Heterogeneity of CD4+ and CD8+ T-cell Responses to Cytomegalovirus in HIV-Infected and HIV-Uninfected Men Who Have Sex With Men

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Studies of T-cell immunity to human cytomegalovirus (CMV) primarily reflect anti-CMV pp65 or immediate early antigen 1 (IE-1) activity. We assessed responses of T cells from human immunodeficiency virus (HIV)–negative and HIV-infected men to peptide pools spanning 19 CMV open reading frames selected because they previously correlated with total CMV-specific T-cell responses in healthy donors. Cells producing cytokines in response to pp65 or IE-1 together composed <12% and <40% of the total CD4+ and CD8+ T-cell responses to CMV, respectively. These proportions were generally similar regardless of HIV serostatus. Thus, analyses of total CMV-specific T-cell responses should extend beyond pp65 and IE-1 regardless of HIV serostatus.

Keywords. CMV; CD4+ and CD8+ T-cell responses; HIV infection; MACS; pp65; IE-1; intracellular cytokine staining; IFN-γ; TNF-α; IL-2.

Increasing evidence suggests that chronic cytomegalovirus (CMV) infection significantly impacts T-cell immunity in human immunodeficiency virus (HIV) infection and aging [1–3]. Most of this evidence consists of analysis of T cells specific to epitopes derived from pp65 (encoded by region UL83 in the CMV genome) and immediate early antigen 1 (IE-1; encoded by region UL123). CD8+ T cells directed against these antigens are clonally expanded, as measured through class I tetramers or pentamers in individuals expressing HLA-A2 [2–5], and expansion of CD4+ and CD8+ T cells responsive to these antigens has also been demonstrated by intracellular cytokine staining [6, 7]. In fact, CMV-specific T cells can occupy a very large proportion (up to 30% in some cases) of the circulating T-cell pool in HIV-seronegative persons [8, 9]. However, because human CMV is a large virus with a 230-kb genome and >213 open reading frames (ORFs), it may have a large number of T-cell epitopes, and its overall effect on T-cell immunity is unlikely to be reflected by immune reactivity to just pp65 and IE-1. Indeed, Sylwester et al, in a comprehensive analysis of CMV-specific CD4+ and CD8+ T cells in healthy adults aged 19–55 years, found responses to approximately 150 CMV ORFs [9]. In that study, to accurately predict the total CD8+ T-cell response to CMV, measurement of reactivity to 15 specific ORFs was required; for CD4+ T cells this number was 6, including 2 ORFs that overlapped with those for the CD8+ T-cell response, giving a total of 19.

CMV infection is common in people infected with HIV, leading to expansion of CMV-specific T cells in the context of already impaired immunity. For example, Naeger et al reported that, compared with HIV-negative persons, HIV-infected individuals, even those receiving long-term highly active antiretroviral therapy, had significantly higher proportions of CD8+ T cells and, to a lesser extent, CD4+ T cells responding to UL83 and UL123 peptide pools [7].

On the basis of these data, we hypothesized that T-cell responses to pp65 and IE-1 would underestimate the overall T-cell reactivity to CMV in CMV-HIV–coinfected people. Addressing this hypothesis is important for a more accurate understanding of overall T-cell immunity against CMV among HIV-infected and HIV-negative men who have sex with men (MSM), for the design of CMV-based vaccine vectors and for studies relating the immune response against CMV to the aging process, as well as other clinical outcomes. Therefore, we investigated, in both HIV-infected and HIV-negative MSM, the relationship between T-cell responses to UL83 and UL123 peptide pools and those to the remaining 17 CMV ORF peptide pools [Supplementary Table 1 [9]] that accurately predicted the total anti-CMV CD4+ and CD8+ T-cell responses in the study by Sylwester et al described above.

METHODS

Study participants were selected from the Baltimore-Washington, D. C., site of the Multicenter AIDS Cohort Study (MACS;
an ongoing prospective study of the natural and treated histories of HIV infection among MSM. The MACS uses standardized protocols for HIV testing, collection, isolation, and storage of sera and peripheral blood mononuclear cells (PBMCs) in local and national repositories [10, 11]; measurement of T-cell subsets at study visits by standardized flow cytometry; and measurement of plasma HIV RNA concentration by the Roche Amplicor assay, which has a lower limit of detection of 50 copies/mL. For the present study, subjects were eligible if (1) their frailty status (frail or nonfrail) was known, defined as expression (or nonexpression) of the frailty phenotype, described by Fried et al [12], at 2 consecutive MACS study visits, and (2) sufficient cryopreserved PBMCs were available from those study visits. Frail and nonfrail men were matched by age and HIV serostatus (results related to frailty will be reported elsewhere.) Serum anti-CMV immunoglobulin G (IgG) titers were determined by enzyme-linked immunosorbent assay (United Biotech, Mountain View, CA) as previously described [8, 13].

In preliminary experiments, CD4+ and CD8+ T-cell responses to staphylococcal enterotoxin B (SEB; Toxin Tech, FL) and to peptide pools derived from UL28, UL48, UL82, and UL99 were similar between cryopreserved and freshly isolated PBMCs (data not shown) [14]. Therefore, cryopreserved PBMCs were used in this study. Cells were thawed, washed, and cultured overnight in Roswell Park Memorial Institute 1640 medium with 10% heat-inactivated fetal bovine serum (HyClone, Logan, UT) at 37°C with 5% CO2. Cells were seeded at 200 μL/well in a 96-well plate at 2 × 10^6 cells/mL containing costimulatory monoclonal anti-CD28 and anti-CD49d (BD Biosciences, San Jose, CA) at final concentrations of 1 μg/mL each and with culture medium containing either no antigen, SEB (at a final concentration of 0.2 μg/mL), or one of 26 peptide pools (each at 2.0 μg/mL) derived from the 19 CMV ORFs. The cultures were incubated as described above for 6 hours, with the final 5 hours including 10 μg/mL of Brefeldin A (Sigma Aldrich). Cells were stored overnight at 4°C, harvested, washed with phosphate-buffered saline, and stained with LIVE/DEAD Fixable Aqua Dead Cell Stain kit (Invitrogen, Eugene, OR) per manufacturer's instructions. Cells were fixed, permeabilized, and washed using Cytofix/Cytoperm and Fixation/Permeabilization solution kit (BD Biosciences) per manufacturer's instructions.

Following stimulation, cells were incubated at 4°C for 45 minutes with 4 premixed monoclonal antibodies (anti-human interferon γ [IFN-γ]–fluorescein isothiocyanate, CD69–phycoerythrin [PE], CD8–peridinin chlorophyll–Cy5.5, and CD3–allophycocyanin), as well as antibodies to interleukin 2 (IL-2)–PE–Cy7, tumor necrosis factor α (TNF-α)–Alexa Fluor 700, and CD4–V450 (all antibodies, including isotype controls, were from BD Biosciences). Samples were analyzed on an LSRII flow cytometer (BD Biosciences), using FACSDiva software, version 6.13 (BD Biosciences). Analyses were gated on live cells, and CD3+/CD4+ and CD3+/CD8+ cells expressing CD69+ and either IFN-γ, TNF-α, and/or IL-2 were quantified using FlowJo software (Tree-Start, Ashland, OR).

The percentages of cytokine antibody staining in the unstimulated cultures and isotype antibody staining in the stimulated cultures were subtracted from the percentages of positive staining in the cultures stimulated with the CMV peptide pools for each participant. The results were expressed as percentages of CD69+/IFN-γ+, CD69+/TNF-α+, or CD69+/IL-2+ cells among total CD4+ or CD8+ T cells for each of the peptide pools. The Wilcoxon–Mann–Whitney test (SAS NPARIWAY procedure; SAS, version 9.3, SAS Institute, Cary, NC) was used to determine the statistical significance of differences between HIV-infected and HIV-negative participants. The Johns Hopkins Bloomberg School of Public Health Institutional Review Board approved the study; written informed consent was obtained from all participants.

RESULTS

Twenty-two men (10 HIV negative and 12 HIV infected) were included in this study. Their demographic and clinical characteristics are summarized in Supplementary Table 2. Briefly, all were CMV-seropositive white men, and the mean age was 60 years. All HIV-infected participants were receiving effective antiretroviral therapy and had undetectable HIV plasma RNA. HIV-negative and HIV-infected men did not differ significantly with regard to age or education level, but the HIV-infected men had lower CD4+ T-cell counts and higher CD8+ T-cell counts. HIV-infected men had higher anti-CMV IgG titers than HIV-negative men, but this difference was not significant.

Figure 1 illustrates the CD4+ and CD8+ T-cell IFN-γ responses to peptide pools derived from UL83, UL123, and all other ORFs for all 22 men studied. Although there was significant individual variation, in most men, regardless of HIV status, CD4+ and CD8+ T-cell IFN-γ responses to the UL83 and UL123 peptide pools represented only a small fraction of the total T-cell response to CMV peptides. Exceptions were few for CD4+ T cells (participant 4 in the HIV-negative group and participant 7 in the HIV-infected group) and more common for CD8+ T cells (participants 5, 9, and 10 in the HIV-negative group and participants 2, 3, 7, 10, and 11 in the HIV-infected group), for whom the combined response to UL83 and UL123 peptide pools represented ≥50% of the total response. As shown in Supplementary Figure 1, most men had detectable responses to several peptide pools, and these varied widely across individuals for both CD4+ and CD8+ T cells and for HIV-infected and HIV-negative men. Of note, several men demonstrated robust total IFN-γ responses to CMV despite minimal or no response to UL83 or UL123 peptides, particularly in CD4+ T cells from HIV-negative men (participants 1–3, 6, 8, and 9) but also in the other groups (participants 10 and 12 in
Figure 1. CD4+ or CD8+ T-cell interferon γ (IFN-γ) responses to peptide pools derived from UL83/pp65, UL123/IE-1, and the other 17 cytomegalovirus open reading frames combined, in human immunodeficiency virus (HIV)–infected and HIV-negative men who have sex with men.

Table 1. CD4+ and CD8+ T-Cell Responses to Peptide Pool Stimuli, as Measured by Intracellular Cytokine Staining, Among Human Immunodeficiency Virus (HIV)–Infected and HIV-Negative Men Who Have Sex With Men (MSM)

<table>
<thead>
<tr>
<th>Cytokine response by intracellular production</th>
<th>HIV-Negative MSM (n = 10)</th>
<th>HIV-Infected MSM (n = 12)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Percentage of Responding T Cells</td>
<td>Percentage of Total Response</td>
</tr>
<tr>
<td></td>
<td>CD4</td>
<td>CD8</td>
</tr>
<tr>
<td>Interferon γ</td>
<td></td>
<td></td>
</tr>
<tr>
<td>UL83</td>
<td>0.1</td>
<td>0.5</td>
</tr>
<tr>
<td>UL123</td>
<td>0.0</td>
<td>1.3</td>
</tr>
<tr>
<td>Otherb</td>
<td>5.1</td>
<td>5.3</td>
</tr>
<tr>
<td>Tumor necrosis factor α</td>
<td></td>
<td></td>
</tr>
<tr>
<td>UL83</td>
<td>0.1</td>
<td>0.2</td>
</tr>
<tr>
<td>UL123</td>
<td>0.0</td>
<td>0.5</td>
</tr>
<tr>
<td>Otherb</td>
<td>4.2</td>
<td>3.8</td>
</tr>
<tr>
<td>Interleukin 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>UL83</td>
<td>0.1</td>
<td>0.0</td>
</tr>
<tr>
<td>UL123</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Otherb</td>
<td>1.8</td>
<td>0.9</td>
</tr>
</tbody>
</table>

All data are median values.

a CMV open reading frame (ORF) from which peptide pools were derived.
b All 17 other cytomegalovirus ORFs.
the HIV-infected group, for CD4\(^+\) T cells; participant 6 in the HIV-negative group, for CD8\(^+\) T cells; and participant 5 in the HIV-infected group, for CD8\(^+\) T cells).

Patterns of CD4\(^+\) and CD8\(^+\) T-cell responses, as measured by IFN-\(\gamma\) production (Figure 1), as well as the responses measured by production of TNF-\(\alpha\) or IL-2, are summarized in Table 1. The total proportion of T cells that responded to any of the CMV peptide pools ranged from 0.2% to 40.3%. Overall, the sum of CD4\(^+\) T-cell responses to UL83 and UL123 represented only a very small portion of the total CMV-specific CD4\(^+\) T-cell response measured, usually <10%, in both HIV-infected and HIV-negative men. CD8\(^+\) T-cell responses to UL83 and UL123 peptide pools constituted a greater proportion of the total response, but the proportion was still <40%. Because only 19 of the previously identified 150 immunogenic ORFs [9] were included in this study, these figures may overestimate the proportion of cells reacting to UL83 and UL123. IFN-\(\gamma\) and TNF-\(\alpha\) responses were very similar and were more attributable to UL83 and UL123, compared with IL-2 responses. In contrast to the report by Naeger et al [7], no significant differences in responses were observed between HIV-infected and HIV-negative men, except that the IL-2 response of CD4\(^+\) T cells to UL83 in the HIV-infected group was higher than that in the HIV-negative group (\(P = .04\) before adjustment for multiple comparisons, but the difference was not significant after this adjustment). However, such differences were not the focus of the present study, which was not powered to detect them. Taken together, these data indicate that the impact of CMV on T-cell immunity is much broader than what would be seen if only responses to pp65 and IE-1 were measured.

**DISCUSSION**

To our knowledge, this study is the first to evaluate CMV-specific T-cell immunity in HIV-infected people against a broad panel of CMV epitopes, in this case ones that were needed to achieve a correlation coefficient of \(\geq 0.9\) with the total CMV-specific CD4\(^+\) and CD8\(^+\) T-cell responses in healthy adults [9]. Many previous studies have selectively focused on individuals with exceptionally strong CD8\(^+\) T-cell responses against CMV, as they used commercially available tetramers containing the commonly used UL83/pp65 and UL123/IE-1 epitopes. Consistent with findings from those studies, we found that UL83 and UL123 peptide pools induced stronger responses within CD8\(^+\) T cells than within CD4\(^+\) T cells. As mentioned above, significant interindividual variation was observed in both HIV-infected and HIV-negative men, although all participants were CMV seropositive. One possible explanation for some of this interindividual variability could be our recent finding in older adults that the presence of CMV viral DNA in peripheral blood monocytes was associated with an increased number of CMV pp65-specific CD8\(^+\) T cells [8] and with elevated immune activation, as indicated by serum levels of neopterin [13]. The relationship between the presence of CMV DNA in monocytes and CMV-specific T-cell responses in younger men with and those without HIV infection remains to be determined.

The present evaluation has the strength of not being restricted to HLA-A\(^*\) donors or to the CD8\(^+\) T-cell compartment, as is the case for studies based on analyses of class I–restricted tetramers or pentamers. This increases the generalizability of this study. However, this study is limited in that T-cell epitopes derived from only 19 ORFs were tested, and other ORFs including those recently identified by Stern-Ginossar et al [15] were not considered. In addition, CMV infection was not directly tested for, and the sample size was relatively small. Despite these limitations, this study supports the notion that the assessment of CMV-directed T-cell immunity based on responses to epitopes derived from UL83 and UL123 alone is inadequate at best and may be misleading, especially for CD4\(^+\) T-cell responses. Thus, more-detailed characterization of the immune response to CMV is needed for an accurate understanding of the effects of CMV infection on T-cell immunity in people with or without HIV infection.

**Supplementary Data**

Supplementary materials are available at *The Journal of Infectious Diseases* online (http://jid.oxfordjournals.org/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

**Notes**

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**Potential conflicts of interest.** All authors: No reported conflicts.

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