Human Immunodeficiency Virus Type 1 (HIV-1)-Specific CD4⁺ T Cells That Proliferate In Vitro Detected in Samples from Most Viremic Subjects and Inversely Associated with Plasma HIV-1 Levels

Eli Boritz,¹ Brent E. Palmer,² and Cara C. Wilson^{1,2*}

Departments of Immunology¹ and Medicine,² University of Colorado Health Sciences Center, Denver, Colorado

Received 31 March 2004/Accepted 12 July 2004

Diminished in vitro proliferation of human immunodeficiency virus type 1 (HIV-1)-specific CD4⁺ T cells has been associated with HIV-1 viremia and declining CD4⁺ T-cell counts during chronic infection. To better understand this phenomenon, we examined whether HIV-1 Gag p24 antigen-induced CD4⁺ T-cell proliferation might recover in vitro in a group of subjects with chronic HIV-1 viremia and no history of antiretroviral therapy (ART). We found that depletion of CD8⁺ cells from peripheral blood mononuclear cells (PBMC) before antigen stimulation was associated with a 6.5-fold increase in the median p24-induced CD4⁺ T-cell proliferative response and a 57% increase in the number of subjects with positive responses. These p24-induced CD4⁺ T-cell proliferative responses from CD8-depleted PBMC were associated with expansion of the numbers of p24-specific, gamma interferon (IFN- γ)-producing CD4⁺ T cells. Among the 20 viremic, treatment-naïve subjects studied, the only 5 subjects lacking proliferation-competent, p24-specific CD4⁺ T-cell responses from CD8-depleted PBMC showed plasma HIV-1 RNA levels > 100,000 copies/ml. Furthermore, both the magnitude of p24-induced CD4⁺ T-cell proliferative responses from CD8-depleted PBMC and the frequency of p24-specific, IFN- γ -producing CD4⁺ T cells expanded from CD8-depleted PBMC were associated inversely with plasma HIV-1 RNA levels. Therefore, proliferation-competent, HIV-1-specific CD4⁺ T cells that might help control HIV-1 disease may persist during chronic, progressive HIV-1 disease except at very high levels of in vivo HIV-1 replication.

Human immunodeficiency virus type 1 (HIV-1) may avoid immune system-mediated clearance during chronic infection in part by targeting HIV-1-specific CD4⁺ T-helper cells for depletion. T-cell receptor (TCR) signaling favors productive infection of CD4⁺ T cells (28), thus exposing these cells to viral cytopathic effects and recognition by HIV-1 antigen-specific cytotoxic T lymphocytes (CTL). As a consequence, replicating HIV-1 may preferentially activate, infect, and kill HIV-1-reactive CD4⁺ T cells as it disseminates throughout the lymphoid tissues during early disease (17). Consistent with this, HIV-1-specific CD4⁺ T cells have been shown to harbor HIV-1 DNA more frequently than CD4⁺ T cells of other specificities (7). Furthermore, functional defects in CD4⁺ T-cell-dependent, HIV-1-specific immune responses have been observed over the course of chronic HIV-1 disease. For example, HIV-1-specific CD8⁺ T cells have been found to proliferate poorly and express relatively little perforin in subjects with untreated HIV-1 replication (19). Such defects resemble those observed in murine models which lack CD4⁺ T cells (13, 16, 27).

Despite these considerations, HIV-1-specific CD4⁺ T cells are not completely depleted during the early phase of infection. Instead, HIV-1-specific CD4⁺ T cells are often detected by gamma interferon (IFN- γ) production well into chronic, progressive disease (24, 32). Moreover, the vast majority (ap-

proximately 90%) of these cells appear not to harbor HIV-1 DNA (7). This might suggest that some degree of HIV-1-specific CD4⁺ T-cell immunity persists during chronic, progressive disease. Nevertheless, higher frequencies of HIV-1-specific, IFN- γ -producing CD4⁺ T cells do not significantly predict lower viremia among untreated subjects (3). One interpretation of this is that HIV-1-specific, IFN- γ -producing CD4⁺ T cells detected in most untreated subjects fail to contribute to protective HIV-1-specific immune responses.

The lack of association between HIV-1-specific, IFN- γ -producing CD4⁺ T cells and lower HIV-1 replication in vivo may relate to the proliferative defect of these cells. The proliferative defect emerged from studies showing significant frequencies of HIV-1-specific, IFN- γ -producing CD4⁺ T cells in viremic subjects who nevertheless showed weak HIV-1-induced CD4⁺ T-cell proliferation (18, 22, 32). Because HIV-1 suppression on antiretroviral therapy (ART) has been associated with stronger HIV-1-specific CD4⁺ T-cell proliferation from such subjects (1, 2, 18, 22), some have suggested that active HIV-1 replication suppresses proliferation of HIV-1-specific CD4⁺ T cells (18, 22). More recent studies have suggested that the mechanism of suppressed proliferation may be loss of interleukin-2 (IL-2) expression (11, 12, 23, 33). As IL-2 expression in vivo may allow expansion and maintenance of HIV-1-specific CD4⁺ T cells as well as help for HIV-1-specific CD8⁺ T cells (33), HIV-1-induced loss of IL-2 expression may compromise HIV-1-specific immune protection.

Here we further investigate diminished in vitro proliferation of HIV-1-specific CD4⁺ T cells during chronic HIV-1 viremia.

* Corresponding author. Mailing address: University of Colorado Health Sciences Center, Campus Box B-164, 4200 East 9th Ave., Denver, CO 80262. Phone: (303) 315-6659. Fax: (303) 315-7642. E-mail: Cara.Wilson@UCHSC.edu.

In particular, we examine whether this diminished proliferation is reversible and whether residual proliferative responses are associated with clinical laboratory measurements of disease severity. Surprisingly, we find that HIV-1 Gag p24 antigen-specific CD4⁺ T cells from the peripheral blood mononuclear cells (PBMC) of untreated, viremic subjects proliferate readily following CD8⁺ cell depletion of PBMC. This confirms that HIV-1-specific CD4⁺ T cells from viremic subjects retain an intrinsic proliferative capacity not apparent in standard assays. However, in subjects with plasma HIV-1 loads of >100,000 copies/ml, we find no evidence of p24-specific CD4⁺ T-cell proliferative responses by the use of either proliferation or IFN- γ production assays. These results are consistent with a lack of HIV-1-specific CD4⁺ T-helper cell immunity at only the highest levels of chronic HIV-1 replication in vivo.

MATERIALS AND METHODS

Study subjects. A total of 25 HIV-1-infected subjects were recruited from the Adult Infectious Diseases Group Practice at the University of Colorado Hospital. Subjects with plasma HIV-1 RNA levels > 1,000 copies/ml and no current ART were selected for this study. Subjects with suspected acute or recent infection were excluded. A total of 20 subjects with no history of ART (i.e., "ART-naïve" subjects) were selected for detailed evaluation. Negative-control samples were obtained from 10 laboratory workers self-identifying as HIV-1 negative. All subjects gave informed consent to participate in this study, and the study was approved by the University of Colorado Health Sciences Center Institutional Review Board.

Fresh IFN- γ enzyme-linked immunospot (ELISPOT) assays. PBMC were isolated from heparinized blood by density gradient centrifugation. CD8⁺ cells were depleted from PBMC by use of CD8 microbeads (Miltenyi Biotec, Auburn, Calif.) according to the manufacturer's instructions, and CD8-depleted PBMC were resuspended to a concentration of 2×10^6 cells/ml in AIM-V medium (Invitrogen, Carlsbad, Calif.). Cells were plated at 100 μ l/well (2×10^5 cells/well) in 96-well, nitrocellulose-backed plates (Millipore, Bedford, Mass.) that had been coated with a solution of 5 μ g of anti-IFN- γ monoclonal antibody (mouse immunoglobulin G1 [IgG1]) (1-D1K; Mabtech, Nacka, Sweden)/ml of phosphate-buffered saline (PBS), washed three times, and blocked with standard culture medium (RPMI 1640 [Invitrogen], 10% human AB serum [Gemini Bio-Products, Woodland, Calif.], penicillin-streptomycin-glutamine [Invitrogen]). Stimuli used in these assays included 1 μ g of phytohemagglutinin (PHA; Murex Diagnostics, Dartford, United Kingdom)/ml; pooled 20- and 15-amino-acid (aa) peptides spanning the p24 amino acid sequence at 1 μ g of each peptide (HIV-1 HXB2 Gag aa positions 131 to 363; supplied by the National Institutes of Health AIDS Research and Reference Reagent Program)/ml; and dimethyl sulfoxide (DMSO) at 0.2% (vol/vol) as a control for the solvent used in peptide preparations. Monoclonal antibody to human CD28 (eBiosciences, San Diego, Calif.) was included at a final concentration of 1 μ g/ml in p24 peptide pool and DMSO (i.e., medium alone) stimulations. Stimuli were prepared at $2 \times$ concentrations in AIM-V medium and added to triplicate wells of cells at 100 μ l/well. Plates were then incubated for 36 to 40 h at 37°C in 5% CO₂, washed with PBS-0.05% Tween-20, and incubated at 37°C for 2 h with 2 μ g of biotinylated anti-IFN- γ monoclonal antibody (mouse IgG1) (7-B6-1; Mabtech)/ml. Avidin-biotinylated enzyme complex from a Vectastain ABC Elite kit (PK-6100; Vector Laboratories, Burlingame, Calif.) was added at room temperature for 1 h followed by the avidin-biotinylated enzyme complex peroxidase substrate for 5 min. Following development, plates were rinsed with double-distilled water and dried, and spots were counted using a dissecting microscope. The net p24-specific response per million input cells was calculated according to the following formula: [(median p24-induced spot-forming cells (SFCs)/well) - [median medium-induced SFCs/well]] $\times$ 5. Net p24-specific responses of 4 SFCs/well (20 SFCs/million) greater than the median net p24-specific response from the negative-control group were considered positive.

Expanded IFN- γ ELISPOT assays. CD8-depleted PBMC (1×10^7 to 3×10^7) were suspended at 5×10^6 cells/ml in standard culture medium and stimulated with 5 μ g of recombinant HIV-1 Gag p24 antigen (Protein Sciences, Meriden, Conn.)/ml in 6-well polystyrene plates. In some experiments, additional compounds were added to these cultures in an attempt to improve p24-specific CD4⁺ T-cell expansion. These included 1 μ g of CD28 monoclonal antibody/ml, 1 μ g of CD40 ligand trimer (Immunex, Seattle, Wash.)/ml, 50 units of recombinant hu-

FIG. 1. Frequencies of HIV-1 p24-specific, IFN- γ SFCs in CD8-depleted, p24-expanded PBMC of viremic, ART-naïve subjects. PBMC were depleted of CD8⁺ cells and cultured with recombinant p24 antigen and the indicated compounds for 7 days. Expanded cells were then stimulated in IFN- γ ELISPOT assays with pooled p24 peptides or medium alone as a negative control, and the net p24-specific response was calculated by subtracting the medium-stimulated response from the p24-stimulated response. Factors tested included monoclonal CD28 antibody (CD28; open bars), human CD40 ligand trimer (CD40LT; hatched bars), recombinant human interleukin-2 (IL-2; filled bars), recombinant human interleukin-12 (IL-12; open bars), and the nucleoside reverse transcriptase inhibitor didanosine (ddI; hatched bars). All seven subjects were studied using all the indicated factors except for subject UH136, with whom IL-2 and IL-12 were not tested.

man interleukin-2 (IL-2) (teceleukin; Hoffman LaRoche, Nutley, N.J.)/ml, 5 ng of recombinant human interleukin-12 (IL-12; R&D Systems Inc., Minneapolis, Minn.)/ml, and 10 μ M didanosine (ddI; Sigma-Aldrich, Saint Louis, Mo.). Cells were fed with a 0.5 $\times$ volume (1 to 3 ml) of standard culture medium after 2 days of incubation at 37°C in 5% CO₂. After 7 days, the cells were gently scraped from the wells, CD8 was depleted again to remove residual CD8⁺ cells, and the cells were resuspended in standard culture medium and plated at 100 μ l/well in coated, nitrocellulose-backed plates as described above. Cell concentrations were 2×10^4 to 4.5×10^4 cells/well in preliminary studies (Fig. 1) and 4.5×10^4 cells/well subsequently. Stimuli used in these assays included 1 μ g of PHA/ml, pooled p24 peptides at 1 μ g/ml each, individual p24 peptides from the pool at 5 μ g/ml each, and DMSO (medium alone). These stimuli were prepared at $2 \times$ concentrations in standard culture medium and added to triplicate wells of cells at 100 μ l/well. Monoclonal CD28 antibody was included at a final concentration of 1 μ g/ml. Plates were incubated, developed, and counted as for fresh IFN- γ ELISPOT assays. The net p24-specific response per million input cells was calculated according to the following formula: [(median p24-induced SFCs/well) - (median medium-induced SFCs/well)] $\times$ (10⁶/the number of cells originally plated). Net p24-specific responses of 4 SFCs/well (89 SFCs/million) greater than the median net p24-specific response from the negative control group were considered positive.

CFSE proliferation assays. Whole or CD8-depleted PBMC were suspended at 5×10^6 cells/ml in a prewarmed 1.5 μ M solution of carboxyfluorescein diacetate succinimidyl ester (CFSE) (Molecular Probes, Eugene, Oreg.) in Hanks' balanced salt solution. Cells were incubated at 37°C for 15 min, rinsed once in standard culture medium, and resuspended at 10^7 cells/ml in standard culture medium. Cells were then plated at 50 μ l/well (5×10^5 cells/well) in 96-well, round-bottomed polystyrene plates and stimulated with 1 μ g of PHA/ml, 5 μ g of recombinant p24 antigen/ml, or 0.5 μ g of baculovirus control protein (Protein Sciences)/ml. Stimuli were made up at $2 \times$ concentrations in standard culture medium and were then mixed with cells in wells at 50 μ l/well. After incubation for 2 days at 37°C in 5% CO₂, cells were fed with a 0.5 $\times$ volume (50 μ l) of standard culture medium. After 7 days, cells were removed from wells, washed with PBS-1% bovine serum albumin, and stained with CD3-phycoerythrin (CD3-PE; Becton-Dickinson, Franklin Lakes, N.J.), CD4-tricolor (CD4-TC; Caltag, Burlingame, Calif.), and CD8-allophycocyanin (CD8-APC; Becton-Dickinson). Cells were then washed again, fixed, and analyzed on a FACSCalibur flow cytometer (Becton-Dickinson). Gate values for CFSE fluorescence of CD3⁺-

CD4⁺ or CD3⁺-CD8⁺ were set immediately below the value for the large population of events with the highest CFSE fluorescence intensity. The net p24-specific response was calculated by subtracting the percentage of CD3⁺-CD4⁺ or CD3⁺-CD8⁺ cells with reduced CFSE fluorescence after control stimulation from the percentage of CD3⁺-CD4⁺ or CD3⁺-CD8⁺ cells with reduced CFSE fluorescence after p24 stimulation. Responses at least threefold greater than the median response from the negative-control subject group were considered positive.

Intracellular cytokine staining. HIV-1 p24-specific, IFN- γ -secreting CD4⁺ T cells in CD8-depleted, CFSE-labeled, p24-expanded PBMC were identified as described previously (22), with several modifications. After CD8 depletion, CFSE labeling, and 7 days of culturing with p24 antigen as described for CFSE proliferation assays, cells were rinsed in PBS, resuspended in standard culture medium with 1 μ g of CD28 monoclonal antibody/ml, and transferred to 5-ml polypropylene tubes. Cells were stimulated with either pooled p24 peptides (1 μ g/ml each) or DMSO as a negative control (medium alone). Tubes were incubated at a 5° slant at 37°C in 5% CO₂ for 6 to 9 h. To allow intracellular accumulation of cytokines, exocytosis was blocked by the addition of brefeldin A (Golgi Plug; Pharmingen, San Diego, Calif.) after the first 1 to 2 h of incubation. Cells were then stained with CD4-TC for 20 min at 4°C. CD4-stained cells were washed once with PBS–1% bovine serum albumin and fixed for 15 min at room temperature with solution A (Caltag). Fixed cells were then permeabilized with solution B (Caltag) and stained with an allophycocyanin-conjugated monoclonal antibody to IFN- γ (Caltag) for 30 min at 4°C. Cells were then washed, fixed in 1% paraformaldehyde, and analyzed by flow cytometry.

RESULTS

Detection of strong p24-specific, IFN- γ -producing CD4⁺ T-cell responses from viremic subjects in CD8-depleted PBMC following culture with p24 antigen. In a previous study (5), HIV-1 Gag p24-specific CD4⁺ T cells were expanded from ART-treated subjects by culturing PBMC in vitro with recombinant p24 antigen. Expanded, p24 epitope-specific CD4⁺ T-cell responses were then detected by depleting CD8⁺ cells and stimulating with individual overlapping p24 peptides in IFN- γ ELISPOT assays. We used a similar approach to examine p24-induced CD4⁺ T-cell expansion in this study, except that we added to the expansion cultures several factors previously shown or hypothesized to improve HIV-1-specific CD4⁺ T-cell proliferation from untreated, viremic subjects. These factors included anti-CD28 monoclonal antibody, CD40 ligand trimer (CD40LT), and IL-12, all hypothesized to increase costimulation for antigen-specific T cells (8); IL-2, which may reverse T-cell anergy (14); and the nucleoside reverse transcriptase inhibitor didanosine (ddI), which inhibits HIV-1 replication and might thus prevent replicating HIV-1 from deleting HIV-1-specific CD4⁺ T cells in vitro. In addition, PBMC were CD8 depleted before culturing rather than afterwards so that individual CD8⁺ cell depletions would not be required for each of multiple expansion cultures from each subject. For study subjects, we initially selected seven individuals with chronic HIV-1 infection and untreated HIV-1 viremia. The median plasma HIV-1 RNA level (viral load) for the group was 29,365 copies/ml (range, 12,921 to 445,028); the median peripheral blood CD4 count was 468 cells/ μ l (range, 237 to 1,008).

Figure 1 shows that five of the seven subjects tested had p24-specific responses of at least several hundred IFN- γ SFCs per million in these “expanded ELISPOT” assays. Surprisingly, strong responses were observed even when CD8-depleted PBMC were expanded with p24 antigen alone. The median response after expansion with p24 alone was 489 IFN- γ SFCs/million and ranged from 0 to 2,400 SFCs/million. Of the compounds used to improve p24-specific CD4⁺ T-cell prolifera-

tion, only CD40LT further increased responses above those detected from CD8-depleted, otherwise untreated expansion cultures (median, 711 IFN- γ SFCs/million; range, 0 to 3244). However, CD40LT failed to increase greatly the p24-specific responses from the two subjects who failed to respond to p24 antigen alone. We noted that the two subjects lacking p24-specific CD4⁺ T-cell responses in these assays showed by far the highest plasma HIV-1 RNA levels in the group (445,028 and 207,115 copies/ml for subjects UH138 and UH153 compared to 12,921 to 32,104 copies/ml for the other subjects).

Expansion of p24-specific, IFN- γ -producing CD4⁺ T cells from CD8-depleted PBMC of viremic, ART-naïve subjects. For further studies of the apparent p24-specific CD4⁺ T-cell expansion from CD8-depleted PBMC of viremic subjects, we selected a group of 20 untreated subjects with HIV-1 viremia. Because intermittent or interrupted ART has been previously associated with alterations in HIV-1-specific CD4⁺ T-cell responses (21, 26), we included only subjects with no history of ART (i.e., ART-naïve subjects). Subjects with suspected acute or recent (within 6 months of the study date) infection were excluded. The CD4 counts of these subjects ranged from 6 to 986 cells/ μ l, with a median of 371. Their plasma viral loads ranged from 1,040 to >750,000 copies/ml, with a median of 39,520. Though seroconversion dates for most of these subjects were not available, 16 of 20 (80%) had CD4 counts below 500 cells/ μ l, suggesting chronic infection with progressive disease. Though the remaining four subjects had higher CD4 counts, we anticipated that their viremia would be associated with diminished HIV-1-specific CD4⁺ T-cell proliferation, as has been previously reported (18, 22).

Because the IFN- γ ELISPOT responses from expanded, CD8-depleted PBMC shown in Fig. 1 ranged only up to 2,400 SFCs/million (probably corresponding to less than 1% of all CD4⁺ T cells in the assay), we wanted to confirm that culturing of CD8-depleted PBMC with p24 antigen truly increased the magnitude of the p24-specific response. To accomplish this, we used ELISPOT assays to compare the frequencies of HIV-1 p24-specific IFN- γ SFCs in CD8-depleted PBMC before and after expansion with p24 antigen. Figure 2 shows the results for the 15 subjects tested in both fresh and expanded ELISPOT assays. Before expansion, the median p24-specific response from the group of viremic subjects was 25 SFCs/million (range, 0 to 1,278). After expansion, the median response increased more than 20-fold (median, 644 SFCs/million; range, 0 to 2,978). Only one subject showed a decreased response after expansion, and the increase in responses was statistically significant ($P = 0.0190$ [paired t test]). Therefore, p24 expansion culture of CD8-depleted PBMC was associated with increased frequencies of HIV-1 p24-specific, IFN- γ -producing cells from this group of viremic, ART-naïve subjects.

Increased p24-induced CD4⁺ T-cell division in PBMC of viremic, ART-naïve subjects after depletion of CD8⁺ cells. To determine whether these increased frequencies of p24-specific, IFN- γ -producing cells were associated with CD4⁺ T-cell division, p24-induced CD4⁺ T-cell proliferation was analyzed directly by CFSE dye dilution. PBMC from viremic, ART-naïve subjects were labeled with CFSE with or without prior CD8 depletion and then stimulated with recombinant p24 antigen or baculovirus control protein. After 7 days of culture, CFSE fluorescence of CD3⁺-CD4⁺ cells was analyzed by flow cytom-

FIG. 2. Increased frequencies of HIV-1 p24-specific, IFN- γ SFCs from CD8-depleted PBMC of viremic, ART-naïve subjects. Fresh CD8-depleted PBMC (Fresh) or CD8-depleted PBMC expanded with recombinant p24 antigen for 7 days (Expanded) were stimulated in IFN- γ ELISPOT assays with pooled p24 peptides or medium alone as a negative control. Net p24-specific responses were determined by subtracting the median medium-induced SFCs/million input cells from the median p24-induced SFCs/million input cells. Subjects showing detectable p24-specific responses in the fresh assay are grouped on the left side of the graph.

etry (see Fig. 3A for representative p24 stimulation data). Net p24-induced responses were then calculated as the percentages of p24-stimulated cells with reduced CFSE fluorescence minus the percentages of control stimulated cells with reduced CFSE fluorescence. The net p24-induced CD4⁺ T-cell proliferative responses of whole and CD8-depleted PBMC from each subject were then compared (Fig. 3B).

Figure 3B shows that p24-induced CD4⁺ T-cell division was readily detected in the CD8-depleted PBMC of most viremic, ART-naïve subjects tested. Subjects with positive p24-specific responses in expanded ELISPOT assays showed positive p24-induced CD4⁺ T-cell proliferation in CFSE dilution assays. By contrast, none of the subjects who failed to respond in expanded ELISPOT assays showed a positive proliferative response (compare to Fig. 2). Furthermore, p24-induced CD4⁺ T-cell proliferation was often stronger in CD8-depleted than in whole PBMC. When whole PBMC were used, 7 of 16 subjects showed positive p24-induced CD4⁺ T-cell proliferation results, with a median response of 0.23%. When CD8-depleted PBMC were used, the number of subjects with positive p24-induced CD4⁺ T-cell proliferation results increased by 57% (to 11) and the median response increased more than 6.5-fold (to 1.50%). Among the 11 subjects showing some positive proliferative response, CD8 depletion increased the median response from 0.82 to 5.96%. Importantly, the results for five subjects showed negative responses from both whole and CD8-depleted PBMC, and those for three subjects with strong responses from whole PBMC showed weaker positive responses from CD8-depleted PBMC. Despite this heterogeneity, p24-induced CD4⁺ T-cell proliferation was never lost after CD8 depletion and was often increased.

It remained possible that p24-specific CD4⁺ T cells in the CD8-depleted PBMC of these viremic, ART-naïve subjects were stimulating non-p24-specific bystander cells to proliferate without themselves dividing. To test this, CD8-depleted, CFSE-

labeled, p24-expanded PBMC from several subjects were analyzed further for p24 specificity by staining for antigen-induced intracellular IFN- γ . Following culturing with p24 antigen for 7 days, CD8-depleted, CFSE-labeled PBMC were stimulated with pooled p24 peptides or medium alone as a

FIG. 3. HIV-1 p24-induced CD4⁺ T-cell proliferation in whole and CD8-depleted PBMC of viremic, ART-naïve subjects. CFSE-labeled PBMC, either whole (PBMC) or CD8 depleted (CD8d), were stimulated with recombinant p24 antigen or baculovirus control protein and analyzed by flow cytometry after 7 days of culture. (A) CFSE fluorescence of CD4⁺ T cells from representative p24-stimulated cultures. Results for three HIV-1-infected subjects (subjects UH195, UH178, and UH189) and one negative-control subject (subject N4) are shown in density plots gated on resting and blasting CD3⁺ lymphocytes. CD4⁻ events are omitted for simplicity. (B) Net p24-induced CD4⁺ T-cell proliferative responses from whole or CD8-depleted PBMC for each ART-naïve subject tested in both assays. The percentages of p24- or control-stimulated, CD3⁺-CD4⁺ cells with reduced CFSE fluorescence were determined by flow cytometry, and the net p24-induced percentages were calculated by subtracting the control-stimulated percentages from the p24-stimulated percentages. The horizontal line above the x axis indicates the minimum positive response.

FIG. 4. HIV-1 p24-specific, IFN- γ -producing CD4⁺ T cells from CD8-depleted, p24-expanded PBMC of viremic, ART-naïve subjects that divided in culture. CD8-depleted, CFSE-labeled PBMC expanded with p24 antigen as described for Fig. 3 were divided into two cultures that were then stimulated with medium alone or with pooled p24 peptides, permeabilized, stained for IFN- γ , and analyzed by flow cytometry. Density plots were gated on resting and blasting CD4⁺ lymphocytes. Results are shown for four ART-naïve subjects. The percentage of CD4⁺ cells with both reduced CFSE fluorescence and intracellular IFN- γ is shown in the upper left corner of each plot.

negative control, stained for intracellular IFN- γ , and analyzed by flow cytometry. As expected, p24-specific, IFN- γ -producing CD4⁺ T cells were detected in the expansion cultures for all four subjects tested (Fig. 4). Although a very small number of these IFN- γ -producing cells were CFSE^{high}, most showed reduced CFSE fluorescence, suggesting that they had proliferated. Therefore, p24-specific CD4⁺ T cells from many of these ART-naïve, viremic subjects showed no irreversible proliferative defect but rather divided readily after p24 stimulation of CD8-depleted PBMC.

Lower p24-induced CD4⁺ T-cell proliferation in whole PBMC is not associated with bystander proliferation by CD8⁺ T cells. Several recent studies have shown that HIV-1-specific CD4⁺ T cells from viremic subjects produce IL-2 much less frequently than IFN- γ and that viral suppression in vivo is associated with more frequent IL-2 production (11, 12, 23, 33). We therefore investigated whether CD8⁺ T cells, by consuming low levels of IL-2 that these cells might produce and then proliferating as bystanders, might inhibit HIV-1-specific CD4⁺ T-cell proliferation for viremic subjects. In an analysis of CFSE fluorescence of CD3⁺-CD8⁺ cells from whole, p24-stimulated PBMC, we did find strong CD8⁺ T-cell proliferation with several subjects following p24 antigen stimulation (Fig. 5A and B). Nevertheless, if CD8⁺ T cells were inhibiting p24-induced

CD4⁺ T-cell proliferation by consuming IL-2, we hypothesized that CD8⁺ T-cell proliferation from whole PBMC would be strongest in subjects showing greatly improved CD4⁺ T-cell proliferation after CD8 depletion. In fact, we observed the opposite: strong p24-induced CD8⁺ T-cell proliferation from whole PBMC was detected only when p24-induced CD4⁺ T-cell proliferation was detectable within the same culture. This association was statistically significant among members of the total subject group ($P = 0.0079$ [Mann-Whitney test]) (Fig. 5C) and remained significant when analysis was limited to subjects showing positive responses in at least one CD4⁺ T-cell assay ($P = 0.0424$ [Mann-Whitney]). Therefore, if CD8⁺ T cells inhibited p24-induced CD4⁺ T-cell proliferation in the whole PBMC of most of these viremic, ART-naïve subjects by consuming IL-2, they did not appear to use that IL-2 to proliferate.

Sensitivity of proliferation-dependent assays for p24-specific CD4⁺ T cells using CD8-depleted PBMC of viremic, ART-naïve subjects. Table 1 shows the HIV-1 Gag p24-specific CD4⁺ T-cell responses of all of the viremic, ART-naïve subjects studied along with each subject's CD4 count and viral load at the time of study. The compiled data confirm that CFSE dilution assays detected p24-induced CD4⁺ T-cell proliferation with greater sensitivity when CD8-depleted PBMC rather than whole PBMC were used. When whole PBMC were used, 7 of 16 (44%) subjects tested showed p24-induced CD4⁺ T-cell proliferation (defined as a net 0.6% of CD3⁺-CD4⁺ cells with decreased CFSE fluorescence). By contrast, 14 of 19 subjects (74%) showed positive p24-induced CD4⁺ T-cell proliferative responses when CD8-depleted PBMC were used. Not all subjects tested using CD8-depleted PBMC were also tested using whole PBMC, but the positive-response frequency seen for CD8-depleted PBMC from only those subjects also tested using whole PBMC still increased (11 of 16; 69%).

Table 1 also shows that p24-specific CD4⁺ T-cell expansion from CD8-depleted PBMC was consistently detected for every subject in whom p24-specific CD4⁺ T-cell precursors were confirmed to be present by use of fresh IFN- γ ELISPOT assays. Even using p24-specific CD4⁺ T-cell proliferative responses measured from whole PBMC, high frequencies of p24-specific IFN- γ SFCs detected in fresh ELISPOT assays were associated with stronger p24-induced proliferation (Spearman rank correlation coefficient [R] = 0.7585; $P = 0.0055$). Nevertheless, several subjects studied here showed strong responses in fresh ELISPOT assays but negative proliferative responses with whole PBMC. By contrast, both CFSE labeling and expanded IFN- γ ELISPOT assays using CD8-depleted PBMC detected p24-specific CD4⁺ T-cell responses for every subject showing a fresh ELISPOT response. Both of these assays also detected p24-specific CD4⁺ T-cell responses for several subjects (subjects UH156, UH168, UH198, UH206, and UH207) showing low or undetectable responses in fresh ELISPOT assays.

IFN- γ ELISPOT assays using CD8-depleted PBMC expanded with p24 antigen were sensitive enough to detect multiple p24 epitope-specific CD4⁺ T-cell responses for many of the viremic, ART-naïve subjects tested. In our previous study, we detected multiple p24 epitope-specific CD4⁺ T-cell responses from members of a group of subjects with chronic HIV-1 disease, but all of these subjects had been receiving effective ART for years. Table 1 shows that 8 of 20 subjects studied here

FIG. 5. HIV-1 p24-induced CD8⁺ T-cell proliferation in whole PBMC is associated with p24-induced CD4⁺ T-cell proliferation in whole PBMC. (A) CFSE fluorescence of CD4⁺ T cells (left panels) and CD8⁺ T cells (right panels) from p24-stimulated PBMC of two viremic, ART-naïve subjects. Results for one subject with CD4⁺ T-cell proliferation in whole PBMC (subject UH185) and for one without (subject UH202) are shown. Density plots were gated on resting and blasting CD3⁺ lymphocytes. (B) Net p24-induced proliferative responses of CD4⁺ T cells (CD4) and CD8⁺ T cells (CD8) from whole PBMC for each ART-naïve subject tested. The percentages of p24- or control-stimulated CD3⁺-CD4⁺ or CD3⁺-CD8⁺ cells with reduced CFSE fluorescence were determined by flow cytometry, and the net p24-induced percentages were calculated by subtracting the control-stimulated percentages from the p24-stimulated percentages. The results for seven subjects with positive CD4⁺ T-cell proliferation results are grouped on the left. (C) HIV-1 p24-induced CD4⁺ T-cell prolifer-

ation expanded IFN- γ ELISPOT assays showed CD4⁺ T-cell responses to at least three different p24 epitopes. Interestingly, two of these (subjects UH168 and UH200) had among the lowest CD4 counts (162 and 182 cells/ μ l) in the group, meeting criteria for the diagnosis of AIDS.

Lack of p24-induced CD4⁺ T-cell responses from CD8-depleted PBMC associated with plasma HIV-1 RNA levels > 100,000 copies/ml. Although CD8⁺ cell depletion of PBMC allowed p24-induced CD4⁺ T-cell proliferation to be detected with most of the viremic, ART-naïve subjects studied, five showed no p24-induced CD4⁺ T-cell responses from CD8-depleted PBMC in any assay used. Table 1 shows that these five all had plasma HIV-1 RNA levels of >100,000 copies/ml, a value previously associated with a lack of HIV-1-specific, IFN- γ -producing T-cell responses after viral suppression on ART (20). By contrast, only 1 of the 15 subjects with results showing expansion of p24-specific CD4⁺ T cells from CD8-depleted PBMC had such a high viral load. To examine more rigorously the inverse association of plasma HIV-1 RNA level and p24-induced CD4⁺ T-cell expansion from CD8-depleted PBMC, subjects were stratified by viral load into a >100,000 copies/ml group and a $\leq$ 100,000 copies/ml group, and p24-induced CD4⁺ T-cell expansion results in IFN- γ ELISPOT and CFSE proliferation assays of CD8-depleted PBMC were compared by the Mann-Whitney nonparametric test. As shown in Fig. 6, viral loads of >100,000 copies/ml significantly predicted lower p24-induced CD4⁺ T-cell expansion from CD8-depleted PBMC in both ELISPOT ($P = 0.0193$) (Fig. 6A) and CFSE ($P = 0.0202$) (Fig. 6B) assays. Furthermore, both expanded IFN- γ ELISPOT and CD8-depleted CFSE proliferation results were inversely associated with plasma HIV-1 RNA loads by a Spearman nonparametric correlation (expanded ELISPOT $R = -0.6276$ [$P = 0.0123$]; CD8d CFSE $R = -0.5632$ [$P = 0.0120$]). Therefore, even without the imposition of an arbitrary viral load cutoff in analyzing these data, stronger proliferation-dependent, p24-induced CD4⁺ T-cell responses from CD8-depleted PBMC were inversely associated with plasma HIV-1 RNA load among these viremic, ART-naïve subjects.

DISCUSSION

The first major finding of this study was that Gag p24 antigen-specific CD4⁺ T cells from most chronically HIV-1-infected, viremic subjects possessed an intrinsic proliferative capacity not revealed by standard assays. This confirms a recent publication according to which exogenous IL-2 was found to restore antigen-induced proliferation of HIV-1-specific CD4⁺ T cells for viremic subjects (12). The second major finding of this study is that HIV-1-specific CD4⁺ T cells were seldom detected for subjects with viral loads > 100,000 copies/ml either by IFN- γ production or by proliferation. Therefore, although replicating HIV-1 does not completely ablate or sup-

pression from whole PBMC predicts stronger p24-induced CD8⁺ T-cell proliferation from whole PBMC. CD8⁺ T-cell proliferative responses in whole PBMC from subjects with positive (+) or negative (-) p24-induced CD4⁺ T-cell proliferation in whole PBMC were compared using the Mann-Whitney nonparametric test. The median response for each group is shown as a horizontal bar.

TABLE 1. HIV-1 p24-specific CD4⁺ T-cell responses of viremic, ART-naïve subjects.

Subject	CD4 count (cells/ μ l)	Plasma HIV-1 RNA (copies/ml)	Net CD4 ⁺ T-cell proliferation (%CFSE ^{low}) ^a		Net IFN- γ -producing CD4 ⁺ T-cell frequency (SFCs/10 ⁶) ^b		No. of peptides targeted ^c
			Whole PBMC ^f	CD8d PBMC ^g	Fresh ^h	Expanded ⁱ	
UH129	208	21,861	0.82	1.73	NA	NA	NA
UH149	595	32,104	NA ^d	NA	380	644	NA
UH154	468	12,921	0.67	3.45	NA	NA	NA
UH156	299	15,379	NA	9.66	25	711	5 (4)
UH168	162	46,704	NA	8.87	—	222	4 (3)
UH185	456	150,000	3.93	12.82	250	1,000	1
UH189	414	8,602	8.02	1.24	45	178	2 (1)
UH195	578	13,300	—	19.84	145	1,511	6
UH198	986	1,040	2.22	43.4	38	2,978	6 (3)
UH200	182	90,400	3.76	0.65	1,278	1,044	10 (7)
UH202	378	58,900	—	12.21	180	NA	6 (5)
UH206	279	37,140	NA	3.11	—	111	2 (1)
UH207	897	20,600	—	1.26	—	933	9 (8)
UH209	384	41,900	9.59	8.47	1,210	2,489	13 (8)
UH219	364	8,630	—	1.85	NA	NA	NA
UH178	143	>750,000 ^e	—	—	—	—	0
UH208	408	177,000	—	—	—	—	0
UH210	88	460,313	—	—	—	—	0
UH212	6	>750,000	—	—	—	—	0
UH221	180	117,000	—	—	NA	NA	NA

^a The median net responses from the negative-control group were 0.0% (whole PBMC) and 0.2% (CD8d PBMC); net responses at least 3-fold greater than the CD8d PBMC median (i.e., $\geq 0.6\%$) were considered positive. Negative responses are shown as dashes.

^b The median net responses from the negative control group were zero SFCs/10⁶ cells (fresh) and 11 SFCs/10⁶ cells (expanded). Net responses of 4 SFCs/well greater than these responses (i.e., $\geq 20/10^6$, for fresh, $\geq 100/10^6$ for expanded) were considered positive. Negative responses are shown as dashes.

^c Numbers of individual 20- or 15-residue p24 peptides targeted by positive responses, determined by IFN- γ ELISPOT as for expanded IFN- γ -producing CD4⁺ T-cell frequency. Numbers in parentheses are the minimum numbers of individual epitope-specific responses detected when responses to two contiguous overlapping peptides are assumed to reflect a single response. Median response, 4 (3); the percentage of responders was 73 (11/15).

^d NA, not attempted.

^e Value exceeded the quantitative range of the assay.

^f Median response, negative; the percentage of responders was 44 (7/16).

^g Median response, 1.85; the percentage of responders was 74 (14/19).

^h Median response, 32; the percentage of responders was 56 (9/16).

ⁱ Median response, 644; the percentage of responders was 73 (11/15).

press proliferation of HIV-1-specific CD4⁺ T cells in many subjects with chronic, progressive disease, our results are consistent with a near absence of HIV-1-specific CD4⁺ T-cell immunity at the highest levels of in vivo HIV-1 replication.

That HIV-1-specific CD4⁺ T cells from chronically viremic subjects are capable of proliferating in vitro adds to our understanding of the HIV-1-specific CD4⁺ T-cell proliferative defect. It had previously remained possible that active replication of HIV-1 irreversibly inhibited HIV-1-specific CD4⁺ T-cell proliferation in viremic subjects. Possible explanations for this included that the HIV-1-specific CD4⁺ T cells had become irreversibly anergic or that they were programmed to undergo apoptosis after activation. In combination, the results obtained in the present study and in that of Iyasere et al. (12) demonstrate that not all HIV-1-specific CD4⁺ T cells irreversibly lose proliferative capacity during chronic viremia. HIV-1-specific CD4⁺ T cells might therefore retain in vivo proliferative function during chronic viremia, although this remains to be explored.

Although the present report confirms the conclusions of Iyasere et al., it differs from that study in two important ways. First, we have used CD8⁺ cell depletion of PBMC rather than exogenous IL-2 to uncover HIV-1-specific CD4⁺ T-cell proliferation in viremic subjects. The improved HIV-1 antigen-induced proliferation of CD4⁺ T cells treated with exogenous IL-2 observed in the Iyasere et al. study follows from the

importance of IL-2 for T-cell proliferation in vitro and the poor IL-2 production of HIV-1-specific CD4⁺ T cells during HIV-1 viremia (4, 11, 12, 23, 33). However, an important limitation to the use of exogenous IL-2 in studying CD4⁺ T-cell function is that it might compensate for a complete lack of endogenous, HIV-1 antigen-induced IL-2 production in viremic subjects. By contrast, any IL-2-driven proliferation in our study must have required an endogenous IL-2 source. Therefore, if T-cell proliferation in vitro requires IL-2, our results suggest that HIV-1-specific, IL-2-producing CD4⁺ T cells may persist in most subjects with chronic HIV-1 viremia, although the frequencies of these cells may be below the detection limit of standard flow cytometry methods. As IL-2 production may allow CD4⁺ T cells to provide "help" for antigen-specific CD8⁺ T cells, IL-2-dependent CD4⁺ T-cell responses in vitro might be expected to predict effective immune responses in vivo. Consistent with this, we found p24-induced CD4⁺ T-cell proliferative responses measured for CD8-depleted PBMC in vitro to be inversely associated with plasma HIV-1 RNA levels among the viremic, ART-naïve subjects studied here.

How does CD8 depletion of PBMC improve p24-induced CD4⁺ T-cell proliferation in viremic subjects? The mechanism may involve increased IL-2 availability in vitro. CD8⁺ cells, including both CD3⁺ T and natural killer lineages, are known to express IL-2 receptors (29, 30), and their removal may

FIG. 6. Plasma HIV-1 RNA levels of >100,000 copies/ml predict lack of proliferation-dependent, p24-induced CD4⁺ T-cell responses from CD8-depleted PBMC. Subjects were divided into two groups: one consisted of subjects with viral loads greater than 100,000 copies/ml (>10⁵), and the other consisted of subjects with viral loads less than or equal to this value (≤10⁵). HIV-1 p24-induced CD4⁺ T-cell responses in expanded IFN- γ ELISPOT assays (A) or CFSE proliferation assays from CD8-depleted PBMC (B) were then compared between the two groups by the Mann-Whitney nonparametric test. The median value for each group is shown as a horizontal bar.

therefore increase IL-2 concentrations available for CD4⁺ T cells. Although this could occur in any in vitro proliferation assay, it might have been particularly noticeable in this study because chronically viremic subjects often show increased CD8⁺ T-cell numbers (25), increased CD8⁺ cell responsiveness to IL-2 (29), and skewing of HIV-1-specific CD4⁺ T cells to an IL-2-deficient, "effector memory" state (11, 23, 33). Of note, additional exogenous IL-2 added to CD8-depleted, p24-stimulated PBMC failed to increase p24-specific responses further (Fig. 1), suggesting that CD8 depletion and exogenous IL-2 might improve p24-induced CD4⁺ T-cell proliferation by a common mechanism. Nevertheless, we found that subjects showing greatly improved p24-induced CD4⁺ T-cell proliferation following CD8⁺ cell depletion of PBMC generally did not show strong p24-induced CD8⁺ T-cell proliferation from whole PBMC. Although this may mean that CD8⁺ T cells from these subjects consumed IL-2 without proliferating, it may also suggest that CD8 cell depletion of PBMC improved p24-induced CD4⁺ T-cell proliferation in viremic subjects by other mechanisms. For example, CD8 depletion probably increases CD4⁺ T-cell contacts with antigen-presenting cells (APCs) as well as the density of HIV-1-specific CD4⁺ T cells in vitro.

CD8⁺ T cells or natural killer cells may also lyse p24-reactive CD4⁺ T cells or p24-presenting APCs in vitro, particularly those infected with HIV-1 (31). Finally, the CD8⁺ cell population may include a suppressive cell type whose removal increases CD4⁺ T-cell proliferation (6). Distinguishing the contribution and biological significance of these mechanisms will require further study.

The present study also differs from that of Iyasere et al. in that we were unable to detect p24-induced CD4⁺ T-cell proliferation from all viremic subjects tested. Instead, several subjects showed no p24-induced CD4⁺ T-cell proliferation even from CD8-depleted PBMC and also showed no p24-specific CD4⁺ T-cell IFN- γ production in fresh or expanded IFN- γ ELISPOT assays. As these subjects all had viral loads >100,000 copies/ml, our results suggest a greater HIV-1-specific CD4⁺ T-cell defect at very-high-level HIV-1 replication in vivo than at lower viral loads. At viral loads of ≤100,000 copies/ml, HIV-1-specific CD4⁺ T cells produce IFN- γ in vitro and proliferate poorly in standard assays but show an intrinsic proliferative capacity uncovered by either exogenous IL-2 or CD8 depletion of PBMC. At higher viral loads, however, HIV-1-specific CD4⁺ T cells may be undetectable by either proliferation or IFN- γ production in vitro. Importantly, UH185 showed p24-induced CD4⁺ T-cell proliferative and IFN- γ responses despite a viral load of 150,000 copies/ml, and subjects with such high viral loads have shown p24-specific CD4⁺ T-cell IFN- γ production in previous studies (3). Therefore, the viral load value of 100,000 copies/ml does not accurately predict a lack of in vitro p24-specific CD4⁺ T-cell responses in all subjects. Nevertheless, Oxenius et al. showed that HIV-1-specific, IFN- γ -producing T-cell responses were seldom detected with ART-treated subjects with pretreatment viral loads > 100,000 copies/ml (20). Although that study used subjects who were no longer viremic, it supports the association between the highest levels of in vivo HIV-1 replication and an absence of HIV-1-specific CD4⁺ T-cell responses in vitro.

Why subjects with viral loads of >100,000 copies/ml might lack HIV-1-specific CD4⁺ T-cell responses in vitro remains unclear. On the one hand, persistent HIV-1 replication to the highest levels in vivo might cause complete depletion of HIV-1-specific CD4⁺ T cells. This could occur by progressive, preferential spread of HIV-1 through the HIV-1-specific CD4⁺ T-cell population (7). Furthermore, as chronic, high-level antigen exposure may cause ablation of T cells targeting even non-T-cell-tropic viruses, HIV-1-specific CD4⁺ T cells might be depleted without being infected (9). On the other hand, HIV-1-specific CD4⁺ T cells may persist in subjects with HIV-1 loads of >100,000 copies/ml without proliferating or producing IFN- γ in vitro. This might occur by a progressive loss of cytokine production (9) or by an insensitivity to T-cell receptor stimulation (10) with sustained HIV-1 replication. HIV-1 replication to levels of >100,000 copies/ml might also have interfered with HIV-1-specific CD4⁺ T-cell responses indirectly, as an APC dysfunction at the highest levels of HIV-1 replication could leave HIV-1-specific CD4⁺ T cells functionally intact but insufficiently stimulated in vitro. These possibilities will need to be addressed in future studies.

Overall, the results presented in this study are significant because they demonstrate the intrinsic proliferative capacity of HIV-1-specific CD4⁺ T cells from subjects with chronic HIV-1

viremia. Thus, replicative senescence and activation-induced apoptosis in vitro do not completely explain the diminished HIV-1-specific CD4⁺ T-cell proliferative responses often observed in viremic subjects. Furthermore, our results show that proliferation-competent, HIV-1-specific CD4⁺ T-cell responses measured in samples from viremic subjects are inversely associated with plasma HIV-1 RNA levels. Although other studies have found similar associations among subjects with chronic, progressive disease (15), our study is unusual in that proliferation-competent, HIV-1-specific CD4⁺ T-cell responses were detected for a sizable majority of subjects. Therefore, if proliferation-competent, HIV-1-specific CD4⁺ T cells contribute to immune control of HIV-1 in vivo, our results suggest that some degree of this HIV-1-specific CD4⁺ T-cell-dependent immune protection may often persist well into progressive HIV-1 disease. Additional studies will be required to address how these cells might persist in the face of chronic HIV-1 replication as well as how well in vitro assays like those used here predict in vivo protective functions.

ACKNOWLEDGMENTS

We thank the study subjects for their generous participation. We also thank the staff of the University of Colorado Hospital Infectious Diseases Group Practice for help with subject enrollment and phlebotomy. Reagents for this study were provided by the DAIDS Vaccine and Prevention Research Program and the National Institutes of Health AIDS Reagent Program.

This work was supported by National Institutes of Health grant P01AI48238.

REFERENCES

- Al-Harthi, L., J. Siegel, J. Spritzler, J. Pottage, M. Agnoli, and A. Landay. 2000. Maximum suppression of HIV replication leads to the restoration of HIV-specific responses in early HIV disease. *AIDS* 14:761–770.
- Angel, J. B., K. G. Parato, A. Kumar, S. Kravcik, A. D. Badley, C. Fex, D. Ashby, E. Sun, and D. W. Cameron. 2001. Progressive human immunodeficiency virus-specific immune recovery with prolonged viral suppression. *J. Infect. Dis.* 183:546–554.
- Betts, M. R., D. R. Ambrozak, D. C. Douek, S. Bonhoeffer, J. M. Brenchley, J. P. Casazza, R. A. Koup, and L. J. Picker. 2001. Analysis of total human immunodeficiency virus (HIV)-specific CD4⁺ and CD8⁺ T-cell responses: relationship to viral load in untreated HIV infection. *J. Virol.* 75:11983–11991.
- Boaz, M. J., A. Waters, S. Murad, P. J. Easterbrook, and A. Vyakarnam. 2002. Presence of HIV-1 Gag-specific IFN-gamma+IL-2+ and CD28+IL-2+ CD4 T cell responses is associated with nonprogression in HIV-1 infection. *J. Immunol.* 169:6376–6385.
- Boritz, E., B. E. Palmer, B. Livingston, A. Sette, and C. C. Wilson. 2003. Diverse repertoire of HIV-1 p24-specific, IFN-gamma-producing CD4⁺ T cell clones following immune reconstitution on highly active antiretroviral therapy. *J. Immunol.* 170:1106–1116.
- Clerici, M., E. Roilides, C. S. Via, P. A. Pizzo, and G. M. Shearer. 1992. A factor from CD8 cells of human immunodeficiency virus-infected patients suppresses HLA self-restricted T helper cell responses. *Proc. Natl. Acad. Sci. USA* 89:8424–8428.
- Douek, D. C., J. M. Brenchley, M. R. Betts, D. R. Ambrozak, B. J. Hill, Y. Okamoto, J. P. Casazza, J. Kuruppu, K. Kunstman, S. Wolinsky, Z. Grossman, M. Dybul, A. Oxenius, D. A. Price, M. Connors, and R. A. Koup. 2002. HIV preferentially infects HIV-specific CD4⁺ T cells. *Nature* 417:95–98.
- Dybul, M., G. Mercier, M. Belson, C. W. Hallahan, S. Liu, C. Perry, H. Herpin, L. Ehler, R. T. Davey, J. A. Metcalf, J. M. Mican, R. A. Seder, and A. S. Fauci. 2000. CD40 ligand trimer and IL-12 enhance peripheral blood mononuclear cells and CD4⁺ T cell proliferation and production of IFN-gamma in response to p24 antigen in HIV-infected individuals: potential contribution of energy to HIV-specific unresponsiveness. *J. Immunol.* 165:1685–1691.
- Fuller, M. J., and A. J. Zajac. 2003. Ablation of CD8 and CD4 T cell responses by high viral loads. *J. Immunol.* 170:477–486.
- Grossman, Z., M. B. Feinberg, and W. E. Paul. 1998. Multiple modes of cellular activation and virus transmission in HIV infection: a role for chronically and latently infected cells in sustaining viral replication. *Proc. Natl. Acad. Sci. USA* 95:6314–6319.
- Harari, A., S. Petitpierre, F. Vallelian, and G. Pantaleo. 2004. Skewed representation of functionally distinct populations of virus-specific CD4 T cells in HIV-1-infected subjects with progressive disease: changes after antiretroviral therapy. *Blood* 103:966–972.
- Iyasere, C., J. C. Tilton, A. J. Johnson, S. Younes, B. Yassine-Diab, R. P. Sekaly, W. W. Kwok, S. A. Migueles, A. C. Laborico, W. L. Shupert, C. W. Hallahan, R. T. Davey, Jr., M. Dybul, S. Vogel, J. Metcalf, and M. Connors. 2003. Diminished proliferation of human immunodeficiency virus-specific CD4⁺ T cells is associated with diminished interleukin-2 (IL-2) production and is recovered by exogenous IL-2. *J. Virol.* 77:10900–10909.
- Janssen, E. M., E. E. Lemmens, T. Wolffe, U. Christen, M. G. von Herrath, and S. P. Schoenberger. 2003. CD4⁺ T cells are required for secondary expansion and memory in CD8⁺ T lymphocytes. *Nature* 421:852–856.
- Kroemer, G., and C. Martinez. 1992. Interleukin-2, a pro-autoimmune lymphokine that interferes with post-deletional tolerance. *Semin. Immunol.* 4:167–179.
- Malhotra, U., R. J. Bosch, E. Chan, R. Wang, M. A. Fischl, A. C. Collier, and M. J. McElrath. 2004. Association of T cell proliferative responses and phenotype with virus control in chronic progressive HIV-1 disease. *J. Infect. Dis.* 189:515–519.
- Matloubian, M., R. J. Concepcion, and R. Ahmed. 1994. CD4⁺ T cells are required to sustain CD8⁺ cytotoxic T-cell responses during chronic viral infection. *J. Virol.* 68:8056–8063.
- McMichael, A. J., and S. L. Rowland-Jones. 2001. Cellular immune responses to HIV. *Nature* 410:980–987.
- McNeil, A. C., W. L. Shupert, C. A. Iyasere, C. W. Hallahan, J. A. Mican, R. T. Davey, Jr., and M. Connors. 2001. High-level HIV-1 viremia suppresses viral antigen-specific CD4⁺ T cell proliferation. *Proc. Natl. Acad. Sci. USA* 98:13878–13883.
- Migueles, S. A., A. C. Laborico, W. L. Shupert, M. S. Sabbaghian, R. Rabin, C. W. Hallahan, D. Van Baarle, S. Kostense, F. Miedema, M. McLaughlin, L. Ehler, J. Metcalf, S. Liu, and M. Connors. 2002. HIV-specific CD8⁺ T cell proliferation is coupled to perforin expression and is maintained in nonprogressors. *Nat. Immunol.* 3:1061–1068.
- Oxenius, A., D. A. Price, S. J. Dawson, H. F. Gunthard, M. Fischer, L. Perrin, E. Ramirez, C. Fagard, B. Hirschel, G. Scullard, J. N. Weber, A. R. McLean, R. E. Phillips, and HIV Swiss Cohort Study. 2002. Residual HIV-specific CD4 and CD8 T cell frequencies after prolonged antiretroviral therapy reflect pretreatment plasma virus load. *AIDS* 16:2317–2322.
- Oxenius, A., D. A. Price, G. F. Huldrych, S. J. Dawson, C. Fagard, L. Perrin, M. Fischer, R. Weber, M. Plana, F. Garcia, B. Hirschel, A. McLean, and R. E. Phillips. 2002. Stimulation of HIV-specific cellular immunity by structured treatment interruption fails to enhance viral control in chronic HIV infection. *Proc. Natl. Acad. Sci. USA* 99:13747–13752.
- Palmer, B. E., E. Boritz, N. Blyveis, and C. C. Wilson. 2002. Discordance between frequency of human immunodeficiency virus type 1 (HIV-1)-specific gamma interferon-producing CD4⁺ T cells and HIV-1-specific lymphoproliferation in HIV-1-infected subjects with active viral replication. *J. Virol.* 76:5925–5936.
- Palmer, B. E., E. Boritz, and C. C. Wilson. 2004. Effects of sustained HIV-1 plasma viremia on HIV-1 gag-specific CD4⁺ T cell maturation and function. *J. Immunol.* 172:3337–3347.
- Pitcher, C. J., C. Quittner, D. M. Peterson, M. Connors, R. A. Koup, V. C. Maino, and L. J. Picker. 1999. HIV-1-specific CD4⁺ T cells are detectable in most individuals with active HIV-1 infection, but decline with prolonged viral suppression. *Nat. Med.* 5:518–525.
- Rabson, A. 1995. Enumeration of T-cells subsets in patients with HIV infection. *AIDS Clin. Care* 7:1–3.
- Rosenberg, E. S., M. Altfeld, S. H. Poon, M. N. Phillips, B. M. Wilkes, R. L. Eldridge, G. K. Robbins, R. T. D'Aquila, P. J. Goulder, and B. D. Walker. 2000. Immune control of HIV-1 after early treatment of acute infection. *Nature* 407:523–526.
- Shedlock, D. J., and H. Shen. 2003. Requirement for CD4 T cell help in generating functional CD8 T cell memory. *Science* 300:337–339.
- Stevenson, M., T. L. Stanwick, M. P. Dempsey, and C. A. Lamonica. 1990. HIV-1 replication is controlled at the level of T cell activation and proviral integration. *EMBO J.* 9:1551–1560.
- Vanham, G., L. Kestens, G. Penne, G. Leroux-Roels, and P. Gigase. 1994. The interleukin-2 receptor subunit expression and function on peripheral blood lymphocytes from HIV-infected and control persons. *Immunol. Lett.* 39:163–168.
- Vie, H., M. Bonneville, P. Sondermeyer, J. F. Moreau, and J. P. Souillou. 1986. Limiting dilution analysis (LDA) of cells responding to recombinant interleukin-2 without previous stimulation: evidence that all responding cells are lymphokine-activated potent effectors. *Immunology* 57:351–357.
- Weinberg, A., D. A. Wohl, R. J. Barrett, and C. van der Horst. 2001. Inconsistent reconstitution of cytomegalovirus-specific cell-mediated immunity in human immunodeficiency virus-infected patients receiving highly active antiretroviral therapy. *J. Infect. Dis.* 184:707–712.
- Wilson, J. D., N. Imami, A. Watkins, J. Gill, P. Hay, B. Gazzard, M. Westby, and F. M. Gotch. 2000. Loss of CD4⁺ T cell proliferative ability but not loss of human immunodeficiency virus type 1 specificity equates with progression to disease. *J. Infect. Dis.* 182:792–798.
- Younes, S. A., B. Yassine-Diab, A. R. Dumont, M. R. Boulassel, Z. Grossman, J. P. Routy, and R. P. Sekaly. 2003. HIV-1 viremia prevents the establishment of interleukin 2-producing HIV-specific memory CD4⁺ T cells endowed with proliferative capacity. *J. Exp. Med.* 198:1909–1922.