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Effects of Sustained HIV-1 Plasma Viremia on HIV-1 Gag-Specific CD4+ T Cell Maturation and Function

Brent E. Palmer, Eli Boritz, and Cara C. Wilson

An in vitro proliferative defect has been observed in HIV-1-specific CD4+ T cells from infected subjects with high-level plasma HIV-1 viremia. To determine the mechanism of this defect, HIV-1 Gag-specific CD4+ T cells from treated and untreated HIV-1-infected subjects were analyzed for cytokine profile, proliferative capacity, and maturation state. Unexpectedly high frequencies of HIV-1-specific, IL-2-producing CD4+ T cells were measured in subjects with low or undetectable plasma HIV-1 loads, regardless of treatment status, and IL-2 frequencies correlated inversely with viral loads. IL-2-producing CD4+ T cells also primarily displayed a central memory (T_{CM}; CCR7+CD45RA-) maturation phenotype, whereas IFN-γ-producing cells were mostly effector memory (T_{EM}; CCR7-CD45RA+). Among Gag-specific, IFN-γ-producing CD4+ T cells, higher T_{EM} frequencies and lower T_{CM} frequencies were observed in untreated, high viral load subjects than in subjects with low viral loads. The percentage of HIV-1 Gag-specific CD4+ T cells correlated inversely with HIV-1 viral load and directly with Gag-specific CD4+ T cell proliferation, whereas the opposite relationships were observed for HIV-1-specific CD4+ T_{EM} cells. These results suggest that HIV-1 viremia skew Gag-specific CD4+ T cells away from an IL-2-producing T_{CM} phenotype and toward a poorly proliferating T_{EM} phenotype, which may limit the effectiveness of the HIV-1-specific immune response. The Journal of Immunology, 2004, 172: 3337–3347.

Loss of T cell function has been associated with sustained viral replication in several chronic viral infections. When lymphocytic choriomeningitis virus, a virus normally controlled by the murine immune system, is introduced into perforin-defective mice unable to control viral replication, lymphocytic choriomeningitis virus-specific T cells progressively lose the ability to produce IL-2, then TNF-α, and finally IFN-γ (1). Similarly, during chronic hepatitis B virus (HBV) infection, HBV-specific CD4+ T cell proliferation is often lost; however, it can be restored when HBV replication is suppressed with lamivudine (2). In chronic, progressive HIV-1 infection, HIV-1-specific CD8+ T cells detected by MHC class I tetramer staining gradually lose the capacity to produce IFN-γ in the face of persistent viral replication (3), and defects in CD8+ T cell proliferation and perforin expression have also been associated with progressive disease (4–6). These observations suggest that certain viruses may induce functional changes in virus-specific T cells if viral replication is not initially controlled by the immune system.

Functional changes are also apparent in HIV-1-specific CD4+ T cells of HIV-1-infected subjects with chronic, progressive disease. The loss of CD4+ T cell proliferation to HIV-1 and other recall Ags in subjects with untreated HIV-1 disease was one of the earliest reported immunologic manifestations of HIV-1 infection (7, 8). Initially, the loss of proliferation was believed to result from the deletion of HIV-1-specific CD4+ T cells (9). However, recent studies have used intracellular IFN-γ staining to demonstrate significant frequencies of HIV-1-specific CD4+ T cells in most HIV-1-infected subjects, regardless of disease stage (10–13). In studies simultaneously using HIV-1-specific lymphoproliferative assays, we and others have found a discordance between the frequency of HIV-1-specific, IFN-γ-producing CD4+ T cells and HIV-1-specific CD4+ T cell proliferation in subjects with HIV-1 viremia. Although viremic subjects and those on effective antiretroviral therapy had similar frequencies of HIV-1-specific, IFN-γ-producing CD4+ T cells, HIV-1-specific lymphoproliferative responses were significantly lower in viremic subjects (12). HIV-1-specific lymphoproliferative responses were also found to diminish during periods of plasma viremia associated with structured treatment interruptions (13). These findings suggested that unchecked HIV-1 replication might lead to suppression of HIV-1-specific CD4+ T cell proliferation and that this dysfunction could be reversed upon suppression of HIV-1 replication. However, the mechanism of this proliferative dysfunction had not been fully investigated.

We hypothesized that the differences in HIV-1-specific CD4+ T cell proliferation observed between treated and untreated HIV-1-infected subjects might parallel differences in CD4+ T cell maturation phenotype. Recent studies in both humans and mice indicated that T cells of different maturation states were functionally distinct (14, 15). One model divided T cells into subpopulations based on expression of CCR7, a chemokine receptor involved in homing to secondary lymphoid organs, and CD45RA, a cell surface molecule involved in T cell activation (16, 17). T cells that expressed both CD45RA and CCR7 were termed naive, whereas those that had down-regulated CD45RA were termed central memory (T_{CM}). Memory T cells that lacked expression of CCR7 and CD45RA were termed effector memory (T_{EM}) and upon regaining CD45RA expression were considered to be terminally differentiated (T_{EM,TD}). T_{EM} generally produced more IFN-γ than IL-2,
whereas T_{cm} produced more IL-2 than IFN-γ and had greater prolif-erative capacity (16, 18). Therefore, we hypothesized that HIV-1-infected subjects with strong HIV-1-specific CD4$^+$ T cell proliferative responses would have relatively high frequencies of HIV-1-specific CD4$^+$ T cells, whereas the HIV-1-specific CD4$^+$ T cell populations in subjects with weaker proliferative responses would be enriched in T_{cm} cells.

To investigate this hypothesis, we compared the cytokine profiles, proliferative capacities, and maturation phenotypes of HIV-1 Gag-specific CD4$^+$ T cells from two groups of CD4-matched HIV-infected subjects: those effectively treated with antiretroviral therapy vs untreated subjects with plasma viremia. We also examined the phenotypic and functional characteristics of HIV-1 specific CD4$^+$ T cells from another group of untreated subjects clinically assessed as having slowly progressive HIV-1 disease and low viral loads. Lastly, we compared the characteristics of HIV-1 specific CD4$^+$ T cells with those specific for CMV, another virus chronically present in most of our HIV-1-infected subjects. The results suggest that the phenotypic maturation state and associated functional attributes of HIV-1-specific CD4$^+$ T cells differ from those specific for CMV, vary according to disease stage and treatment status, and are heavily influenced by the degree of HIV-1 replication.

Materials and Methods
Study population
A total of 40 HIV-1-infected study subjects were selected from a cohort of HIV-1-infected individuals followed in the Adult Infectious Diseases Group Practice at the University of Colorado Health Sciences Center. HIV-1-infected subjects enrolled in this study were prospectively assigned to one of three clinical cohorts: highly active antiretroviral therapy (HAART) suppressed (HS), treatment naive (TN), or untreated with slowly progressive disease (SP). Efforts were made to CD4 match the HS and TN groups based on having a screening peripheral CD4 count in the range of 350–800 cells/μL. Ag-specific maturation studies were performed on a subset of 31 subjects. Inclusion criteria for the HS cohort (n = 13) included receiving a combination of three or more antiretroviral agents with suppression of plasma viral load to <20 copies HIV-1 RNA/ml plasma for ≥6 mo (median CD4$^+$ T cell count = 574 cells/μL; range, 340–1240); TN subjects (n = 11) were treatment naive with a median viral load of 18,415 copies HIV-1 RNA/ml plasma (range, 2,520–126,267), a median CD4$^+$ T cell count of 518 cells/μL (range, 394–754), and a median documented duration of infection of 1 year (range, 1–6 years). Subjects were prospectively enrolled into the SP cohort (n = 7) based on clinical evaluation/physician recommendation of slow progressor status and documentation of CD4 counts and HIV-1 viral load values over time. The majority of SP subjects (n = 5) had been infected for many years with low viral setpoint values and maintenance of stable CD4 counts without therapeutic intervention. Two SP subjects, however, were more recently infected but had high CD4 counts and extremely low HIV-1 plasma RNA setpoints, consistent with slow progression based on natural history studies (19). The median CD4$^+$ T cell count and viral load for the slow progressor group (n = 7) was 931 cells/μL (range, 400–1071) and 750 copies HIV-1 RNA/ml plasma (range, <200–2589), respectively, and the median documented duration of infection was 8 years (range, 2–21 years). The SP group’s median CD4 count was significantly higher (p = 0.01) and the plasma viral load was significantly lower (p = 0.0008) than for the TN group. HIV-1 seronegative subjects (n = 11) were normal healthy adult volunteers. All study subjects participated voluntarily and gave informed consent. The study was approved by the University of Colorado Health Sciences Center Institutional Review Board.

Collection and preparation of PBMCs
Blood from HIV-1 seropositive and seronegative adults was collected in Vacutainer tubes containing sodium heparin (BD Vacutainer, San Diego, CA). Within 2 h of venipuncture, PBMCs were isolated from whole blood by density gradient centrifugation on Ficoll. PBMCs for intracellular staining were rested overnight in RPMI 1640 with 10% human serum at 3 × 10^6 cells/ml. CFSE staining and whole blood maturation staining were performed immediately.

Whole blood surface staining
Whole blood (100 μl/stain) was stained with pure anti-CCR7 mAb (BD Biosciences, San Diego, CA) for 30 min at 4°C. Cells were washed once with PBS containing 1% BSA and stained with the secondary biotin-labeled anti-mouse IgM for 30 min at 4°C. Cells were washed once with PBS-BSA and stained with anti-CD4 tricolor (Caltag Laboratories, Burlingame, CA), anti-CD3 allophycocyanin (Caltag Laboratories), anti-CD45RA FITC (BD Biosciences), and streptavidin-PE for 30 min at 4°C. RBs were lysed by a 5-min incubation at room temperature with 5 ml of FACS LYSE (BD Biosciences). Cells were spun down, washed once more with PBS-BSA, and resuspended in 1% formaldehyde.

Flow cytometric detection of Ag-induced intracellular cytokines and maturation marker surface staining
The frequency of Ag-specific IFN-γ and IL-2-secreting CD4$^+$ T cells in PBMCs was determined using a previously reported method, with minor modifications (10). Briefly, 1.5–3 × 10^6 PBMCs were placed in 12 × 75-mm culture tubes containing 3 μg/ml anti-CD28 and anti-CD95 Abs (BD Biosciences) in RPMI 1640 10% human serum and one of the following stimulation conditions; pooled HXB2 strain HIV-1 Gag peptides (15 aa overlapping by 11 aa, 1 μg/ml final of each peptide (122 peptides); AIDS Reagent Program, Rockville, MD), CMV lyset (1/10 dilution, derived from G-lg-lung cell line infected with CMV strain AD169, virus titer 2 × 10^5 PFU/ml; provided by A. Weinberg, University of Colorado Health Sciences Center) or untreated control G-lg-lung cells, staphylococcal enterotoxin B (1 μg/ml; Sigma-Aldrich, St. Louis, MO), or medium alone. The cultures were incubated at a 5% slant at 37°C in a humidified 5% CO₂ atmosphere for 6 h with Brefeldin A (BD Biosciences) added after the initial 2 h. Cells were surface stained with unlabelled anti-CCR7 mAb (BD PharMingen, San Diego, CA) for 30 min at 4°C, washed once with PBS containing 1% BSA, and stained with the secondary anti-mouse IgM label with biotin for 30 min at 4°C. Cells were washed once with PBS-BSA and stained with anti-CD4 (Caltag Laboratories), anti-CD45RA (BD Biosciences), and streptavidin-PE for 30 min at 4°C. Cells were fixed and held overnight at 4°C. The next morning, cells were permeabilized and stained with anti-IFN-γ and anti-IL-2 mAbs (Caltag Laboratories) for 30 min at 4°C. Permeabilized cells were washed and resuspended in 1% formaldehyde. Samples were analyzed on a FACSCaliber flow cytometer (BD Biosciences). Generally, 400,000–1,000,000 events in the lymphocyte gate were collected.

Analysis of Ag-specific cytokine-positive cells
CellQuest software (BD Biosciences) was used to determine the frequencies of Ag-specific CD4$^+$ T cells by subtracting the percentage of IFN-γ or IL-2$^+$CD4$^+$ T cells from PBMCs stimulated with control Ag from the percentage of PBMCs stimulated with Ag. The maturation state of Ag-specific lymphocytes was determined by the expression of CCR7 and CD45RA. Cytokine-positive cells coexpressing surface CD4 were gated upon, and the percentage of those cells expressing CCR7 and CD45RA was determined. To ensure the most accurate analysis of the Ag-specific cytokine-producing lymphocytes, the percentage of CCR7$^+$ and CD45RA$^+$ cells from unstimulated PBMCs was subtracted from the percentage of cells stimulated with Ag.

Proliferation of Ag-specific CD4$^+$ T cells
PBMCs were labeled with CFSE (Molecular Probes, Eugene, OR) by incubating 6 × 10^6 cells in a 1.5-μM solution of CFSE in HBSS for 20 min at 37°C followed by two washes, before incubation with Ag. PBMCs (1 × 10^6 PBMC/ml) were cultured with HIV-1 Gag p24 Ag (5 μg/ml; NYS strain; Protein Sciences provided by the National Institutes of Health. Division of AIDS, Vaccine and Prevention Program, Bethesda, MD). HIV-1 infected Gag 15 mers (0.25 μg/ml of each overlapping peptide), CMV Ag (1/100 final dilution), or relevant controls for 6 days in RPMI 1640 10% human serum at 37°C in a humidified 5% CO₂ atmosphere. On day 6, cells were gently removed from the wells, washed with PBS containing 1% BSA, stained with anti-CD4 Tricolor (BD PharMingen), anti-CD8 allophycocyanin (BD PharMingen), and anti-CD3 PE (BD PharMingen) for 30 min at 4°C. Cells were washed again and resuspended in 1% formaldehyde. The frequency of cultured Ag-specific CD4$^+$ and CD8$^+$ T cells that divided under each stimulation condition was determined by subtracting the percentage of CFSE$^{low}$ cells from unstimulated cultures from the percentage of CFSE$^{low}$ PBMCs stimulated with Ag.
Statistical analysis

The Mann-Whitney test and the Kruskal-Wallis test with pairwise comparison (Dunn multiple comparison test) were used to determine significance of differences between subject groups. A Spearman correlation test was performed to analyze the associations among Ag-specific lymphoproliferation, IFN-γ and IL-2 frequencies, percentage of cells expressing each maturation phenotype, and plasma viral load.

Results

Association between HIV-1 Gag-specific cytokine production and plasma HIV-1 viral load

The frequencies of HIV-1 Gag-specific IFN-γ- and IL-2-producing CD4+ T cells from 14 HS subjects with undetectable viral loads, 11 TN subjects, and 7 SP subjects were determined using intracellular cytokine staining as described above (Fig. 1). The median frequencies of Gag-specific IFN-γ-producing CD4+ T cells were 0.13% (range, 0–0.64%) for the TN subjects, 0.07% (0.04–0.33%) for the SP, and 0.015% (0–0.08%) for the HS group (Fig. 1A). ANOVA (Kruskal-Wallis test) revealed that TN with progressive disease and the highest plasma viral loads had significantly higher frequencies of Gag-specific IFN-γ-producing CD4+ T cells than did treated HS subjects \( (p < 0.05) \). TN subjects also had higher median Gag-specific IFN-γ-producing CD4+ T cell frequencies than did SP subjects, although these differences did not reach statistical significance. When the frequency of Gag-specific IFN-γ-producing CD4+ T cells for each HIV-1-infected subject was plotted against the respective plasma HIV-1 RNA viral load, a significant positive correlation was noted \( (r = 0.50, p = 0.002) \) (Fig. 1B).

Conversely, the highest frequencies of Gag-specific IL-2-producing CD4+ T cells were measured in PBMCs from subjects with the lowest viral loads. The median frequencies of Gag-specific IL-2-producing CD4+ T cells were 0.08% (range, 0–0.26) for the HS subjects, 0.06% (0.04–0.09) for the SP subjects, and 0.02% (0–0.24%) for TN subjects (Fig. 1C). ANOVA revealed a significant difference in frequencies between the HS and TN groups \( (p < 0.05) \) and differences in frequencies between the HS and SP groups that trended toward significance. When the frequency of Gag-specific IL-2-producing CD4+ T cells from each HIV-1-infected subject was plotted against the respective plasma HIV-1 viral load, an inverse correlation was noted \( (r = -0.42, p = 0.01) \) (Fig. 1D).

The frequencies of Gag-specific IFN-γ- and IL-2-producing CD4+ T cells from individual subjects within each cohort are compared in Fig. 2. The frequencies of Gag-specific IL-2-producing CD4+ T cells were higher than the frequencies of IFN-γ-producing CD4+ T cells in PBMCs from 13 of the 14 HS subjects (Fig. 2A). Conversely, the frequencies of Gag-specific IFN-γ-producing CD4+ T cells were higher than the frequencies of IL-2-producing CD4+ T cells in 9 of the 11 TN subjects with progressive disease and plasma viremia (Fig. 2B). The relationship between IL-2- and IFN-γ-producing CD4+ T cell frequencies was less consistent in the SP group, with three of seven subjects exhibiting higher frequencies of Gag-specific IL-2 than IFN-γ-producing CD4+ T cells and the remainder with higher IFN-γ- than IL-2-producing CD4+ T cells (Fig. 2C). These data show that HIV-1 Gag-specific CD4+ T cells predominantly produced IL-2 in treated subjects without measurable plasma viremia, whereas IFN-γ-producing CD4+ T cells predominated in subjects with unchecked HIV-1 replication.

Inverse relationship between HIV-1 p24-specific CD4+ T cell proliferation and plasma HIV-1 viral load

We previously reported that many subjects successfully treated with HAART had measurable HIV-1 p24-specific lymphoproliferative responses, whereas most subjects with active viral replication and chronic progressive disease displayed a p24-specific lymphoproliferative defect (12). To evaluate whether subjects in the current study also displayed this pattern, we assessed the p24-specific proliferative capacity of CD4+ T cells from a subset of HIV-1-infected subjects from each clinical cohort (five HS, nine TN, and seven SP) and nine HIV-1 seronegative controls by incubating CFSE-labeled PBMCs with p24 Ag for 6 days (Fig. 3). On day 6, CD4+ T cell proliferation was determined by the decrease in fluorescence of CFSE-labeled CD4+ T cells as described in Materials and Methods. Fig. 3A shows a representative example of the CFSE staining of CD4+ T cells stimulated with HIV-1 p24 or control Ag. Consistent with our previous studies, a significant p24-specific proliferative defect was observed in CD4+ T cells obtained from untreated, viremic subjects (Fig. 3B). The median percentage of CFSElow proliferating CD4+ T cells, with the background subtracted, was 2.1% (range, 0–13.1%) in the HS group, 0.27% (range, 0–1.0%) in the TN group, 2.4% (range, 0.93–6.8) in the SP group, and 0.17 (range, 0–2.4%) in HIV-1 seronegative controls (Fig. 3B). The median percentage of proliferating (CFSElow) CD4+ T cells from TN subjects was lower than the

**FIGURE 1.** Frequencies of HIV-1 Gag-specific IFN-γ- and IL-2+ CD4+ T cells from HIV-1-infected subjects receiving HAART with suppressed viral loads (HS), treatment naive with progressive disease (TN), or untreated with slowly progressive disease (SP) (A and C) and correlations with HIV-1 plasma viral load (B and D). Frequencies of Gag-specific cytokine-producing CD4+ T cells were determined by intracellular cytokine staining. Plasma viral loads were determined using the Roche HIV-1 RNA Monitor kit (Roche, Basel, Switzerland). Values for each subject are depicted as separate points and the bars represent the median value for each group. Statistical significance of differences between subject groups was determined by Kruskal-Wallis test with pairwise comparison (A and C) or the Spearman correlation test (B and D).
percentage proliferating from SP ($p < 0.05$) and HS, but was not significantly different from the percentage proliferating from the HIV-1 seronegative control group. These data demonstrated that HIV-1-specific CD4$^+$ T cell proliferative responses in untreated, viremic subjects with progressive disease were much lower than those observed in subjects whose viral loads were either extrinsically or intrinsically suppressed.

To more closely examine the association between HIV-1 p24-specific CD4$^+$ T cell proliferation and HIV-1 replication, the percentage of CD4$^+$ T cells proliferating in response to p24 Ag for each HIV-1-infected subject was plotted against the respective plasma HIV-1 RNA viral load. This analysis negated biases that may have resulted from grouping subjects according to clinical characteristics. When all HIV-1-infected subjects were included in this analysis, a significant inverse correlation was observed between p24-specific proliferation and plasma viral load ($r = -0.43$, $p = 0.05$, data not shown). This significant inverse relationship was maintained when only untreated subjects were included in the analysis ($r = -0.66$, $p = 0.0095$, data not shown), suggesting that this finding did not simply represent a treatment effect.

**Characterization of the total memory T cell population**

Because T cell maturation state has been linked to T cell function, we studied the maturation state of the total CD4$^+$ T cell compartment in HIV-1-infected subjects in each clinical cohort described above ($n = 36$) and in seronegative control individuals ($n = 11$). Surface expression of CD45RA and CCR7 was examined on the total CD4$^+$ T lymphocyte population in whole blood (Fig. 4), and the phenotype ascribed to a particular maturation state of the
CD4+ T cells was designated as previously described (16). Fig. 4A shows a representative example of the CD4+ T cell maturation staining in whole blood. No significant differences ($p = 0.83$) were noted between subject groups in the percentage of total CD4+ T cells expressing a naive phenotype (CD45RA+, CCR7+) (Fig. 4B). The percentage of CD4+ T cells expressing central memory phenotype (TCM, CD45RA-, CCR7+) ranged from 13.9% to 71.4% (Fig. 4C). Although there were no statistically significant differences ($p = 0.12$) between any of the groups in the median percentages of CD4+ TCM, there was a trend toward a relative decrease in TCM in TN subjects, with a median value of 37.2 vs 46.5% for the HS group and 47.2% in seronegative controls. The percentages of CD4+ T cells with effector memory phenotype (TEM, CD45RA-, CCR7+) were generally lower than percentages of TCM in all cohorts (Fig. 4D). No significant differences ($p = 0.35$) were observed in the median percentages of TEM in any of the four cohorts, although a slightly higher median TEM percentage was measured in the TN group (16.5%) relative to the HS, SP, and seronegative cohorts (median percentages of 11.6, 11.8, and 11.7%). The TEMTD population (CD45RA+, CCR7-) made up the smallest percentage of the total CD4+ T cell population, and no statistically significant differences ($p = 0.48$) or trends were observed between groups in the percentage of CD4+ T cells expressing this maturation phenotype (Fig. 4E). In summary, there were no statistically significant differences in the maturation phenotypes of the total CD4+ T cell compartments between any of the subject groups evaluated; however, there was a trend toward lower percentages of TCM and higher percentages of TEM in subjects with high viral loads and progressive disease.

**HIV-1 viral load influences Gag-specific CD4+ T cell maturation**

We next directly examined the maturation state of the HIV-1 Gag-specific CD4+ T cells, a memory population that generally makes up a small fraction of the total CD4+ T cell pool. To determine the maturation state of HIV-1-specific CD4+ T cells, PBMCs were cultured for 6 h with HIV-1 Gag overlapping 15-mer peptides, and expression of CCR7 and CD45RA on Gag-specific IFN-γ or IL-2+ CD4+ T cells was then examined (representative example, Fig. 5). To ensure that enough events could be collected to allow a statistically valid analysis, maturation markers were only evaluated on PBMCs from the subset of subjects with Gag-specific IFN-γ-producing CD4+ T cells from five HS, six TN, and seven SP subjects were determined and compared in Fig. 6, A–C. The percentage of Gag-specific, IFN-γ+ CD4+ T cells with TCM phenotype was found to be significantly lower for the TN group (median, 10.25%; range, 5.8–25.5%) than for either the HS group (37.9%; range, 33.9–58.4%; $p < 0.0001$) or the SP group (39.5%; range, 28.1–67.0%; $p < 0.01$) (see Fig. 6A). Conversely, the median percentage of Gag-specific, IFN-γ+ CD4+ T cells with TEM phenotype was significantly higher in the TN group (79.6%; range, 65.8–93.2%) than in either the HS group (52%; range, 24.2–66%; $p < 0.0001$) or the SP group (35.5%; range, 24.9–69.1%; $p < 0.01$) (Fig. 6B). Gag-specific, IFN-γ+ CD4+ T cells with EmTD phenotype were rare or undetectable in most subjects, with no significant differences ($p = 0.88$) among groups in the percentages of these cells.

As shown in Fig. 1, most TN subjects had extremely low or undetectable frequencies of Gag-specific, IL-2-producing CD4+ T cells, thus weakening the comparative analysis. As a result, using inclusion criteria similar to those above of frequencies ≥0.04%, the maturation analysis of Gag-specific, IL-2-producing CD4+ T cells was limited to eight HS, three TN, and six SP subjects. In contrast with the findings in Gag-specific, IFN-γ+ CD4+ T cells, most Gag-specific, IL-2-producing CD4+ T cells in all subjects expressed a CM phenotype (Fig. 6D). No significant differences in the median percentages of Gag-specific, IL-2-producing CD4+ TCM, TEM or TEMTD were found among the groups of HIV-1-infected subjects studied (Fig. 6, D–F).

**Associations among HIV-1 Gag-specific CD4+ T cell maturation state, CD4+ T cell proliferation, and HIV-1 plasma viral load**

To better understand the influence of HIV-1 replication on CD4+ T cell maturation and function, the associations among HIV-1 Gag p24-specific CD4+ T cell proliferation as determined by CFSE staining, memory maturation phenotypes of Gag-specific IFN-γ-producing CD4+ T cells, and plasma viral load were explored in a subset of HIV-1-infected subjects. Fig. 7, A and B, depict the correlations between p24-specific CD4+ T cell proliferation and percentage of Gag-specific, IFN-γ+ CD4+ TCM and TEM in a subset of 14 subjects (two HS, five TN, and seven SP) with significant
cytokine secreting CD4 T cells from HIV-infected subjects. The relationship between the maturation state of Gag-specific IFN-γ and IL-2-producing CD4+ T cells from the TN subjects exhibited a lower percentage of Cm and higher percentage Em phenotype than did cells from HS and SP subjects (Fig. 8A). This skewing toward TEm in viremic subjects was similar but less pronounced than that observed for HIV-1-specific IFN-γ-producing CD4+ T cells (Fig. 6, A and B). No statistically significant differences in the maturation states of CMV-specific IL-2-producing CD4+ T cells among the three groups of HIV-infected subjects were noted (Fig. 8E).

In comparing the function and phenotype of HIV-1 Gag-specific vs CMV-specific CD4+ T cells in all HIV-1-infected subjects, several major differences were noted. First, frequencies of CD4+ T cells specific for CMV were higher than those specific for HIV-1 Gag. This was true for frequencies measured by IFN-γ production (Figs. 1A and 8A; p = 0.0004) and IL-2 production (Figs. 1B and 8B; p = 0.0398). These differences in virus-specific CD4+ T cell frequencies may in part reflect the greater number of potential immune targets in the CMV lysate, which presumably contains both structural and nonstructural CMV proteins. Second, median CMV-specific proliferation among all subjects was significantly higher than median HIV-specific proliferation (Figs. 3A and 8C; p = 0.0058). Third, HIV-1- and CMV-specific IFN-γ-producing CD4+ T cells showed different patterns of maturation. In 11 subjects with significant frequencies of both CMV and HIV-1 Gag-specific CD4+ T cells, percentages of Tem were significantly higher in the CMV than HIV-1-specific IFN-γ-producing CD4+ T cell populations.
Discussion

The goal of this study was to delineate the mechanisms underlying the proliferative dysfunction of HIV-1-specific CD4⁺ T cells that have been observed in the setting of HIV-1 plasma viremia (11–13). To determine how HIV-1 replication affects CD4⁺ T cell phenotype and function, we evaluated HIV-1-specific CD4⁺ T cells from several clinical cohorts of HIV-1-infected subjects: subjects with untreated progressive disease and associated high level plasma viremia, CD4-matched subjects effectively treated with HAART with suppressed viral loads, and subjects with slow disease progression and associated lower viral loads and higher CD4⁺ counts. The two low viral load cohorts, treated subjects and slow progressors, were evaluated separately to determine whether extrinsic (i.e., antiretroviral therapy) and intrinsic causes of viral suppression differentially influenced T cell phenotype and function. We observed both functional and phenotypic differences in HIV-1 Gag-specific CD4⁺ T cells depending on the disease stage, treatment status, and viral load of the HIV-1-infected subjects studied.

We found that untreated subjects with elevated viral loads had relatively high frequencies of Gag-specific, IFN-γ⁺ CD4⁺ T cells and relatively low frequencies of Gag-specific, IL-2⁺ CD4⁺ T cells, whereas the converse was true for subjects with low viral loads. These relationships were evident in statistically significant differences between clinical subject groups. Importantly, correlations between viral load and frequencies of Gag-specific, cytokine-producing cells (positive for viral load vs IFN-γ⁺ and negative for viral load vs IL-2⁺) were observed when the data for all subjects were analyzed without grouping based on treatment status or disease progression. The high frequencies of IFN-γ⁺ cells in subjects with higher viral loads are consistent with some prior studies of HIV-1-specific CD4⁺ T cells. In the CD4⁺ compartment, Pitcher et al. (10) found that Gag-specific IFN-γ⁺ cells were more frequent in untreated than in treated subjects. However, although Betts et al. (20) reported a positive correlation between viral load and frequencies of Gag-specific, IFN-γ⁺, CD8⁺ T cells in infected subjects, they and others have not consistently found...
such a relationship between viral load and HIV-specific CD4$^+$ T cell frequencies (11–13, 20, 21). Nevertheless, the IFN-γ data presented here, taken alone, could be interpreted as showing that high-level HIV-1 viremia simply boosts the frequencies of HIV-1-specific CD4$^+$ T cells.

On the contrary, the results of intracellular IL-2 staining experiments in the present study suggest a more complex relationship between HIV-1 viremia and HIV-1-specific CD4$^+$ T cell frequencies. In particular, we observed that subjects with low or undetectable viral loads, regardless of treatment status, had higher frequencies of Gag-specific, IL-2$^+$ cells than did TN subjects with higher viral loads. This suggests that high-level viremia may skew the cytokine profiles of Gag-specific CD4$^+$ T cells away from IL-2 and toward IFN-γ production. Consistent with this, Boaz et al. (22) found that frequencies of Gag-specific CD4$^+$ T cells producing both IL-2 and IFN-γ, rather than those producing IFN-γ alone, correlated inversely with viral load and were markers of nonprogressive disease. Although this finding could be interpreted to show a protective effect of IL-2 production compared with IFN-γ, alone in suppressing viral replication, the present study also found elevated Gag-specific, IL-2$^+$ CD4$^+$ T cell frequencies in subjects treated with antiretroviral therapy. Because many of these subjects would presumably have had high-level viremia and progressive disease without treatment, our results suggest that high frequencies of Gag-specific, IL-2-producing cells may be an effect rather than a cause of viral suppression. Importantly, however, our results do not exclude the possibility that a subset of HIV-1-specific, IL-2-producing CD4$^+$ T cells, particularly in subjects with slowly progressive disease and low viral loads, may play a direct or indirect role in the immunologic control of HIV-1 replication, as has been previously suggested (11, 22).

Consistent with our previous study and other published reports (11–13, 23), we observed an inverse relationship between plasma HIV-1 RNA levels and proliferation, with diminished HIV-1-specific CD4$^+$ T cell proliferation in untreated progressors and strong proliferation limited to subjects with low viral loads. Because the ratios of Gag-specific, IFN-γ$^+$ cell frequencies to Gag-specific, IL-2$^+$ cell frequencies were inverted in these different subject groups, we hypothesized a relationship between proliferative capacity and cytokine profile. Furthermore, we hypothesized that low viral loads might allow for strong HIV-1-specific CD4$^+$ T cell proliferation because elevated viremia drives HIV-1-specific CD4$^+$ T cells toward a maturation state in which their cytokine profile and other functional characteristics are inconsistent with strong proliferation.

A number of recent studies have described both CD4$^+$ and CD8$^+$ T cell function in relation to maturation or differentiation phenotype (16, 24). The phenotypes of these T cell populations have been differentiated by analyzing the expression of a number of surface molecules, such as CCR7, CD45RA, CD28, and CD27 (16, 25, 26). Sallustio et al. (16) revealed two functionally distinct populations of human memory CD4$^+$ T cells based on expression of CCR7 and CD45RA. These two CD45RA$^-$ memory CD4$^+$ T cell populations responded differently to polyclonal stimulation, with CD4$^+$ T cell proliferation (CCR7$^+$) producing more IL-2 than CD4$^+$ T cell proliferation (CCR7$^-$), which were superior at producing IFN-γ and IL-4/IL-5. It has also been shown that CD8$^+$ T cells have an increased proliferative potential when compared with TEm, presumably due to increased production of IL-2 (18). Although proliferation of CD4$^+$ T cell memory subsets is clearly dependent on the type of stimulus and cytokine environment (24), it stands to reason that the ability to produce IL-2 might also enhance the proliferative capacity of virus-specific CD4$^+$ T cells in vivo. This maturation model holds that differentiation primarily occurs linearly as follows: naive (CD45RA$^+$CCR7$^+$) to TEm ($^+$CCR7$^+$), producing more IL-2 than CD4$^+$ T cell proliferation (CCR7$^+$), which were superior at producing IFN-γ and IL-4/IL-5. There is also evidence that CD8$^+$ T cells have an increased proliferative potential when compared with TEm, presumably due to increased production of IL-2 (18).

Based on this paradigm and our previous findings, we hypothesized that subjects with high viral loads would have increased frequencies of poorly proliferating CD4$^+$ T cells, whereas subjects with low viral loads would have increased frequencies of proliferation competent Tcm cells. When the maturation states of the total CD4$^+$ T cell populations in the HIV-1-infected groups and seronegative controls were evaluated, no statistically significant differences in the surface expression of CCR7 and CD45RA were observed. However, a trend toward increased TEm and decreased Tcm percentages among untreated subjects with active viral replication compared with the other HIV-1-infected groups and controls was noted. Significant differences in maturation phenotype of HIV-1-specific CD4$^+$ T cells might not be detected in the total CD4$^+$ T cell population, because HIV-1-specific CD4$^+$ T cells constitute only a small fraction of the total CD4$^+$ T cell compartment.

To address the hypothesis that viral load influenced HIV-1-specific CD4$^+$ T cell maturation and that differences in CD4$^+$ T cell memory populations accounted for the variation seen in T cell function among subject cohorts, we investigated the maturation phenotype of HIV-1 Gag-specific CD4$^+$ T cells according to their production of IFN-γ and IL-2. Consistent with the findings of Sallustio et al. (16), we noted that the Cm phenotype was more frequent among IL-2- than IFN-γ-producing CD4$^+$ T cells, whereas the Em phenotype was more frequent among IFN-γ- than IL-2-producing cells. More interestingly, when maturation phenotypes.
of HIV-1-specific IFN-γ-producing CD4⁺ T cells were compared between subject cohorts, significantly lower Cm and higher Em phenotype expression was observed in TN, viremic subjects than in either treated subjects or those with slow disease progression and lower viral loads. Further analyses revealed significant correlations between maturation phenotype and levels of plasma HIV-1 viremia, with virus load inversely related to Cm phenotype and directly related to Em phenotype. One limitation of this analysis was the necessary restriction of evaluating maturation phenotype only in those subjects with significant frequencies of either IL-2⁺ or IFN-γ-producing cells, thus decreasing sample size. Yet, despite these sample size limitations, the associations of maturation phenotype with HIV-1 viral load were quite strong, suggesting that the degree of HIV-1 replication significantly determined the maturation or differentiation state of HIV-1-specific CD4⁺ T cells.

The maturation profiles of virus-specific memory T cells and the role that different subsets may play in mediating virologic control have been recently described in several chronic viral infections (14, 15, 18, 27, 28). There is increasing evidence that chronic infection with different viruses may induce distinct subsets of virus-specific T cells, which may be more or less effective at controlling viral replication (15, 18). In particular, a number of reports showed that CMV-specific CD8⁺ T cells tended to accumulate at later stages of differentiation than did HIV-1-specific CD8⁺ T cells (15, 27). Virus-specific CD4⁺ T cells play a critical role in orchestrating antiviral immune responses, yet few studies of humans have evaluated the maturation profiles of these cells. Harari et al. (28) evaluated CCR7 expression on CMV- and HIV-1-specific IFN-γ-producing cells in primary and chronic HIV-1 infection and found that the majority of Ag-specific cells lacked CCR7 expression, consistent with an effector phenotype. In this study, we compared frequencies and phenotypes of CMV- and HIV-1-specific
CD4+ T cells in several cohorts of HIV-1-infected subjects. Our observation that a higher percentage of CMV-specific than HIV-1-specific IFN-γ-producing CD4+ T cells expressed both Em and EmTD phenotypes mirrors differences in reported maturation profiles of HIV-1- and CMV-specific CD8+ T cells (15, 27).

Overall, the major finding of our study is that HIV-1 viremia may drive HIV-1-specific CD4+ T cells to an Em state in which they produce little IL-2 and proliferate poorly. This finding may help to clarify previous studies linking HIV-1-specific CD4+ T cell proliferation and IL-2 production with low viremia and non-progressive disease (22, 23). These studies and ours are still consistent with HIV-1-specific immune protection mediated by HIV-1-specific CD4+ T cells that produce IL-2, proliferate, and express a Cm phenotype. In fact, if CD4+ TEm do contribute to effective HIV-1-specific immunity, then the skewing away from this maturation phenotype observed in HIV-1-specific CD4+ T cells from untreated subjects with progressive disease might reflect a key pathogenic mechanism in chronic HIV-1 infection. Nevertheless, the relatively high frequencies of these cells detected in subjects whose viral loads were controlled extrinsically (i.e., by HAART) suggest that TEM cells may arise as a consequence of viral suppression without necessarily helping to cause that suppression. Additional studies will be required to determine whether and how HIV-1-specific CD4+ T cells that produce IL-2, proliferate, and express a Cm phenotype might influence HIV-1-specific immune protection.

An important limitation of this study is that cytokine profiles, proliferation, and maturation state were only examined in CD4+ T cells from the peripheral blood. As a result, we cannot say with certainty that HIV-1-infected subjects with elevated viral loads have more TEM and fewer TEM in their total body HIV-1-specific, CD4+ T cell pools. It is possible that in subjects with active HIV-1 replication, HIV-1-specific CCR7+ cells could home to the lymph nodes, where the majority of the viral replication takes place, leaving mostly Em cells in the peripheral blood. However, recent studies of SIV-specific CD8+ T cells in monkeys (29) and HIV-1-specific CD8+ T cells in humans (30) have found qualitatively and quantitatively similar responses in the blood and lymph nodes, making this explanation of our findings less likely.

Therefore, we consider it more likely that active HIV-1 replication causes a change in the proportions of TEM and TEM in the total body HIV-1-specific CD4+ T cell pool. This may occur because Ag burden influences T cell maturation. This theory is consistent with studies of virus-specific CD8+ T cells showing that certain memory phenotypes predominate when Ag loads are absent or low (14). It is also consistent with a study by Harari et al. (28) that reported higher percentages of p55-specific CCR7+ than CCR7+ cells in subjects with high HIV-1 viremia as well as higher frequencies of CMV-specific CCR7+ CD4+ T cells in subjects with detectable plasma CMV DNA. Alternatively, the differences in T cell maturation between the different clinical groups might be due to differences in cytokine environments. Many studies have described increased production of proinflammatory cytokines in HIV-1-infected subjects with active viral replication (31, 32), and there is in vitro evidence that certain cytokines may drive the generation of CD4+ TEM from a pool of TEM, even in the absence of antigenic stimulation (24). Thus, it is possible that the abnormal cytokine milieu found in untreated HIV-1-infected subjects may cause skewed maturation of HIV-specific CD4+ T cells. This would also explain the slight skewing of CMV-specific CD4+ T cell maturation that we have seen in subjects with untreated progressive disease and higher viral loads.

Taken together, these data support the hypothesis that chronic HIV-1 replication influences the maturation state of virus-specific CD4+ T cells. Furthermore, these results may help to explain the etiology of the CD4+ T cell proliferative “defect” observed during chronic HIV-1 infection and its association with HIV-1 replication. Although the associations observed between proliferation and maturation phenotype were indirect in nature, our data suggest that the observed loss of HIV-1-specific lymphoproliferative responses in viremic subjects may in part reflect a physiologic change from a predominance of proliferation-competent IL-2–producing CD4+ TEM cells to TEM cells with decreased proliferative potential. Furthermore, chronic HIV-1 replication may either push HIV-1-specific CD4+ T cells to, or block cells from, progressing out of the Em state, although our evaluation of HAART-treated subjects suggests that this maturation skewing may be reversed with treatment. Our results further indicate that HIV-1-specific CD4+ T cell maturation phenotypes, and associated functional characteristics, are strongly influenced by the degree of HIV-1 replication. The role that different CD4+ T cell maturation subsets play in mediating virologic control, and the clinical consequences of these virus-driven changes in CD4+ T cell maturation phenotype, remain to be determined in future studies.

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References


