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T Cell Recognition Patterns of Immunodominant Cytomegalovirus Antigens in Primary and Persistent Infection

Naeem Khan,1* Donna Best, † Rachel Bruton, † Laxman Nayak, ‡ Alan B. Rickinson, † and Paul A. H. Moss †

Replication of human cytomegalovirus is controlled by a vigorous CD8 T cell response. The persistent nature of infection is believed to periodically stimulate T cell responses resulting in considerable expansions of virus-specific CD8 T cells over time. In this study, we describe the magnitude and breadth of CD8 T cell responses against the immunodominant viral Ags, IE-1 and pp65, in acute and long-term infection using the IFN-γ ELISPOT assay. Simultaneously, we have identified several novel MHC class I restricted CD8 T cell epitopes. Acute phase responses in immunocompetent donors appear to be extremely focused as early as 1 week post diagnosis with dominant peptide-specific responses observed against both proteins. These dominant responses remain detectable at all later time points over a 4-year follow-up. Interestingly the IE-1 responses show an increase over time whereas the pp65 responses do not, which contrasts with data showing that responses against both Ags are elevated in elderly individuals. We also observe the rapid emergence of an effector memory phenotype for virus-specific CD8 T cells as observed in persistent infection. Over time the revertant CD45RApos effector cell population is also expanded, and this is more evident in the preferential expansion of IE-1 responses. We postulate that periodic low-level virus reactivation after the acute infection phase preferentially stimulates these responses whereas pp65-specific T cell expansions probably occur during the infrequent episodes of lytic viral replication or secondary infection. The Journal of Immunology, 2007, 178: 4455–4465.

Human CMV is a widespread β-herpesvirus that usually manifests as an asymptomatic primary infection followed by lifelong persistence in the immunocompetent host. The full pathogenic nature of virus carriage is evident in immunocompromised individuals such as neonates and stem cell transplant (SCT)2 recipients (1–3). Studies in mice infected with murine CMV (mCMV), which shares biological properties with human CMV, have highlighted the pivotal role of cytotoxic CD8 T cells in the prevention of virus replication and associated disease (4, 5).

The precise antigenic determinants of CMV have been under investigation for over a decade now with a long-standing consensus that the T cell response is dominated by one or two CMV proteins (6–9). This was thought to be attributed to the immunosubversive tactics of CMV that interfere with class I MHC presentation of virus-derived peptide fragments for recognition by CD8 T cells (reviewed in Ref. 10). However, two recent studies have redefined our knowledge of the CMV “immunome.” Khanna’s group used computer-based algorithms to predict CMV-encoded HLA class I binding nonameric peptides and tested their ability to induce IFN-γ responses. Their results showed a broad and multispecific T cell response with up to 14 Ags from across the virus replicative cycle recognized (11), suggesting that immune evasion does not prevent the maturation of a diverse response in vivo. More recently, Picker and colleagues (12) have tested peptides corresponding to every protein encoded by the CMV genome. In this mammoth study, over 150 open reading frames were immunogenic for CD8 and/or CD4 T cells. However, these studies and others using recombinant CMV viruses (13) do suggest that IE-1 and pp65 represent two of the most important Ags. These are viral proteins from different ends of the replicative cycle: IE-1 expression occurs during the immediate early (IE) phase and depends on limited reactivation during persistence whereas pp65 is expressed during the late (L) phase and requires virus replication. Very recently IE-1-specific responses have been implicated as crucial for protection against CMV in heart and lung transplant recipients whereas pp65 responses are not (14). However, pp65-specific CD8 T cell clones have been shown to be of biological value in adoptive transfer studies in SCT (15, 16).

We have reported an increase in CMV-specific CD8 T cell responses against both IE-1 and pp65 with age suggesting an accumulation of memory cells over time (17, 18), which is also observed in murine CMV infection (19). However, these analyses were performed using MHC-peptide tetramers incorporating CMV peptides that were applicable to a limited set of HLA class I types (HLA-A1, -A2, -A68, -B7, -B8, and -B35). This would have excluded a number of individuals with other common HLA types and may not be truly representative of the global pp65 and IE-1 responses in these donors. Moreover, the vast majority of data concerning CMV immunity is derived from long-term infected hosts in whom the T cell response is in the memory phase. Our knowledge of the primary CD8 T cell response and its transition to memory is mainly restricted to immunosuppressed subjects such as renal transplant recipients and neonates, but these have also been limited to a few T cell epitopes and a single Ag, pp65 (20, 21).
FIGURE 1. Representative IFN-γ ELISPOT responses in healthy virus carriers against IE-1 and pp65 15-mer peptide pools. A, IFN-γ response profile of selected CMV seropositive individuals. Data derived from two young (Y07 and Y23) and two elderly seropositive donors (E02 and E10) is shown. Donor PBMC were incubated with peptide pools for 16 h at 37°C in standard IFN-γ ELISPOT assays. Gray and black bars represent responses against the IE-1 and pp65 peptide pools, respectively. Data is shown as SFC per 100,000 cells. B, The summarized immunogenic regions of IE-1 and pp65 in long-term virus carriers. Charts show the combined number of responders from 33 young and 28 elderly donors against each 15-mer peptide pool used in ELISPOT screening. Pools encompassing known MHC class I or class II restricted T cell epitopes are highlighted with first three amino acid abbreviations above the marked pool, with the restriction element shown in brackets. All responses represent ELISPOT wells containing at least 10 spots per 100,000 PBMC, after subtracting the background response against DMSO.
Therefore, a comparison using peptide pools was deemed necessary to minimize possible HLA bias and also provide information about the relative immunodominance of epitope-specific responses. We report global IE-1 and pp65-specific T cell responses in the memory phases of young and elderly donors and also the acute phase in primary CMV-infected immunocompetent subjects, following the response patterns over time.

Materials and Methods

Donors and samples

All healthy volunteers gave formal written consent to donate blood for this study. In addition three patients with primary CMV infection were identified among heterophile Ab-negative adult mononucleosis patients by the presence of CMV-specific IgM Abs, with subsequent isotype switching to IgG, and high CMV DNA loads in plasma. Ethical approval for this study was granted from the South Birmingham Health Authority Local Research Ethics Committee. Heparinized blood was taken by venipuncture and used immediately to isolate PBMC. Cells were used for testing CMV responses immediately or cryopreserved in 10% DMSO. One aliquot of PBMC was used for DNA extraction to allow HLA typing, which was performed by the local center of the U.K. Blood Transfusion Service (Birmingham, U.K.).

Peptides and Ags

Fifteen amino acid peptides (15-mers) overlapping by five amino acids spanning the entire IE-1 and pp65 protein sequences were synthesized (Alta Biosciences) and reconstituted in DMSO to a final concentration of 10 μg/ml. Peptide pools containing five or six peptides were prepared in RPMI 1640 medium, with each peptide at a final working concentration of 100 μg/ml. To test for total CD4 responses we prepared cell lysates of CMV-infected fibroblasts. Mock-infected fibroblasts were used for control lysates.

ELISPOT assay for IFN-γ

Polyvinylidene difluoride-backed plates (96-well; Millipore) were coated with 7.5 μg/ml anti-IFN-γ Ab 1-DIK (Mabtech) at room temperature for 4 h and blocked with 10% FCS for 1 h. Then 1 × 10^5 PBMC were added to wells in 100 μl volume per well and incubated with either CMV lysate, mock lysate, DMSO, or PHA. Ten microliters of each peptide pool was added to separate wells to give a final peptide concentration of 10 μg/ml. After incubation for 16 h at 37°C in 5% CO2, the cells were discarded, and the wells were washed six times with PBS containing 0.05% Tween 20. This was followed by incubation with 1 μg/ml biotinylated anti-IFN-γ Ab 7-B6–1-biotin (Mabtech) for 3 h at room temperature. Wells were washed again and incubated with streptavidin-conjugated alkaline phosphatase (Mabtech) for another 1 to 2 h. Individual cytokine-producing cells were identified as dark spots after a 10- to 30-min reaction with 5-bromo-4-chloro-3-indolyl phosphate and NBT by means of an alkaline phosphatase conjugate substrate kit (Bio-Rad). Spots were counted using an automated reader, (AID-Diagnostika), and results displayed as number of spot-forming cells (SFC) per 10^5 PBMC.

Antibody staining and flow cytometry

PBMC were resuspended in 100 μl of growth medium (RPMI 1640 containing 10% FCS, 2 mM L-glutamine, and 100 IU/ml penicillin-streptomycin solution) and stimulated with 10 μl of each peptide pool, DMSO or PHA, for 6 h at 37°C in 5% CO2. After the first 2 h of this incubation, we added Brefeldin A (Sigma-Aldrich) to a final concentration of 10 μg/ml. Afterward cells were washed with PBS.
containing 0.1% BSA and 2 mM EDTA) twice and then incubated with mAbs (mAb) for surface markers using FITC-conjugated anti-CD4 (BD Biosciences), ECD-conjugated anti-CD3 (Beckman Coulter) and PC5-conjugated anti-CD8 (Beckman-Coulter) for 20 min at 4°C. For membrane phenotyping, cells were incubated with a FITC-conjugated mAb specific for CD27, CD28, CD45RA, or CCR7 instead. Following washing, cells were fixed and permeabilized (Intraprep reagent; Coulter Pharmaceutical) according to the manufacturer’s instructions and then incubated with a PE-conjugated anti-IFN-γ mAb for 30 min at room temperature. Appropriate mouse PE-conjugated isotype controls were used in separate tubes. Cells were washed and analyzed immediately using a Coulter XL Epics flow cytometer by gating on lymphocytes based on side and forward scatter profiles. Subsequent analyses were performed using WinMDI software downloaded from The Scripps Institute website (http://facs.scripps.edu/software.html).

**In vitro T cell expansion and cytotoxicity assays**

PBMC were stimulated with 1 μg/ml peptide for 2 h at 37°C, and then plated out in 24-well tissue culture plates at 2–3 × 10⁶ cells per well and returned to 37°C incubation. Cultures were supplemented with rIL-7 (Peprotech) after 5 days. On day 8 cells were fed with fresh medium and rIL-2 (Chiron). From day 15 onward, cytotoxicity assays were performed using partially matched EBV-transformed lymphoblastoid cell line (LCLs) as target cells after loading with 5 μg/ml peptide or overnight infection with recombinant modified virus ankar (MVA) expressing either IE-1 or pp65 at multiplicity of infection of 5:1. Target cells were also ⁵¹Cr-labeled (Amersham Biosciences) and were seeded at 2500 cells per well in triplicate. Effector cells (expanded cultures) were added at different ratios and incubated with targets at 37°C for 5–6 h. Supernatants were harvested and then analyzed using a Gamma Counter (Packard). Lysis values were calculated with the formula: (test release/spontaneous release/maximal release/spontaneous release) × 100.

**Results**

**Distribution of pp65 and IE-1 ELISPOT responses in young and elderly individuals**

We selected the IFN-γ ELISPOT assay for initial screening due to the 96-well plate format and the increased sensitivity over cytoplasmic staining and flow cytometry (22). PBMC from 61 healthy volunteers (33 young, ages 24–50 years, and 28 elderly, ages 60–85 years) were screened using pools of pentadecameric (15-mer) peptides spanning the full length of IE-1 and pp65. A total of 60 of the 61 CMV seropositive donors made IFN-γ ELISPOT responses to at least one of these proteins. Fig. 1A shows representative examples of young and elderly donors that were either...
very focused on 1 or 2 peptides only, or had broader immunity with up to 10 or more different peptide-specific responses. After pooling the ELISPOT data, we compared the distribution of responses across the proteins between the two study populations (Fig. 1B). Interestingly, most of the pools for IE-1 (17/19) and pp65 (21/22) induced IFN-γ responses in one or more of the donors tested. For both proteins there appeared to be some commonly immunogenic regions; IE-1 pools 4, 8, and 13, and pp65 pools 5, 11, 17, and 20 induced responses in young and elderly donors. This was not expected as these pools encompass published MHC class I and class II epitopes (reviewed in Ref. 23). There were differences in the frequency of immunogenic pools between the young and elderly groups, which are probably due to HLA haplotypic variation of the donors studied. In addition we identified a number of immunogenic pools that did not contain known epitopes and so these were further investigated (Fig. 1B, unmarked pools).

**Breadth and magnitude of pp65 and IE-1 peptide-specific CD8 T cell responses in young and elderly individuals**

We then selected the individual immunogenic pools for each of 18 young and 18 elderly donors and stimulated PBMC from these donors for 6 h and then measured IFN-γ-producing cells by flow cytometry. These were confirmed in the vast majority of cases to be CD8⁺ T cell responses. Our next objective was to ascertain whether the T cell responses against CMV became more focused with aging toward fewer peptides. Fig. 2A shows that usually the response against IE-1 was focused on fewer peptides than against pp65 in both young (mean 1.33 peptides vs 3.91 peptides) and elderly donors (mean 1.78 peptides vs 4.11 peptides). Between the age groups this represented a slight increase in IE-1 and pp65 responses, but this did not reach statistical significance (IE-1: p = 0.282, pp65: p = 0.96). In general, the breadth of responses was very similar between young and elderly donors suggesting that the number of epitope-specific responses may be maintained throughout long-term infection.

For each donor we calculated the cumulative protein-specific CD8 T cell response for a comparison between the age groups (see Fig. 2B). In this manner we observed extremely high frequencies of protein-specific CD8 T cells; IE-1 specific responses ranged 0.2%–20% and pp65-specific responses ranged from 0.25%–27% of the CD8 T cell subset. The mean CD8 T cell response against IE-1 was 1.97% in young and 5.54% in elderly donors, and the response against pp65 was 2.7% in young and 6.4% in elderly donors. In both cases the difference was statistically significant confirming that CD8 T cell responses against IE-1 and pp65 are indeed of higher magnitude in elderly donors irrespective of HLA type. This increase was associated with higher responses both against pools containing known CMV epitopes such as HLA-A*0201 (hereafter referred to as HLA-A2) restricted NLVPVMVATV and against those containing as yet unknown epitopes. We also measured the contribution of the single strongest epitope within each Ag to ascertain whether the responses in elderly donors were more concentrated toward a single peptide. However, there was little difference in epitope focusing with dominant IE-1 and pp65 epitopes representing ~81% and 68% of the total response, respectively, in both young and elderly donors (data not shown).

In some donors (12/18 young and 13/18 elderly) the determined immunogenic peptides elicited CD4 T cell responses but these were only directed against pp65 (Fig. 2C). These responses were often quite strong (>1% of CD4 subset) but importantly lower in frequency than the CD8 response in the vast majority of donors tested. In most cases a single peptide (such as those marked in Fig. 1B) represented the only pp65 response within an individual. pp65-specific CD4 T cell responses in the elderly donors were higher (mean 1.44%) than young donors (0.77%) but this did not reach statistical significance (p = 0.242). In contrast, CD4 T cell responses against IE-1 were infrequent and very low frequency (<0.1% of the CD4 subset) which was also the case when stimulations were prolonged to 18 h. Therefore a detailed comparison was not feasible.

**Table I. Novel CMV epitopes identified by ELISPOT screening of subjects with acute and persistent infection**

<table>
<thead>
<tr>
<th>Pool</th>
<th>Epitope Sequence</th>
<th>Coordinates</th>
<th>HLA Allele</th>
<th>Range of Responses (Percentage of CD8)</th>
<th>Responders/Donors Tested</th>
</tr>
</thead>
<tbody>
<tr>
<td>IE1</td>
<td>ATTFLQTMMLR⁺⁺⁺</td>
<td>32–41</td>
<td>A*6801</td>
<td>10</td>
<td>1/4</td>
</tr>
<tr>
<td>2</td>
<td>KENVQGLSL</td>
<td>42–50</td>
<td>B*4001</td>
<td>0.35–6.2</td>
<td>3/6</td>
</tr>
<tr>
<td>8</td>
<td>DFERBFPFNY⁺⁺⁺⁺⁺</td>
<td>198–206</td>
<td>B*4402</td>
<td>0.7–2.9</td>
<td>2/7</td>
</tr>
<tr>
<td>9</td>
<td>FPEITNGCSQA⁺⁺⁺⁺⁺⁺⁺</td>
<td>221–231</td>
<td>B*5501</td>
<td>0.77–5.7</td>
<td>5/5</td>
</tr>
<tr>
<td>14</td>
<td>EVISMVRM</td>
<td>334–342</td>
<td>A*6801</td>
<td>0.4–3.5</td>
<td>2/4</td>
</tr>
<tr>
<td>16</td>
<td>BAQAIAYTL</td>
<td>381–389</td>
<td>B*4402</td>
<td>0.18–3.5</td>
<td>4/7</td>
</tr>
</tbody>
</table>

pp65  

| 2    | DTPVLPHETR       | 31–40       | A*6801     | 2.5                                    | 1/4                     |
| 3    | QPSLILVSQLY      | 52–61       | B*3501     | 0.45–1.7                               | 2/6                     |
| 8    | YTPGGTFTCH⁺⁺⁺⁺⁺⁺⁺ | 61–70       | A*6801     | 0.25–2.2                               | 2/4                     |
| 6    | VIHAGKQM⁺⁺⁺⁺⁺⁺⁺⁺⁺ | 148–156     | A*2601     | 0.45                                    | 1/3                     |
| 9    | QYVKVYLESF       | 222–231     | A*2402     | 0.3–0.84                               | 2/5                     |
| 10   | VTLGSDVEELDTMTMR⁺⁺⁺⁺⁺⁺⁺ | 244–258 | B*5701     | 0.8                                    | 1/2                     |
| 13   | QAIRETVELR       | 331–340     | B*3501     | 0.24–3.2                               | 2/6                     |
| 15   | SEHPTFTSQQY      | 364–373     | B*4402     | 0.15–0.34                              | 2/7                     |
| 17   | TPRVSGGAMA       | 417–427     | B*5501     | 1.3                                     | 1/5                     |

CD4 responses (Percentage of CD4)  

| 9    | DVPSGKLHMVTGLGS  | 234–239     | ND         | 0.32                                   | 1/9                     |
| 10   | KLFHRVLGSDVEED   | 239–253     | ND         | 0.3                                    | 1/9                     |
| 12   | DVEELDITRNPFQPPT⁺⁺⁺⁺⁺⁺⁺ | 249–263 | ND         | 0.08–1.1                               | 2/9                     |
| 12   | VAFTSHEHGFCLCPK  | 294–308     | ND         | 0.14–1                                 | 4/9                     |
| 15   | SEHPTFTSQQYRQSK  | 364–378     | ND         | 0.29                                   | 1/9                     |
| 15   | LEYRHTHDRIDEGGA  | 379–393     | ND         | 0.23                                   | 1/9                     |

a The known polymorphic residues for IE-1 are underlined.  
b Response identified in primary CMV infection.  
c Minimal epitope not defined.
FIGURE 4. Prospective analysis of IE-1 and pp65 peptide-specific responses following acute CMV infection. Three individuals with symptomatic primary CMV infection, P01 (A) and P02 (B), from multiple time points, and P03 (C) from a single time point (indicated by weeks post diagnosis), were tested. PBMC were incubated with IE-1 and pp65 peptide pools in ELISPOT plates overnight at 37°C as described in Materials and Methods. All IFN-γ responses are shown as SFC/100,000 PBMC and are calculated after subtracting the background response against DMSO.
Mapping of novel responses in CMV seropositive hosts

In most cases the responses to individual pools could be predicted based on the HLA type of the donor and our knowledge of published IE-1 and pp65 HLA class I restricted epitopes. In other cases it appeared that undefined epitopes were accountable for the observed ELISPOT responses. We thus determined the immunogenic peptide(s) for a number of such pools (those inducing >40 SFC/10⁵ PBMC) by testing individual peptides to induce IFN-γ production that we detected by cytoplasmic staining and flow cytometry. A typical example shown in Fig. 3A represents the response by a HLA-B55⁺ donor against IE-1 peptides D9 (FPKTT NGCSQAMAAL), which measured over 4% of CD8 T cells. The use of shorter peptide fragments allowed definition of minimal T cell epitopes inducing the IFN-γ response (Fig. 3B). These activities were then expanded in vitro by peptide stimulation in the presence of rIL-2 and then tested for specificity in cytotoxicity assays. Fig. 3C shows that these cultures demonstrated significant lysis of HLA-matched peptide-pulsed LCL targets and but not control peptide-pulsed LCL targets. We also confirmed the recognition of endogenous processed Ag by the use of MVA-IE-1- and MVA-pp65-infected targets. IE-1 peptide (D9) stimulated T cell lines only recognized MVA-IE-1-infected LCL but not MVA-pp65-infected LCL. These T cell lines were then tested against partially HLA-matched LCL to determine HLA restriction, which in this case was confirmed as HLA-B55. Overall, six novel IE-1-derived and nine novel pp65-derived CD8 T cell reactivities were discovered in this study and most were longer than the standard nine amino acids. These were restricted by various HLA alleles, namely HLA-A24, -A68, -B35, -B40, -B44, and -B55. We also identified several CD4 T cell epitopes for which the HLA class II restrictions were not defined. The complete peptide epitope data derived from selected donors with persistent infection and primary CMV infection (responses described later) are summarized in Table I. A number of these responses were of very high frequency, exceeding 1% of the CD8 subset in many cases. These frequencies were comparable to those of published CMV epitopes. Intriguingly the high magnitude responses were detected in donors not expressing common alleles such as HLA-A2 (data not shown). For example, HLA-A24-restricted responses against the QYV epitope were undetectable in HLA-A24⁺ donors coexpressing HLA-A2 and responses against the novel HLA-B40 (KEV) and B44 (EEA) epitopes were also undetectable in donors that coexpressed HLA-B7 or HLA-B8. This suggests that T cell responses restricted through HLA-A2, HLA-B7, and HLA-B8 exert immunodominance over responses restricted by other HLA types and reach very high frequencies. However in the absence of HLA-A2/-B7/-B8 the

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Table II. Dynamics of peptide-specific responses in primary CMV infection

<table>
<thead>
<tr>
<th>Donor</th>
<th>IE-1 Epitopes</th>
<th>pp65 Epitopes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>B7-CRV</td>
<td>B7-RPH</td>
</tr>
<tr>
<td>P01</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 wk</td>
<td>0.35</td>
<td>0.9</td>
</tr>
<tr>
<td>4 wk</td>
<td>1.5</td>
<td>0.55</td>
</tr>
<tr>
<td>16 wk</td>
<td>2.4</td>
<td>0.3</td>
</tr>
<tr>
<td>104 wk</td>
<td>4.9</td>
<td>0.32</td>
</tr>
<tr>
<td>208 wk</td>
<td>4.5</td>
<td>0.13</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>IE-1 Epitopes</th>
<th>pp65 Epitopes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>B44-DEL</td>
<td>B55-FPK</td>
</tr>
<tr>
<td>P02</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 wk</td>
<td>1.8</td>
<td>0.1</td>
</tr>
<tr>
<td>24 wk</td>
<td>6.1</td>
<td>0.25</td>
</tr>
<tr>
<td>104 wk</td>
<td>10</td>
<td>0.42</td>
</tr>
<tr>
<td>160 wk</td>
<td>10.5</td>
<td>0.7</td>
</tr>
</tbody>
</table>

a Values indicate percentage of CD8 T cells producing IFN-γ after 6 h of stimulation with synthetic peptide. ND, not detectable.

FIGURE 5. Visualization of CMV epitope-specific CD8 T cells in primary CMV infection. PBMC from primary CMV-infected immunocompetent donors P01 (A) and P02 (B) were stimulated for 6 h with 1 µg/ml synthetic peptides (HLA-A68-restricted ATT, HLA-A68-restricted FVF, HLA-B*0702-restricted CRV, HLA-B*0702-restricted TPR) at 37°C and stained for surface CD8 expression (PC5) and then cytoplasmic IFN-γ (PE). All events are gated on lymphocytes. Values indicated in top right quadrants denote the percentage of CD8 T cells that are epitope-specific.
responses restricted by other HLA molecules are no longer suppressed and can also be of high magnitude.

**pp65 and IE-1 responses in symptomatic primary CMV infection**

To study immunity after acute infection, we tested immunocompetent donors with symptomatic primary CMV infection for responses against IE-1 and pp65 peptide pools from 1 week post diagnosis, and the results are shown in Fig. 4. Donor P01 (HLA-A3, -A24, -B7, -B44) displayed good levels of reactivity against both IE-1 and pp65 Ags with a number of dominant responses (≥40 SFC/10⁵ PBMC) that were evident at the 1 week bleed (Fig. 4A). The IE-1 response was dominated by a single pool that was confirmed by flow cytometry to be CD8⁺ and then mapped to be against the HLA-B7-restricted CRV peptide. The pp65 response was dominated by two peptide pools that were also CD8⁺, which were later mapped to HLA-B7-restricted peptides: RPH and TPR (Table II). These peptides dominated the response at all bleeds during a 4-year period. In addition, there were several other subdominant responses observed but at later time points these were mostly undetectable. In donor P02 (HLA-A26, -A68, -B7, -B55), we also observed dominant responses at the first bleed against two IE-1 and several pp65 pools (Fig. 4B). These persisted at later time points while the weaker subdominant responses observed at week 1 were mostly absent later on. These responses were confirmed as CD8⁺, three of which were HLA-A68 restricted while others were HLA-26, -B44, and -B55 restricted (Table II). Importantly most of these responses were against novel epitopes (see Table I). We also analyzed a third donor, P03 (HLA-A3, -A68, -B7, -B13) but this was relatively late at 4 weeks post diagnosis (Fig. 4C). This subject was not available for follow-up study and so subsequent analyses could not be performed.

**Kinetics of IE-1 and pp65 responses after primary CMV infection**

From the ELISPOT data (Fig. 4), it appeared that the dominant IE-1 peptide response(s) actually increased over the follow-up period whereas dominant pp65 peptide responses declined albeit to still detectable levels. This pattern may have been attributed to differences in CD8 counts that we observed at different bleeds. We
subsequently visualized responding cells by flow cytometry, and Fig. 5 shows the increase in IE-1-specific CD8 T cell responses over time in both donors. Donor P01 showed a 10-fold increase in CD8 T cells responding to the HLA-B7-restricted CRPV peptide and donor P02 showed over 6-fold increases against both the novel HLA-A68-restricted ATT peptide and the novel HLA-B55-restricted FVK peptide (Table II). The reverse trend was observed for pp65 responses; HLA-B7-restricted TPR-specific and HLA-A68-restricted FVF-specific CD8 T cells declined during the follow-up period in donor P01 and donor P02, respectively. This divergent pattern corroborated our ELISPOT results using peptide pools but contrasted with our published MHC peptide tetramer-guided data showing that both IE-1- and pp65-specific CD8 T cell responses in long-term virus carriers increase with age. In these donors, CD4 T cell responses could be detected only against pp65 peptides but at a very low frequency (<0.1% of CD4 subset); however, we did detect stronger responses (0.3–1%) against a lysate of CMV-infected fibroblasts (data not shown). Unlike with the CD8 responses, there was little fluctuation over time.

Phenotypic analysis of CMV-specific CD8 T cells in primary infection

CMV-specific CD8 T cells are skewed mainly toward the CCR7<sup>neg</sup>“effector memory” subset (17, 24, 25) described by Salustro and coworkers (26). However, these studies describe responses in the memory phase of the response and provide little information regarding the evolution of phenotypic change within different CMV peptide-specific CD8 T cell populations. Thus virus-specific CD8 T cells from each available time point post diagnosis were characterized for expression of CD45RA, CD27, CD28, and CCR7. Fig. 6 shows a representative example of this analysis. Both IE-1- and pp65-specific CD8 T cells were predominantly CCR7<sup>neg</sup>, CD28<sup>neg</sup>, CD45RA<sup>neg</sup> indicative of activated effector memory cells at 1 week post diagnosis. This phenotype gradually changed to that of a revertent phenotype; CCR7<sup>neg</sup>, CD28<sup>neg</sup>, CD45RA<sup>neg</sup>, suggesting that responses were becoming more differentiated over time. Interestingly, we observed a greater degree of differentiation in the IE-1-specific response over time which correlated with an increase in the frequency as described above. This was illustrated by the lower levels of CD27 expression by IE-1-specific CD8 T cells at later time points. In contrast, pp65-specific responses did not show such dramatic loss of CD27 expression. This contrasts with persistent infection where we have described similar levels of differentiation for both IE-1 and pp65 responses (18).

Discussion

Understanding the maturation of CD8 T cells from acute to chronic phases of infection is of paramount importance in the field of adaptive immunity. We have described the T cell response against human CMV in the primary and long-term memory phases of virus carriage using peptide pools representing the two principal immunodominant Ags, IE-1 and pp65. In so doing we have identified a number of novel T cell epitopes from these proteins that induce high frequency responses.

The frequency of CMV-specific T cells is probably underestimated with our approach because staining with MHC-peptide tetramers has shown higher frequencies than IFN-γ staining for a number of different host-pathogen studies (27). However, protein-spanning peptides are not limited by HLA haplotype and also provide a functional readout. From the literature, elderly individuals are believed to accumulate CMV-specific CD8 T cells that are apparently dysfunctional in vitro (18, 28) though it appears there is a similar increment in functional virus-specific cells with aging. This occurs without focusing of the response toward fewer peptides, suggesting that responses established early after infection may persist over time. An important difference between the age groups will be reduced naïve CD8 T cell numbers in elderly donors. This suggests that the increased CMV-specific response may not be as dramatic when considered only as a proportion of the CD8 memory population.

Notably pp65 appears to encode more T cell epitopes than IE-1 and certainly induces stronger CD4 responses as shown by others (14), although we cannot rule out secretion of other cytokines by CD4 T cells. The apparently lower immunogenicity of IE-1 may be explained by sequence variation between natural strains of virus in each donor and the AD169 encoded IE-1 peptides used in this study. Several T cell epitopes of IE-1 contain polymorphic amino acids (29, 30). Indeed in some donors there appears to be a preference for certain residues within these epitopes (such as HLA-B8-restricted ELR) with lack of recognition for others, suggesting that IE-1 responses may be underestimated in our study (unpublished observations).

Healthy seropositive subjects can either have extremely focused or very broad immune responses against CMV, regardless of age. It is possible that donors with very focused responses to IE-1 and pp65 show broader patterns of responses against other CMV proteins. There also appeared to be interesting patterns of responses between donors expressing certain alleles; HLA-A2/B7/B8<sup>pos</sup> donors made CD8 T cell responses almost exclusively through these alleles against immunodominant epitopes. Conversely donors not expressing these HLA types made epitope-specific responses that were restricted by a broader range of HLA molecules. In our donors there is no obvious deficit in health but it has been shown that a broader antigenic repertoire is strongly associated with protection from CMV disease in HIV infection (31) with the magnitude of the T cell response being less important.

We also investigated the transition of T cells from primary to persistent phases of virus carriage. Our longitudinal analysis was limited to two immunocompetent donors recovering from symptomatic primary CMV infection so we express caution in data interpretation. At 1 week post diagnosis, responses against both Ags in these donors were dominated by a few peptides with a number of much weaker subdominant responses also detected. At later time points only dominant responses were detectable suggesting that suppression of responses against subdominant determinants may be occurring, possibly by competition for Ags at the level of the APC and for cytokines such as IL-15 (32, 33). Another factor could be TCR avidity for Ags as this appears to shape the clonality of CD8 T cells specific for immunodominant CMV and EBV epitopes (34). The antigenic repertoire of CD8 T cells was also strongly influenced by HLA type in these donors with HLA-B7-restricted responses dominating the profile of two donors. Thus patterns in long-term memory were also evident in our limited cases of primary CMV infection, suggesting that immunodominance is established very early after initial virus encounter and can be HLA associated. This may be attributed to higher TCR avidities for epitopes presented by allomorphs such as HLA-B7 and also unequal levels of different MHC-peptide complexes on the surface of infected cells or APCs. Such disparities may reflect differences in epitope generation after Ag processing. Because IE-1 and pp65 responses were predominantly CD8<sup>+</sup> in all the primary cases, a role for other Ag in the CD4 response is implied because CD4 T cells did produce IFN-γ after stimulation with a CMV lysate. However, these responses were at much lower frequency than peptide-specific CD8 responses as reported previously (35),
This is interesting because CD4 T cells may be critical in limiting viral replication after primary infection in immunosuppressed patients (20).

Another intriguing observation was that IE-1-specific CD8 T cell responses continuously increased in the post acute infection phase whereas the pp65-specific CD8 T cell response progressively declined. A similar pattern has been described in HIV-infected subjects experiencing acute CMV retinitis, although pp65 responses were elevated in the long-term recovery period (31). Furthermore, IE-1-specific CD8 immunity also correlates with protection in solid organ transplant recipients (14) reinforcing the importance of IE-1 as a biologically relevant target Ag in humans.

The selective increase of IE-1-specific CD8 T cells contrasts with our published data describing amplified responses to IE-1 and pp65 with aging, suggesting that both Ags are sufficiently presented to stimulate cognate T cells over time. During primary CMV infection, naive CD8 T cells for various CMV Ag including IE-1 and pp65 were stimulated by Ag that is cross-presented on the surface of professional APCs and expand to high frequencies as we have observed. Subsequently, viral reactivation would involve IE-1 Ag presentation favoring the selective expansion of IE-1-specific CD8 T cells. Recognition of virus-infected cells at this stage by IE-1-specific T cells would limit viral gene expression proceeding toward the late phase and thus hamper pp65-specific responses. IE-1 (pp89)-specific CD8 T cells also accumulate over time in mCMV infection (19) but the response against pp105 (late Ag), the mCMV sequence homolog of pp65, does not increase over time which was the case with pp65-specific responses in our study. However, pp105 does not enjoy access to the Ag presentation pathway after virus penetration before or in the absence of viral gene expression (36), which may explain why this Ag is not immunodominant in mice, as pp65 is in humans. During instances of productive reactivation or re-infection it is likely that pp65-specific T cells are preferentially expanded due to efficient processing and presentation of pp65 before viral gene expression (6, 37, 38). Because pp65 is an exogenous source of Ag, this would also rationalize the greater immunogenicity of pp65 for CD4 T cells. We speculate that our subjects may not have experienced productive reactivation or re-infection with CMV; indeed viral DNA was undetectable in PBMC from any of the post diagnosis time points (data not shown). With a longer follow-up period, such events may occur and translate into increased pp65-specific responses.

Many studies have highlighted the differences in membrane phenotype of CD8 T cells specific for CMV and other persistent and nonpersistent viruses (24, 25, 39). Our study of immunocompetent subjects confirms the rapid emergence of highly differentiated effector memory CD8 T cells. These Ags expressed at opposite ends of the replicative cycle reflect the marked focusing of responses that are probably established very early on. However, the kinetics of CD8 T cell responses for these Ags expressed at opposite ends of the replicative cycle reflect the different modes of Ag presentation, which probably depend on levels of viral activity occurring over the lifetime of the host.

Disclosures
The authors have no financial conflict of interest.

References


