HIV-1-Specific Memory CD4⁺ T Cells Are Phenotypically Less Mature Than Cytomegalovirus-Specific Memory CD4⁺ T Cells

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*J Immunol* 2004; 172:2476-2486; doi: 10.4049/jimmunol.172.4.2476

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HIV-1-Specific Memory CD4⁺ T Cells Are Phenotypically Less Mature Than Cytomegalovirus-Specific Memory CD4⁺ T Cells

Feng Yun Yue,* Colin M. Kovacs,** Rowena C. Dimayuga,‡ Paul Parks,‡ and Mario A. Ostrowski2*†

HIV-1-specific CD4⁺ T cells are qualitatively dysfunctional in the majority of HIV-1-infected individuals and are thus unable to effectively control viral replication. The current study extensively details the maturational phenotype of memory CD4⁺ T cells directed against HIV-1 and CMV. We find that HIV-1-specific CD4⁺ T cells are skewed to an early central memory phenotype, whereas CMV-specific CD4⁺ T cells generally display a late effector memory phenotype. These differences hold true for both IFN-γ- and IL-2-producing virus-specific CD4⁺ T cells, are present during all disease stages, and persist even after highly active antiretroviral therapy (HAART). In addition, after HAART, HIV-1-specific CD4⁺ T cells are enriched for CD27⁻CD28⁻expressing cells, a rare phenotype, reflecting an early intermediate stage of differentiation. We found no correlation between differentiated state, if compared with CMV-specific CD8⁺ antiretroviral therapy (HAART). In addition, after HAART, HIV-1-specific CD4⁺ T cells are enriched for CD27⁺CD28⁻expressing cells, a rare phenotype, reflecting an early intermediate stage of differentiation. We found no correlation between differentiation phenotype of HIV-1-specific CD4⁺ T cells and HIV-1 plasma viral load or HIV-1 disease progression. Surprisingly, HIV-1 viral load affected the maturational phenotype of CMV-specific CD4⁺ T cells toward an earlier, less-differentiated state. In summary, our data indicate that the maturational state of HIV-1-specific CD4⁺ T cells cannot be a sole explanation for loss of containment of HIV-1. However, HIV-1 replication can affect the phenotype of CD4⁺ T cells of other specificities, which might adversely affect their ability to control those pathogens. The role for HIV-1-specific CD4⁺ T cells expressing CD27⁺CD28⁻ after HAART remains to be determined. The Journal of Immunology, 2004, 172: 2476–2486.

CD4⁺ T cells hold promise as immune effectors against HIV-1. Studies by Rosenberg et al. (1) have shown that individuals with strong HIV-1-specific CD4⁺ T cell proliferative responses to HIV-1 p24 Ag are able to better control their viremia (e.g., plasma viremia, <10,000 copies/ml) than those with diminished or absent responses. Most HIV-1-infected individuals show poor or absent proliferative responses to HIV-1, even on successful antiretroviral therapy (2–4). Although IFN-γ-producing CD4⁺ T cells directed against HIV-1 are detectable in individuals at all stages of disease (5–8), the intensity of the HIV-1-specific IFN-γ⁺ CD4⁺ T cell response does not correlate with viral load or disease progression (6, 7). These findings indicate that the majority of HIV-1-infected individuals have circulating HIV-1-specific memory CD4⁺ T cells that are functionally defective. Abnormal maturation of HIV-1-specific memory T cells may potentially lead to an inability of the effector immune responses to clear virus. A number of recent studies using subset marker analysis have now shown that, in HIV-1-infected individuals, HIV-1-specific CD8⁺ T cells exist predominantly in a preterminally differentiated state, if compared with CMV-specific CD8⁺ T cells in the same individuals (9–12). This delayed phenotype correlates with decreased perforin and IFN-γ expression, which may permit virus to evade CD8⁺CTL responses (9, 13). It has thus been postulated that skewed maturation of HIV-1-specific CD8⁺ T cells may represent a pathogenic mechanism whereby CD8⁺ T cells are prevented from achieving full effector function (9, 11, 12) allowing continued viral replication. It is unknown whether a similar difference in maturational phenotype exists for HIV-1-specific memory CD4⁺ T cells in comparison to memory CD4⁺ T cells of other specificities.

Based on studies of proliferative potential, telomere length, and effector cytokine expression of naive and memory T cells in murine and human cells, a working model of memory T cell differentiation is beginning to emerge (10, 14–17). In murine models, it has been suggested that memory T cells follow a progressive pathway of differentiation that leads to different end points, depending on the initial priming stimulus (14–16, 18, 19). Phenotypic states ranging from central memory to fully differentiated effector function can result. Central memory cells are thought to reflect a strategic reserve of memory cells that can be rapidly recruited upon restimulation to generate potent effector cells. Fully differentiated effector memory cells have migratory capacity to home to peripheral tissues and have greater ability to produce cytokines (IL-2, IFN-γ, and TNF-α). Central memory cells have been shown to express the markers CD45RA, the chemokine receptor CCR7, which allows recruitment to lymph nodes, and the costimulatory molecules CD27 and CD28. Fully differentiated effector memory cells have been shown to lose surface expression of CD45RA, CCR7, CD27, and CD28, but gain expression of CCR5, a chemokine receptor that allows recruitment to sites of inflammation. An intermediate state of differentiation has also been demonstrated in CD8⁺ T cells, characterized by the phenotype CD8⁺CD27⁻CCR7⁻CCR5⁺ (10). However, the pathways of memory T cell generation for human CD4⁺ T cells have not yet been well characterized.

In immunocompetent individuals, CMV infection is maintained in a clinically and virologically latent form due to persistent immune pressure (20, 21). It has been assumed that CMV-specific

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Received for publication May 27, 2003. Accepted for publication December 11, 2003.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 The study was funded through grant support supplied by the Ontario HIV Treatment Network. F.Y.Y. and M.A.O. are career scientists of the Ontario HIV Treatment Network.

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0022-1767/04/$02.00
CD4+ T immune responses are critical in controlling CMV replication, because CMV disease is often seen in AIDS or in immunosuppressed organ transplant recipients (20, 21). Thus, a comparison of phenotypes of CMV-specific vs HIV-1-specific memory CD4+ T cells might yield insights into potential mechanisms of loss of immune control against HIV-1 infection. Using the currently described models of T cell maturation, we have characterized the phenotype of CMV- and HIV-1-specific CD4+ T cells in a cohort of HIV-1-uninfected and HIV-1-infected individuals. The study was conducted directly on ex vivo whole-blood samples, which provides a more sensitive staining than does the use of purified PBMCs (10).

Materials and Methods

Study participants

Twenty-nine HIV-1-infected and five HIV-1-uninfected individuals were recruited for this study (see Table I). Six individuals were recently infected by HIV-1 within 6 mo of study (diagnosed by recent seroreactivity to HIV-1) and were asymptomatic at time of blood draw. Fourteen individuals had chronic progressive HIV-1 infection. This was defined as documented HIV-1 infection for >1 year, with evident CD4+ T cell decline, and/or viral load of >10,000 copies/ml (branched DNA (bDNA))3. All were asymptomatic, except for participant 8 who developed CMV colitis. Two HIV-1-infected individuals had slowly progressive HIV-1 infection (slow progression (slow P)), which we defined as having HIV-1 infection <7 years, a <50 CD4 T cell count decline per year, and a viral load of <10,000 copies/ml (bDNA). Six individuals with long-term nonprogression (LTNP), defined as chronic infection for at least 7 years, no evidence of CD4+ T cell decline, and a viral load of <10,000 copies/ml, were studied. Except for participant 7, all were antiretroviral naive at time of study. Participant 7 was studied after being placed on highly active antiretroviral therapy (HAART) for 1 year after an acute seroconversion illness. Participants 4 and 6 were studied before and after 6 mo of HAART treatment. ND, not done; NA, not applicable; f, frequency.

Abbreviations used in this paper: bDNA, branched DNA; slow P, slow progression; LTNP, long-term nonprogression; HAART, highly active antiretroviral therapy; SEB, staphylococcal enterotoxin B.

Table I. Clinical, virologic, and immunologic characteristics of participants a

<table>
<thead>
<tr>
<th>Participant</th>
<th>Clinical Diagnosis</th>
<th>CD4 Count (×103)</th>
<th>Viral Load (Copies/ml)</th>
<th>IFN-γ-Secreting Cells, % of Total CD4 HIVp55</th>
<th>CMV HIVp55</th>
<th>IL-2-Secreting Cells, % of Total CD4 HIVp55</th>
<th>CMV HIVp55</th>
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<tr>
<td>P1</td>
<td>Recent sero</td>
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<td>P2</td>
<td>Recent sero</td>
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<td>300</td>
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<tr>
<td>P3</td>
<td>Recent sero</td>
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<td>Recent sero</td>
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</tr>
<tr>
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<td>Recent sero</td>
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<td>P7</td>
<td>Recent sero/on Rx × 1 year</td>
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<td>ND</td>
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<td>Chronic P/CMV colitis</td>
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<td>ND</td>
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<td>ND</td>
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<td>0.41</td>
<td>ND</td>
<td>ND</td>
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</tr>
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<td>ND</td>
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<td>ND</td>
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<td>ND</td>
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<tr>
<td>P23</td>
<td>Slow P</td>
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<td>P26</td>
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<tr>
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<td>LTNP</td>
<td>3,900</td>
<td>100</td>
<td>0.08</td>
<td>0.41</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td>P28</td>
<td>LTNP</td>
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<td>0.08</td>
<td>0.41</td>
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<td>ND</td>
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<td>P29</td>
<td>LTNP</td>
<td>4,100</td>
<td>100</td>
<td>0.08</td>
<td>0.41</td>
<td>ND</td>
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</table>

a Abbreviations used in this paper: bDNA, branched DNA; slow P, slow progression; LTNP, long-term nonprogression; HAART, highly active antiretroviral therapy; SEB, staphylococcal enterotoxin B.
FIGURE 1. Characterization of CD4\(^+\) T cell differentiation subsets. A, CD27 and CD28 staining of freshly obtained whole blood is illustrated next to possible models of Ag-specific CD8\(^+\) and CD4\(^+\) T cell differentiation, starting from early-differentiated cells, which differentiate into late-differentiated cells, following a stage of intermediate cells. Note that, on CD8\(^+\) T cells, CD28 appears to be down-regulated first, whereas for CD4\(^+\) T cells, CD27 appears to be down-regulated first. Percentages of CD4 or CD8\(^+\) T cells in each quadrant are shown in the upper right corner. (Figure legend continues)
Source of Ags
We used the following Ags: baculovirus-derived p55 of HIV-1 (Protein Sciences, Meriden, CT) was used at 5 μg/ml; baculovirus protein control at 0.15 μg/ml was used as a negative control as done previously (7) (Protein Sciences); CMV lysate and control lysates were tested at various titrations and then used at 1/200 final dilution (Virion, Rüschlikon, Switzerland); and staphylococcal enterotoxin B (SEB) was used at a final concentration of 200 ng/ml (Toxin Technologies, Sarasota, FL.).

Flow cytometry and intracellular cytokine determination
The procedure for intracellular staining of cytokines in fresh blood samples was performed according to the protocols of BD Biosciences (Mountain View, CA) (http://www.bdbiosciences.com). Briefly, fresh heparinized blood samples were incubated with Ag for 6 h in the presence of brefeldin A and 1 μg/ml anti-CD49d Ab for costimulation (BD Biosciences), and then transferred to an 18°C water bath. The following day, blood was stained by a panel of conjugated Abs (FITC, PE, PerCP, and allophycocyanin). The following Abs in various combinations were used: CD27-FITC, CD28-allophycocyanin, CD4-PerCP, CCR5-PE, CCR7-PE, CD3-PE, and respective isotype controls. CCR7 staining involved a three-step staining procedure in which blood was incubated with the 2H4 clone anti-human CCR7 Ab, then biotinylated rat anti-mouse IgM, followed by streptavidin-PE according to the protocols of BD Biosciences. The samples were then fixed, and RBC were lysed using FACS lysis solution (BD Biosciences). Cells were washed, fixed, and permeabilized in FACS permeabilization buffer (BD Biosciences) and then stained for cytokines, IFN-γ-PERCP, or IL-2-PE. Cells were then washed and resuspended in 1% paraformaldehyde/PBS and then analyzed the following day on a FACSCalibur (BD Biosciences). All Abs were obtained from BD Biosciences. Data were acquired by CellQuest software (BD Biosciences) and analyzed using FlowJo (Tree Star, San Carlos, CA). From 100,000 to 200,000 events in the lymphocyte gate were acquired per sample.

Functional assays
CFSE labeling was performed by incubating PBMCs with 4 mM CFSE (Molecular Probes, Eugene, OR) in RPMI 1640 for 10 min at 37°C, before quenching with ice-cold 10% FCS RPMI 1640 and washing. The cells were then incubated with SEB (5 μg/ml) (Sigma-Aldrich, St. Louis, MO) or immobilized anti-CD3 Ab (10 μg/ml; BD Biosciences) or PHA (1 μg/ml; Sigma-Aldrich) for 5 days before staining.

Statistical analysis
To determine whether two groups were statistically different for a given variable, we used the Wilcoxon rank sum test (two-tailed) or Student’s t test. For correlations of different variables within a group, we calculated Spearman’s correlation coefficient and tested whether it was statistically different from 0 by using an asymptotic normal approximation.

Results
Characterization of CD4+ T cell differentiation phenotypes
Using four color intracellular cytokine flow cytometry and a panel of cell surface markers (CD27, CD28, CCR7, and CCR5), we have characterized the phenotype of virus-specific CD4+ T cells in HIV-1-infected and -uninfected individuals. We elected to study directly, whole-blood samples, because we have had the most sensitive and cleanest staining with these types of samples in comparison with the use of purified PBMC or frozen PBMC. In addition, we have found that Ficol can interfere with chemokine and CD27/CD28 surface staining of ex vivo samples (data not shown). As demonstrated previously (10), we find that CD8+ T cells can be primarily separated into three distinct populations: CD27−CD28−, CD27+CD28−, and CD27+CD28+. These correlate with early, intermediate, and late phenotypes. CD4+ T cells demonstrate a somewhat reversed pattern of expression with the majority of CD4+ T cells expressing CD27-CD28-, CD27+CD28+, and CD27+CD28+ (Fig. 1A). A small population expressing CD27+CD28− represents <2% of CD4+ T cells (see Fig. 1A). To further characterize these subsets, we examined proliferative activity and capacity by Ki67 staining and CFSE functional assays, respectively. Previous studies, looking at CD8+ T cells, indicated that the most mature CD8− T cells had the poorest proliferative capacity compared with those of earlier differentiation states (9, 10). In HIV-1-uninfected individuals, we observed highest frequencies of Ki67 cells in the CD27+CD28− subset and lowest frequencies in the CD27−CD28− subset, with intermediate frequencies in the CD27−CD28+ and CD27+CD28− (subsets (see Fig. 1B), indicating that ex vivo CD27−CD28−, CD27−CD28+, and CD27−CD28− cells are more active in the cell cycle than are CD27+CD28−. Interestingly, in HIV-1-infected individuals, CD4+ T cells of the CD27−CD28− and CD27+CD28− subsets tended to show highest Ki67 frequencies (data not shown). To assess proliferative capacity, ex vivo PBMC were CFSE labeled and then stimulated for 5 days with the following mitogens: SEB, anti-CD3, or PHA (Fig. 1C). A gradient of proliferative capacity was observed with the CD27+CD28− subset having the greatest proliferative capacity, followed by CD27−CD28−, and then CD27−CD28+ and CD27+CD28− showing the least (see Fig. 1C). Thus, ex vivo CD27+CD28−CD4+ T cells are not as active in cell cycle but have the greatest proliferative capacity after in vitro stimulation, whereas ex vivo CD27+CD28+ CD4+ T cells are actively cycling but have the poorest proliferative capacity after in vitro TCR stimulation. Geginat et al. (19) previously demonstrated that effector memory CD4+ T cells have a greater capacity to produce high levels of cytokines such as IFN-γ and IL-4 after mitogen stimulation, when compared with central memory cells. To address this, ex vivo whole blood was stimulated with mitogen (PHA) for 6 h (in presence of brefeldin A), and then examined for IFN-γ production within various CD27+CD28− subsets (see Fig. 1D). Although all CD4+ T cell subsets could produce IFN-γ in response to mitogen, a greater proportion of CD27−CD28− CD4+ T cells could produce higher levels of IFN-γ based on mean fluorescence intensity, thus confirming the findings by Geginat et al. (19). Together, these findings are consistent with the notion that CD4+ T cells differentiate from CD27−CD28+ cells to CD27−CD28−, with CD27+CD28+, CD27−CD28−, and CD27−CD28+ cells representing intermediate stages. The expression patterns suggest that the majority of CD4+ T cells lose surface expression of CD27 first in the differentiation pathway toward the CD27−CD28− memory phenotype, with a minor population losing CD28 first.
Characterization of IFN-γ-producing CMV- and HIV-1-specific memory CD4+ T cell differentiation phenotypes in HIV-1-infected and -uninfected individuals

Because CMV-specific memory CD4+ T cells are easily detectable in CMV IgG Ab-positive individuals, we initially determined the differentiation phenotype of these memory cells in five HIV-1-uninfected individuals (P30–P34, listed in Table I). Using intracellular flow cytometry, we identified Ag-specific memory CD4+ T cells by gating on CD4+ T cells that express IFN-γ after brief exposure to the specific Ag in question. The number of Ag-specific cells were quantitated by calculating the frequency of cytokine-producing cells in Ag-stimulated conditions and subtracting the number of cytokine-producing cells in control Ag-stimulated conditions. Only samples that had background IFN-γ staining of <0.04% of total CD4+ T cells during control Ag-stimulated conditions were studied. A representative experiment from participant 30 is shown in Fig. 2A. We find that, in all HIV-1-uninfected individuals, CMV-specific CD4+ T cells are enriched for CD27+CD28+, CD27+CD28−, CCR7-negative cells, indicating a tendency to a mature effector phenotype. Data of CMV-specific cells from these five individuals is found in Fig. 3.

We next examined the phenotype of CMV- and HIV-1-specific CD4+ T cells in a cohort of HIV-1-infected individuals with various clinical stages of HIV-1 infection (Table I). Except for participant 7, all were antiretroviral naive. All were CMV IgG seropositive and CMV IgM seronegative, and only participant 8 had evidence of clinical CMV disease manifested as biopsy-proven CMV colitis. Representative experiments from four HIV-1-infected participants are illustrated in Fig. 2B. Similar to HIV-1-uninfected individuals, CMV-specific CD4+ T cells were highly enriched for CD27+CD28+, or CD27+CD28−, CCR7-negative cells, suggesting intermediate-to-late stages of differentiation. For every HIV-1-infected individual studied, HIV-1-specific CD4+ T cells were enriched for CD27+CD28−, or CD27−CD28+, CCR7-negative cells, suggesting intermediate-to-late stages of differentiation. Although there was some variability in the relative proportions of early-, intermediate-, and late-expressing cells based on

![FIGURE 2. Characterization of virus-specific CD4+ T cells. Representative data from a CMV-seropositive human volunteer (participant 30) (A) and from four HIV-1-infected individuals (B) are shown. HIV-1- and CMV-specific cells were identified by culturing fresh whole-blood samples in the presence of CMV lysate, HIV-1 gag p55 Ag, or control Ags (see Materials and Methods) for 6 h, and brefeldin A, and then assessed for IFN-γ expression by intracellular flow cytometry. The frequencies of Ag-specific cells seen in the second column are indicated as percentages of total CD4+ T cells and are calculated after subtraction from control Ag stimulation (always <0.04% of CD4+ T cells) in the first column. Using four-color flow cytometry, cells are gated in the lymphocyte gate; IFN-γCD4+ expressing cells are then gated and examined for CD27 and CD28 expression (third column). Percentages of cells for all quadrants are described in the upper right corner. Cells are also gated for CD4, CD3, and IFN-γ, and then assessed for CCR7 or CCR5 expression (fourth and fifth columns). Positive CCR7 and CCR5 expression is based on isotype matched controls, and CCR7 and CCR5 staining of total CD4+ T cell population in each sample. The percentages of Ag-specific cells expressing CCR7 or CCR5 are indicated above the gates for each sample in fourth and fifth columns.]
CD27/CD28 staining or expression of CCR7 between individuals, CMV-specific CD4^+ T cells always demonstrated a later stage of differentiation compared with HIV-1 CD4^+ T cells within any individual. Summary data from all participants are illustrated in Fig. 3. For the entire cohort of HIV-1-infected individuals, HIV-1-specific CD4^+ T cells were significantly enriched in early CD27^+CD28^- expressing cells compared with CMV specific (mean, 62 vs 20%, respectively; p < 0.05); and CMV-specific CD4^+ T cells were
significantly enriched for late CD27<sup>+</sup>CD28<sup>-</sup>-expressing cells compared with HIV-specific (56 vs 13%, respectively; \( p < 0.05 \)). Similarly, CMV-specific CD4<sup>+</sup> T cells in HIV-1-uninfected individuals were significantly less mature than HIV-1-specific CD4<sup>+</sup> T cells in HIV-1-infected individuals (see Fig. 3A). CMV-specific CD4<sup>+</sup> T cells in HIV-1-infected individuals tended to be less mature than CMV-specific CD4<sup>+</sup> T cells in HIV-1-infected individuals based on CD27<sup>-</sup>CD28<sup>-</sup> expression (33 vs 56% in HIV-1-uninfected vs HIV-1-infected, respectively; \( p < 0.05 \)). HIV-1-specific CD4<sup>+</sup> T cells were also significantly enriched in the CD27<sup>-</sup>CD28<sup>-</sup> population compared with CMV (12 vs 4%, respectively; \( p < 0.05 \)) in the HIV-1-infected cohort. HIV-1-specific CD4<sup>+</sup> T cells expressed significantly greater CCR7 expression compared with CMV in HIV-1-infected or HIV-1-uninfected individuals (60 vs 25 and 20%, respectively; \( p < 0.05 \)) (Fig. 3B).

Also, HIV-1-specific CD4<sup>+</sup> T cells tended to show higher levels of CCR5 expression when compared with CMV-specific CD4<sup>+</sup> T cells in the HIV-1-infected cohort.

Correlations were determined between viral load or CD4 count and maturational phenotype of HIV-1- or CMV-specific CD4<sup>+</sup> T cells in the HIV-1-infected cohort (see Fig. 4 for representative examples). There was no significant correlation between CD4 count or HIV-1 viral load and the maturation phenotype of HIV-1-specific CD4<sup>+</sup> T cells. Surprisingly, there was a significant correlation between HIV-1 viral load and enrichment for CMV-specific CD4<sup>+</sup> T cells of a CD27<sup>-</sup>CD28<sup>-</sup> phenotype (\( p = 0.006 \); Spearman rank correlation).

CMV-specific CD4<sup>+</sup> T cells exhibited a more differentiated phenotype compared with HIV-1-specific cells at all disease stages within a given patient.

**Characterization of IL-2-producing CMV- and HIV-1-specific memory CD4<sup>+</sup> T cell differentiation phenotypes in HIV-1-infected individuals**

To determine whether the differences in maturational phenotype held with Ag-specific cells of other cytokine specificities, we performed a similar analysis in five HIV-1-infected individuals, using IL-2 expression to identify Ag-specific cells (participants 1, 19, 20, 21, and 29). Representative data from participant 1 are shown in Fig. 5A, and summary data from all five participants are shown in B. If cells were stimulated with superantigen, i.e., SEB, IL-2-expressing CD4<sup>+</sup> T were enriched in the early-differentiated fraction, whereas IFN-\( \gamma \)-expressing cells were seen at all stages of differentiation but were most enriched in late-differentiated cells (Fig. 5A). If cells were stimulated with Ag, the frequencies of IL-2-producing HIV-1- and CMV-specific CD4<sup>+</sup> T cells were much lower than those producing IFN-\( \gamma \) within a given individual ranging from 10 to 25% of the frequency of IFN-\( \gamma \)-producing cells. IL-2-producing CMV-specific CD4<sup>+</sup> T cells were enriched in a late phenotype, whereas IL-2-producing HIV-1-specific CD4<sup>+</sup> T cells showed an early phenotype. Similar findings were found in all five participants (see Fig. 5B). Thus, IL-2-producing CMV- and HIV-1-specific memory CD4<sup>+</sup> T cells also show different maturation phenotypes. Correlations were determined between HIV-1 viral load and maturation phenotype of IL-2-producing cells. Although there was a trend to negative correlation between viral load and a mature phenotype in both HIV-1- and CMV-specific IL-2-producing cells, this trend was not statistically significant (data not shown).

**Effect of HAART on IFN-\( \gamma \)-producing CMV- and HIV-1-specific memory CD4<sup>+</sup> T cell differentiation phenotypes**

Participants 2, 4, 5, 6, and 13 (see Table I) were studied before and 6–8 mo after institution of HAART. All individuals developed undetectable viral loads by 2 mo of HAART. Summary data from these individuals are illustrated in Fig. 6. HAART tended to induce a more differentiated phenotype in CMV-specific cells by further enriching for CD27<sup>-</sup>CD28<sup>-</sup> cells and CD27<sup>-</sup>CD28<sup>-</sup> cells, whereas HAART did not increase the proportion of CD27<sup>-</sup>CD28<sup>-</sup>-expressing cells in the HIV-1-specific CD4<sup>+</sup> T cell population. Of note, the HIV-1-specific population had a significant increase in CD27<sup>-</sup>CD28<sup>-</sup>-expressing cells after HAART, a population which generally is <2% of total CD4<sup>+</sup> T cells (see Figs. 1 and 6A). CCR7 expression on HIV-1-specific CD4<sup>+</sup> T cells increased after HAART in four of five individuals and was essentially unchanged on CMV-specific cells in four of five individuals. CCR5 expression was variable after HAART. In summary, HAART tended to push CMV-specific CD4<sup>+</sup> T cells to a more differentiated memory phenotype, whereas HIV-1-specific CD4<sup>+</sup> T cells were enriched for the uncommon CD27<sup>-</sup>CD28<sup>-</sup> phenotype. A similar phenotype was observed in participant 7, who was only studied after 1 year of HAART (data not shown).

**Discussion**

Our data show that, similar to CD8<sup>+</sup> T cells, Ag-specific CD4<sup>+</sup> T cells can also be separated into functional subsets indicating various stages of differentiation, based on CD27 and CD28 expression. Based on our studies of proliferative capacity, cell cycle activity, and cytokine production, we postulate that memory CD4<sup>+</sup> T cells that are CD27<sup>-</sup>CD28<sup>-</sup> and CCR7<sup>-</sup> represent the earliest stage of differentiation, whereas those that are CD27<sup>-</sup>CD28<sup>-</sup>CCR7<sup>-</sup> are in the latest. CD4<sup>+</sup> T cells expressing

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**FIGURE 4.** Correlation between viral load and phenotype of virus-specific CD4<sup>+</sup> T cells in HIV-1-infected individuals. Plasma viral load (bDNA; copies per milliliter) was determined in each participant from the same time point in which viral-specific responses were measured. Values of \( p \) shown in each figure were determined by the Spearman rank correlation. The solid line represents a regression line.
CD27⁻CD28⁻ represent a common intermediate stage of maturation, and those that are CD27⁺CD28⁻ are a minor population, which are also early to intermediate in the differentiation pathway.

Our results show that circulating ex vivo HIV-1- and CMV-specific memory CD4⁺ T cells display differences in their phenotypic stage of differentiation. Similarly to what has been previously shown for HIV-1-specific CD8⁺ T cells (9, 11, 12), HIV-1-specific CD4⁺ T cells are skewed to an early central memory phenotype when compared with those that are CMV specific. This held true for both IFN-γ- and IL-2-producing CD4⁺ T cells and was present during all disease stages and appeared to persist after HAART. Thus, our findings indicate that CD4⁺ T cells directed against HIV-1 have distinct characteristics compared with those directed against CMV. Our findings are also similar to those recently reported by Harari et al. (22), who showed in a cohort of HIV-1-infected progressors, using different markers of T cell differentiation, that CMV-specific CD4⁺ T cells were more enriched in cells showing the late differentiation phenotype of CD45RA⁻CCR7⁻ compared with HIV-1-specific CD4⁺ T cells.

**FIGURE 5.** Characterization of IL-2-producing virus-specific CD4⁺ T cells. Fresh whole blood was stimulated with CMV lysate, HIV-1 p55 Ag, superantigen (SEB), or control Ags (not shown), for 6 h, and then subsequently stained for intracellular IFN-γ and IL-2, and surface stained for CD27 and CD28. **A,** Shown are CD27/CD28 expression of superantigen-stimulated CD4⁺ T cells expressing IFN-γ and IL-2, and CD27/28 expression of IL-2-producing CMV- and HIV-1-specific CD4⁺ T cells taken from participant 1. **B,** Summary results from all five participants are shown. SE and means are shown.
The reasons for these differences in T cell differentiation are currently unclear, and are likely multifactorial. One possibility is that different functional properties are necessary to control each virus. CMV infection targets endothelial cells, lungs, exocrine and endocrine organs, retina, and gut, whereas HIV-1 replication occurs primarily in the lymphoid tissues. Thus, CMV-specific CD4+ T cells that are highly enriched for the CCR7lowCD27−CD28− phenotype can exit the lymphoid tissues and be targeted to CMV-infected tissues. In contrast, HIV-1-specific CD4+ T cells that express more CCR7 would be targeted to lymph nodes where HIV-1 is replicating. Thus, it is possible that these differences in phenotype are necessary in order for the immune response to be targeted toward different locations, depending on where the particular virus is replicating. A second explanation is that HIV-1 can block or direct CD4+ T cell differentiation as an immune evasion strategy. However, there was no correlation between differentiation phenotype of HIV-1-specific CD4+ T cells and viral load or clinical disease state. That is, we did not see an enrichment for CD27−CD28− late effector cells in those individuals who controlled viral replication to low or undetectable levels. Although it is possible that HIV-1-specific CD4+ T cells are perturbed by the virus early during acute infection in all infected individuals, and have a subsequent irreversible block in their maturation, our data indicate that the maturational state of HIV-1-specific CD4+ T cells cannot be a sole explanation for loss of containment of HIV-1. Papagno et al. (23) recently drew similar conclusions.
when comparing HIV-1- and CMV-specific CD8+ T cells. They showed that, although HIV-1-specific CD8+ T cells were less mature than CMV-specific CD8+ T cells, there was no correlation between HIV-1-specific CD8+ T cell phenotype and HIV-1 disease progression. These authors concluded that patient nonprogression could not be the result from a distinct HIV-1-specific CD8+ T cell maturation phenotype. A third possibility that may explain the immature phenotype of HIV-1-specific CD4+ T cells is due to a rapid consumption of terminally differentiated HIV-1-specific CD4+ T cells as a result of HIV-1 infection, either by direct infection or due to high levels of Ag. HAART ranging from 6 mo to 1 year, however, did not influence the maturational state of HIV-1-specific memory CD4+ T cells toward a more mature, CD27+CD28+, effector phenotype, arguing against this latter explanation. A fourth attractive possibility is that these phenotypes simply reflect the activation state of the Ag-specific cells to differing loads of Ag in the body. However, we were unable to find a direct correlation with specific maturation phenotype of HIV-1-specific cells and HIV-1 viral load in our HIV-1-infected cohort. Also, the maturational phenotype of HIV-1-specific cells did not approach that of CMV-specific cells after HAART. Thus, we are unable to entirely explain the different phenotypes of these Ag-specific cells based on activation due to differing levels of Ag alone.

CMV-specific CD4+ T cells in HIV-1-uninfected individuals tended to be somewhat less mature than those in the HIV-1-infected cohort, as a group. The explanations for this observation are unclear, although one could postulate that increased shedding of CMV in the HIV-1-infected cohort might drive differentiation to a more terminal state to control viral replication.

We were surprised to observe that HIV-1 replication correlates with a maturational phenotype of CMV-specific CD4+ T cells toward an earlier differentiated state. The most extreme example of this was in participant 8, who also had active CMV colitis requiring therapy. The CMV-specific CD4+ T cells in this individual were more enriched for CD27+CD28+ (47% of cells) compared with those of other participants and were 100% CCR7 expressing, thus resembling the phenotype of HIV-1-specific T cells. In addition, in two individuals studied, the phenotype of CMV-specific CD4+ T cells was more mature after HAART. This suggests that HIV-1 replication itself can affect the phenotype of CD4+ T cells of other specificities. If CMV-specific CD4+ T cells of a late effector phenotype are required to adequately control CMV, then high levels of HIV-1 viremia may adversely affect the ability of these cells to control CMV by altering their phenotype. Further studies examining the phenotypes of CMV-specific CD4+ T cells in individuals with active CMV disease will be required to address this mechanism of immune failure.

HIV-1-specific CD4+ T cells also expressed high levels of CCR5. An enrichment for a combined CCR5+CCR7+ phenotype seems to be unique for HIV-1. One could postulate that this phenotype might allow CD4+ T cells to circulate between lymph nodes, or that HIV-1 infection itself may up-regulate CCR5 expression on these cells to enhance their infectivity. Against the latter hypothesis, however, was a lack of correlation between HIV-1 viral load and CCR5 expression of virus-specific cells.

We observed a small but significant increase in a population of HIV-1-specific CD27+CD28+CD4+ T cells, which was not observed in CMV-specific CD4+ T cells or in CD4+ T cells from uninfected individuals. This population of cells was dramatically increased after HAART. We found that this subpopulation of cells demonstrated good proliferative capacity (Fig. 1C) and was also associated with increased IL-2-producing ability (Fig. 5B), suggesting an early intermediate phenotype. It is unclear whether this phenotype represents a state of differentiation which is unique to HIV-1 infection or represents a differentiation phenotype that is seen when the levels of Ag are decreasing such as after HAART. Because these cells have lost CD28 expression early on in their differentiation, their costimulatory requirements for reactivation might be higher than the more commonly observed CD27+CD28+ cells. Thus, an enrichment for Ag-specific cells with the CD27+CD28+ phenotype might not optimally maintain HIV-1-specific Th immune responses after initiation of HAART over the long term. Future studies addressing the role of these cells in the composition of the HIV-1-specific CD4+ T cell immune responses in treated individuals and how they correlate with maintenance of CD4+ T cell help will be required.

One caveat to the interpretation of our data, however, is that the Ag-specific cells of interest may not necessarily be circulating in the blood, but rather be within the tissues. That is, HIV-1-specific T cells should generally accumulate in the mucosa and within lymph nodes, whereas CMV-specific T cells should be in mucosa and other organs. Thus, a more accurate comparison might be to compare CMV- and HIV-1-specific T cells found within genital mucosa. Future studies addressing this are clearly warranted.

In summary, our findings show that HIV-1-specific memory CD4+ T cells are phenotypically distinct from those directed against CMV. However, it is unlikely that these maturational differences are directly responsible for a lack of control of HIV-1. We feel that further study of other acute and chronic viral infections may further help to elucidate the explanations for these differences.

Acknowledgments

We thank our patients for their time and commitment. We thank Rupert Kaul, Reifik Saskin, and Mark Luscher for helpful comments and suggestions.

References


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