lichterfeld et al. [23] used a flow-based cytotoxicity assay combined with ICC assays of HIV-specific CTLs to demonstrate a correlation between cytotoxicity and the secretion of both TNF-α and IFN-γ. Our data support this finding in the context of CMV-specific CTLs and suggest that these multiple cytokine–producing CTLs may represent a key component of the viral CD8+ T cell response. We are at present studying the presence of such trifunctional CMV-specific CTL populations as a possible correlate of clinical protection.

Early studies of the immune response to CMV focused on a restricted group of antigens that were considered to be immunodominant—notably, pp65 and IE1. This view has now given way to an understanding that the CMV CD8+ T cell response is much broader than anticipated [9, 48, 49]. This, in turn, raises the important question of whether T cell immune responses to all of these multiple CMV antigens are important for protection against CMV replication and disease. The answer has implications for the choice of CMV antigens to be used in CMV vaccine design, immune monitoring, and immunotherapy. If, as our data imply, IE1-specific CD8+ T cells have lower cytotoxic potential than pp65-specific CD8+ T cells, then it would be of interest to learn whether CD8+ T cell populations recognizing other CMV antigens have similar variations in cytotoxic potential. We are investigating this question.

**References**


