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Infection of CD127$^+$ (Interleukin-7 Receptor$^+$) CD4$^+$ Cells and Overexpression of CTLA-4 Are Linked to Loss of Antigen-Specific CD4 T Cells during Primary Human Immunodeficiency Virus Type 1 Infection

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We recently found that human immunodeficiency virus (HIV)-specific CD4$^+$ T cells express coreceptor CCR5 and activation antigen CD38 during early primary HIV-1 infection (PHI) but then rapidly disappear from the circulation. This cell loss may be due to susceptibility to infection with HIV-1 but could also be due to inappropriate apoptosis, an expansion of T regulatory cells, trafficking out of the circulation, or dysfunction. We purified CD38^{+++}CD4$^+$ T cells from peripheral blood mononuclear cells, measured their level of HIV-1 DNA by PCR, and found that about 10% of this population was infected. However, a small subset of HIV-specific CD4$^+$ T cells also expressed CD127, a marker of long-term memory cells. Purified CD127$^+$CD4$^+$ lymphocytes contained fivefold more copies of HIV-1 DNA per cell than did CD127-negative CD4$^+$ cells, suggesting preferential infection of long-term memory cells. We observed no apoptosis of antigen-specific CD4$^+$ T cells in vitro and only a small increase in CD45RO$^+$CD25$^+$CD127dimCD4$^+$ T regulatory cells during PHI. However, 40% of CCR5$^+$CD38^{+++} CD4$^+$ T cells expressed gut-homing integrins, suggesting trafficking through gut-associated lymphoid tissue (GALT). Furthermore, 80% of HIV-specific CD4$^+$ T cells expressed high levels of the negative regulator CTLA-4 in response to antigen stimulation in vitro, which was probably contributing to their inability to produce interleukin-2 and proliferate. Taken together, the loss of HIV-specific CD4$^+$ T cells is associated with a combination of an infection of CCR5$^+$ CD127$^+$ memory CD4$^+$ T cells, possibly in GALT, and a high expression of the inhibitory receptor CTLA-4.

Preferential infection and loss of human immunodeficiency virus (HIV)-specific CD4$^+$ T cells have been proposed as significant factors leading to the dysfunction of the immune response to HIV-1 infection (15, 31, 47). We recently found that HIV-specific CD4$^+$ T cells from a long-term nonprogressor with unusually low viral replication expressed cell surface CCR5 (91), consistent with another report describing CCR5$^+$ HIV-specific CD4$^+$ T cells in subjects with chronic HIV-1 infection (90). We also observed that, very early in primary HIV-1 infection (PHI), HIV-specific CD4$^+$ T cells expressed cell surface CCR5, together with a high expression of the activation antigen CD38 and the cell cycle marker Ki-67 but greatly reduced expression of CD127 (interleukin-7 receptor [IL-7R]) (94), a marker of long-term memory cells (63). During PHI and in most asymptomatic HIV-positive (HIV$^+$) subjects, coreceptor usage by HIV-1 is largely directed towards CCR5 (10, 98), suggesting that CCR5$^+$ HIV-specific CD4$^+$ T cells will be targeted by the virus during the early stage of the infection. In vitro studies have shown that CD4$^+$ T cells with activated memory phenotypes are preferentially susceptible to infection by HIV-1 (70, 72), while in vivo studies have shown that Ki-67$^+$ CD4$^+$ T cells are productively infected during PHI (96).

Therefore, we hypothesized that activated, proliferating HIV-specific CD4$^+$ T cells coexpressing CCR5 and high levels of CD38 would be prime targets for HIV-1 infection in vivo during PHI. In our previous cross-sectional study, these cells appeared only transiently, exhibiting a rapid decline approximately 2 to 3 weeks following the onset of symptoms of the acute viral illness (94), consistent with cytopathic infection in vivo.

Dramatic losses of CCR5$^+$ CD4$^+$ T cells as a result of cytopathic infection, particularly in the gut, have been reported in primary simian immunodeficiency virus (SIV) infection (40, 45, 82), and a similar loss of gut CD4$^+$ T cells occurs in primary HIV infection (7, 22, 48). If cytopathic infection of CCR5$^+$ CD4$^+$ T cells is particularly localized to gut-associated lym-

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phoid tissue (GALT), then the trafficking of $CCR5^+$ HIV-specific $CD4^+$ T cells to GALT might also contribute to the rapid loss of these cells. The homing of memory $CD4^+$ T cells to GALT is determined by the coexpression of the integrins α4 (CD49d) and β7, which specify binding to the mucosal vascular addressin MAdCAM-1 (42, 59, 87). A previous study of PHI reported a selective loss of $CCR5^+\alpha4\beta7^+$ CD62L-negative$CD45RO^+$ $CD4^+$ T cells from the circulation (34), suggesting that the expression of gut-homing integrins α4 (CD49d) and β7 on $CCR5^+$ HIV-specific $CD4^+$ T cells may determine their fates.

However, their transient appearance could be also be due to normal homeostatic processes, such as apoptosis or feedback regulation, since a similar peak of $CD4^+$ T-cell responses has also been observed in other acute viral infections in both mice (80) and humans (3, 18, 55). HIV-specific $CD4^+$ T cells during PHI were found to contain low levels of Bcl-2 (94), which has previously been associated with a propensity to undergo apoptosis in vitro and in vivo (74). Precursors of long-term memory $CD4^+$ and $CD8^+$ T cells in murine models selectively express IL-7Rα (CD127), which may play a role in sparing these cells from apoptosis by mediating the signaling leading to the reexpression of Bcl-2 (20, 30, 39, 63). However, 80 to 90% of HIV-specific $CD4^+$ T cells lack CD127 during PHI (94), consistent with a predetermined apoptotic fate for most $CCR5^+CD38^{+++}$ $CD4^+$ T cells.

Apart from apoptosis, the down-regulation of a CD4 T-cell response is also believed to be mediated by feedback inhibition exerted by $CD25^+$ $CD4^+$ T regulatory (T reg) cells, which have been shown to be important in the control of CD4-mediated inflammatory diseases (60). $CD25^+$ T reg cells may also express CTLA-4, an important negative signaling molecule (56, 61, 76). The suppressive influence of CTLA-4 signaling has been inferred from the early fatal $CD4^+$ lymphoproliferation observed in CTLA-4 gene knockout mice (78, 85). We previously observed that, compared to the expression of CTLA-4 by T cells from HIV-negative controls, intracellular CTLA-4 expression was greatly increased during PHI, especially in $CD8^+$ T cells, when maximally stimulated with phorbol myristate acetate and ionomycin (J. Zaunders et al., unpublished results).

Therefore, in the current study we aimed to determine the extent to which all of these various factors may exert a negative influence on $CCR5^+CD38^{+++}$ HIV-specific $CD4^+$ T cells during PHI. We measured their rate of infection with HIV-1 DNA, their rate of spontaneous apoptosis, as well as their expression of the gut-homing integrins α4 and β7, and also the possible generation of CTLA-4^+ T regulatory cells during PHI. The results suggest that none of these factors alone leads to the loss of HIV-specific $CD4^+$ T cells during the resolution of PHI but instead point to a complex multifactorial process that results in an impaired response.

MATERIALS AND METHODS

Subjects. A total of 29 subjects, diagnosed with primary HIV-1 infection as previously described (92), were included in this study and then enrolled in the PHAEDRA/CORE 01 observational cohort. All subjects were males whose risk factor was sex with males. The median age was 34 years, the median CD4 count was 583 cells/μl, the median plasma HIV RNA was 111,450, the median number of days since the onset of symptoms was 21 (seven subjects were asymptomatic), and the median number of bands on HIV-1 Western blots was 1.

Six HIV^+ subjects with established infections but undetectable plasma HIV RNA viral loads (<50 copies/ml) and without antiretroviral therapy at the time of study were also included and are referred to as "HIV^+ controllers."

Healthy HIV-negative university and hospital staff members were recruited as controls for this study. The PHAEDRA/CORE 01 study was approved by the St. Vincent's Hospital Ethics Committee, and all subjects gave written informed consent.

T-lymphocyte phenotyping of fresh whole blood. The monoclonal antibodies used were CD3-PerCP-Cy5.5 and -Pacific Blue; CD4-phycoerythrin (PE)-Cy7, -Alexa Fluor 700, and -allophycocyanin (APC); CD8-Alexa Fluor 700 and -APC-Cy7; CD38-PE-Cy7, -APC, and -PE; CCR5-fluorescein isothiocyanate (FITC) gamma interferon (IFN-γ)-APC; CD45RO-FITC; CD25-APC; CD49d-PE; integrin β7-APC; activated caspase-3-PE; CTLA-4-PE; CD19-PE-Cy7; CD56-APC; CD16-APC-Cy7; HLA-DR-FITC; and CD123-PE (Becton Dickinson, San Jose, CA); CD4-energy-coupled dye (ECD), CD45RO-ECD, and IL-7R (CD127)-PE (Beckman Coulter, Hialeah, FL); and CD62L-APC-Cy7 (eBioscience, San Diego, CA). All antibodies were used according to the manufacturers' directions.

The staining of $CD4^+$ T-cell subsets in fresh peripheral blood was performed as previously described (91, 94) on whole blood within 1 h of venepuncture to minimize spontaneous loss of CCR5 expression. T regulatory $CD4^+$ T-cell subsets were analyzed by five-color flow cytometry on an LSR II flow cytometer (Becton Dickinson) using CD3-PerCP-Cy5.5, CD4-PE-Cy7, CD25-APC, CD127-PE, and CD45RO-FITC as described elsewhere (65a). Foxp3 expression was studied in CD3-PerCP-$Cy5.5^+$CD4-PE-$Cy7^+$CD25-$FITC^+$CD127-PElow cells using Foxp3-APC (eBioscience) according to the manufacturer's directions.

The immunophenotyping of gut-homing $CD4^+$ T cells was analyzed by nine-color flow cytometry on the three-laser LSR II by staining with CD3-Pacific Blue, CD4-Alexa Fluor 700, CD8-APC-Cy7, CD45RO-ECD, CD38-PE-Cy7, HLA-DR-PerCP, CCR5-FITC, integrin β7-APC, and CD49d-PE. For analysis, a minimum of 100,000 events were collected. Spectral compensations were set using cells stained individually with the different fluorochrome conjugates and validated by staining peripheral blood mononuclear cells (PBMCs) with combinations of CD3-Pacific Blue, CD4-ECD, CD8-Alexa Fluor 700, CD19-PE-Cy7, CD56-APC, CD16-APC-Cy7, HLA-DR-PerCP, CD123-PE, and CD14-FITC monoclonal antibodies and obtaining expected patterns (data not shown).

Purification of $CD4^+$ T-cell subsets by cell sorting of PBMCs. Cryopreserved PBMCs from three PHAEDRA/CORE 01 subjects and a further three subjects, who had been included in a previous study (68), at presentation of PHI were used in cell sorting experiments. These PBMCs were obtained a median of 8 days after the onset of symptoms. Cryopreserved PBMCs (12×10^6 to 17×10^6) obtained from three subjects were thawed and stained with CD4-APC and CD38-PE. Cells were washed once with phosphate-buffered saline, fixed with 3% paraformaldehyde in phosphate-buffered saline for 30 min on ice, and then sorted using a FACSVantage by running FACSDiva software (version 4.1; Becton Dickinson). Four populations were obtained from PBMCs: $CD38^{+++}CD4^+$ and $CD38dimCD4^+$ (Fig. 1A) as well as CD4-ve lymphocytes and monocytes defined as CD4dim and high side scatter cells. The purity of the cell subpopulations was >98% in all cases.

In further cell sorting experiments, cryopreserved PBMCs (15×10^6 to 23×10^6) from another three subjects with PHI were stained with CD4-APC and CD127-PE (Fig. 1B) and sorted into $CD127^+$ $CD4^+$ and $CD127^-$ $CD4^+$ subpopulations as described above.

HIV-1 DNA quantification. HIV-1 DNA was quantified by a real-time PCR assay specific for HIV-*gag* using the Rotor-Gene 3000 (Corbett Research, Sydney, Australia). HIV-1 DNA was compared with genomic DNA, determined by beta-actin detection as previously described (75). Both real-time assays used sequence-specific fluorogenic TaqMan probes. Standard curves were constructed by using pNL4-3 and purified human DNA (Sigma). The primers and probes used were the HIV-*gag* sense primer 5′-AGTGGGGGGACATCAAGCAGCCATGCAAAT-3′, antisense primer 5′-TACTAGTAGTTCCTGCTATGTCACTTCC-3′, detection probe 5′-6-carboxyfluorescein-ATCAATGAGGAAGCTGCAGAATGGGATAG-6-carboxytetramethylrhodamine-3′, beta-actin sense primer 5′-TCACCCACACTGTGCCCATCTACGA-3′, beta-actin antisense primer 5′-CAGCGGAACCGCTCATTGCCAATGG-3′, and detection probe 5′-6-carboxyfluorescein-ATGCCCTCCCCCATGCCATCCTGCG-6-carboxytetramethylrhodamine-3′. DNA extraction from sorted cells was performed using the High Pure viral nucleic acid reagents (Roche, Castle Hill, Australia) with an extended 16-h protease K digestion incubation at 56°C. To estimate the number of copies of

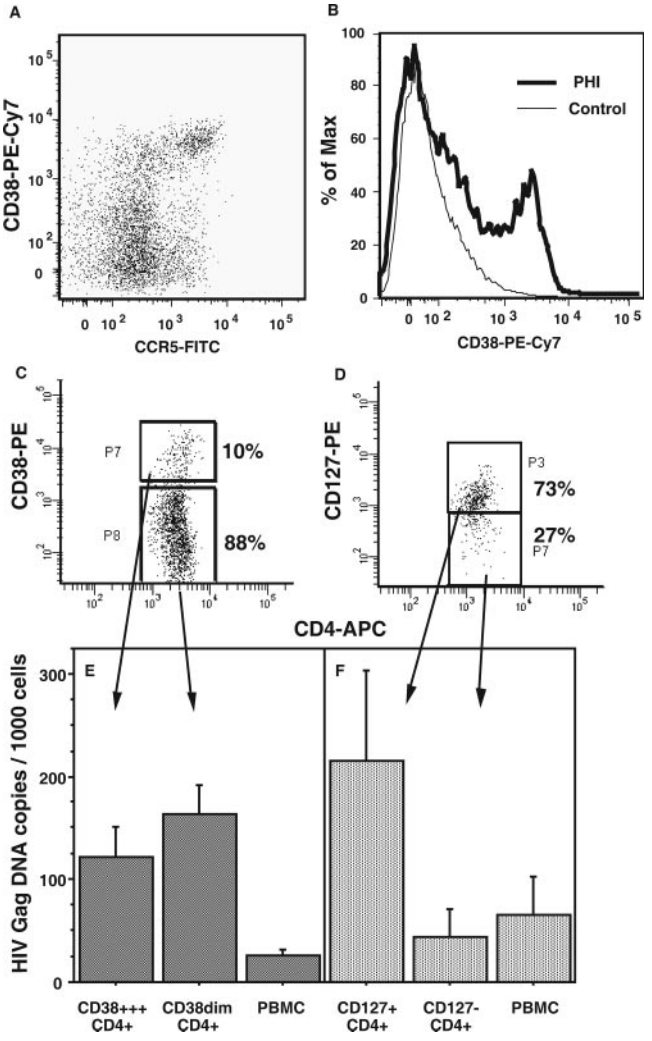

FIG. 1. HIV DNA in $CD38^{+++}$ and $CD127^{+}$ subpopulations of $CD4^{+}$ cells from PBMCs during PHI. A representative dot plot of $CCR5^{+}CD38^{+++}$ $CD4^{+}$ T cells present in fresh whole blood during PHI is shown in panel A, and a representative comparison of CD38 expression on $CD4^{+}$ T cells between a subject during PHI and a healthy adult control subject is shown in panel B. PBMCs were stained with CD4-APC and CD38-PE (C) or CD4-APC and CD127-PE (D) and sorted into different subpopulations. Representative histograms for each of the two sorting protocols are shown. DNA was extracted from purified cells, and the number of copies of HIV DNA per cell in each subpopulation was determined by quantitative PCR and normalized to beta-actin. Each column shows the mean (and standard error) from three independent experiments for each subpopulation of $CD4^{+}$ cells defined by CD38 (E) or CD127 (F) as well as for the corresponding unsorted PBMC samples.

HIV DNA per cell, it was assumed that the yield of DNA extracted was 1 ng per 150 cells (12).

Intracellular cytokine assay. HIV Gag-specific $CD4^{+}$ T cells were identified by using a 6-h whole-blood intracellular cytokine assay with six-color flow cytometry, as previously described (91, 94). Overlapping HIV-1 Gag 15-mer peptides, obtained from the NIH AIDS reference reagents program (catalog no. 11057), were used as a pool of 122 peptides at an individual concentration of 2 μg/ml each. Cytomegalovirus (CMV)-specific $CD4^{+}$ T cells were identified as previously described (91, 94). For analysis, 300,000 events were collected. T lymphocytes were first gated on CD3-PerCP-Cy5.5 versus side scatter and then on CD4-PE-Cy7-positive/CD8-APC-Cy7-negative cells. Finally, IFN-γ–APC^{+} cells were analyzed for staining with either CTLA-4-PE or activated caspase-3-PE.

Statistical analyses. All statistical tests were performed using StatView 5.0 for Macintosh (Abacus Concepts, Berkeley, CA). Results for replicate experiments are shown as means and standard errors. Results for each subject group are shown as medians and interquartile ranges. The Mann-Whitney U test was performed to compare continuous variables between subject groups. A two-sided P value of <0.05 was considered statistically significant.

RESULTS

HIV DNA in purified $CD4^{+}$ T-cell subpopulations. The aim of these experiments was to determine the level of infection of $CCR5^{+}CD38^{+++}$ $CD4^{+}$ T cells during PHI (Fig. 1A) (94). However, CCR5 expression may be lost during PBMC isolation and cryopreservation (91). Therefore, elevated CD38 expression on $CD4^{+}$ T cells during PHI (Fig. 1B), which survives cryopreservation and which on its own is a marker of antigen-specific $CD4^{+}$ T cells from subjects with PHI (94), was used as a surrogate marker for the $CCR5^{+}CD38^{+++}$ HIV-specific $CD4^{+}$ T cells in PBMCs. Purified $CD38^{+++}$ $CD4^{+}$ T cells and CD38dim subsets of $CD4^{+}$ lymphocytes were obtained by cell sorting cryopreserved PBMC samples from three individuals with primary HIV-1 infection (Fig. 1C). The $CD38^{+++}$ $CD4^{+}$ T-cell subpopulation was exclusively $CD45RO^{+}$ memory phenotype cells, while the CD38dim $CD4^{+}$ T-cell subpopulation included both CD38-intermediate CD45RO-negative naïve phenotype cells and CD38-negative $CD45RO^{+}$ resting memory $CD4^{+}$ T cells (data not shown). We also purified CD4-negative lymphocytes and monocytes by cell sorting (data not shown).

DNA was extracted from the different subpopulations, and real-time PCR was used to measure copy numbers of HIV-1 DNA normalized to beta-actin (Fig. 1E). Very few copies ($\leq$1 per 1,000 cells) of HIV-1 DNA were detected in CD4-negative lymphocyte or monocyte subpopulations (data not shown). However, both $CD38^{+++}$ $CD4^{+}$ T cells and CD38dim $CD4^{+}$ T cells contained comparable levels of HIV-1 DNA on a per cell basis, and both purified subpopulations were enriched for HIV-1 DNA relative to the starting PBMCs (Fig. 1E).

The number of HIV-1 DNA copies per cell indicate that if all infected cells contained only one copy of HIV-1 DNA each, then 10 to 15% of $CD4^{+}$ T cells were infected. In a recent study of acute SIV infection, it was shown by single-cell analysis that infected cells contained an average of 1.5 copies per infected cell (45), consistent with previous studies of chronic HIV-1 infection (19, 29). Therefore, there is probably an upper limit of about 10% infected cells in both the activated $CD38^{+++}$ and nonactivated CD38dim $CD4^{+}$ T-cell subpopulations.

The results show that there was no preferential infection of the activated $CD38^{+++}$ $CD4^{+}$ T cells, despite our previous observation that they were predominantly $CCR5^{+}$ in fresh whole blood. Instead, since a large majority of $CD4^{+}$ T cells were CD38dim (Fig. 1A), then the majority of the copies of HIV-1 DNA in PBMCs were in the nonactivated CD38dim subpopulation.

Similarly, low CD127 expression was also used to enrich HIV-specific $CD4^{+}$ T cells (94) in order to measure infection with HIV-1 DNA. Unexpectedly, purified $CD127^{+}$ $CD4^{+}$ T cells (Fig. 1D) were found to contain a disproportionately

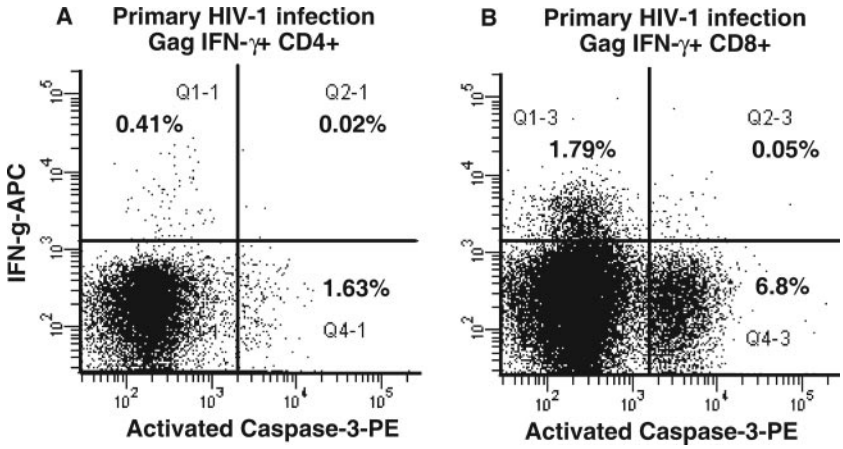

FIG. 2. Lack of apoptosis of Gag-specific $CD4^+$ T cells in vitro during PHI. Following stimulation with Gag peptides in the intracellular cytokine assay, $CD4^+$ (A) and $CD8^+$ (B) T cells were simultaneously stained with monoclonal antibodies to IFN-γ and activated caspase-3. Representative histograms for one subject out of four consecutive subjects with PHI are shown. Also shown are percentages of cells in quadrants.

higher number of copies of HIV-1 DNA, fivefold more on a per cell basis than the more-activated CD127-negative $CD4^+$ T cells (Fig. 1F). Similar to the CD38dim $CD4^+$ T cells, the $CD127^+$ subset makes up the greater part of $CD4^+$ T cells within PBMCs, and therefore the vast majority of copies of HIV-1 DNA were in the $CD127^+$ subset of $CD4^+$ T cells. Conversely, during PHI, 80 to 90% of HIV-specific $CD4^+$ T cells are CD127 negative (94), again suggesting that the majority of HIV-specific $CD4^+$ T cells are not directly infected in vivo.

Combining the results of the two sets of sorting experiments suggests that the $CD38^{+++}$ $CD4^+$ T cells that are infected will be the 10 to 20% that are also $CD127^+$ (94). However, in the current studies, there were insufficient cells available to directly confirm that $CD38^{+++}CD127^+$ $CD4^+$ cells were highly infected; this will be addressed in future studies.

Apoptosis of HIV-specific $CD4^+$ T cells. Since we estimated that only a minority of $CCR5^+CD38^{+++}$ $CD4^+$ T cells were infected with HIV-1 DNA, the loss of these cells from the circulation (94) may not be due to direct cytopathic effect. We previously found that the rate of spontaneous apoptosis, in vitro, of $CD4^+$ T cells from subjects with PHI was slightly elevated compared to that from healthy adult controls (93), and we expected to observe preferential apoptosis of IFN-γ^+ *gag*-specific $CD4^+$ T cells in early PHI, as was previously described for antigen-specific $CD4^+$ T cells in chronic HIV-1 infection (89). Activated intracellular caspase-3 was used as a marker of apoptotic $CD4^+$ T cells, in combination with the intracellular cytokine assay, by using fresh whole-blood samples from subjects in four independent experiments (Fig. 2A). The results in all cases showed that IFN-γ^+ Gag-specific $CD4^+$ T cells were clearly separate from apoptotic activated caspase-3^+ $CD4^+$ T cells. Therefore, we were unable to demonstrate directly a high rate of spontaneous apoptosis of Gag-specific $CD4^+$ T cells that actively produced IFN-γ. Similarly, antigen-specific $CD8^+$ T cells in the same samples were not positive for activated caspase-3, despite a very high level of apoptosis among $CD8^+$ T cells (Fig. 2B).

We also analyzed whether $CD38^{+++}$ cells were caspase-3^+ (data not shown). Apoptotic $CD8^+$ T cells were mostly $CD38^{+++}$, consistent with our previous results (93). In contrast, apoptotic $CD4^+$ expressed only intermediate levels of CD38, again indicating that antigen-specific $CD38^{+++}$ $CD4^+$ T cells do not spontaneously undergo apoptosis in vitro.

Trafficking of $CCR5^+CD38^{+++}$ $CD4^+$ T cells during primary HIV-1 infection. A third possible reason for the rapid loss of $CCR5^+CD38^{+++}$ $CD4^+$ T cells from circulation could be the sequestration in tissues, as shown for CD8 effector cells in mice (44). Since it has recently been reported that gut lymphoid tissue is a major site of HIV-1 during primary infection (7, 22, 48), we examined the coexpression of the gut-homing markers CD49d and integrin β7 on $CD4^+$ T cells during PHI, in particular on the $CD45RO^+$ memory cell and $CCR5^+CD38^{+++}$ HIV-specific subsets of $CD4^+$ T cells, respectively (Fig. 3A). The proportions of $CD45RO^+$ memory $CD4^+$ T cells expressing CD49d and integrin β7 were similar between controls and 12 consecutive subjects presenting during PHI (Fig. 3B), suggesting that there was no preferential loss of this circulating subset of memory $CD4^+$ T cells. However, for the subjects with PHI, a median of 41% of $CCR5^+CD38^{+++}$ $CD4^+$ T cells was found to be positive for the gut-homing markers CD49d and integrin β7, which was generally higher than that for $CD45RO^+$ memory $CD4^+$ T cells (Fig. 3B). These results suggest that a large fraction of the $CCR5^+CD38^{+++}$ $CD4^+$ T cells present during PHI will preferentially traffic to gut-associated lymphoid tissue.

Since the greatest burden of HIV-1 DNA was found in $CD127^+$ $CD4^+$ T cells (Fig. 1F), we also determined the proportion of $CD4^+$ T cells which were $CCR5^+CD127^+$ and whether such cells expressed the gut-homing marker integrin β7 (Fig. 3C). In healthy adult controls, approximately 10% of $CD4^+$ T cells were $CCR5^+CD127^+$, and furthermore, a large fraction of these cells were $\beta7^+$ $CD45RO^+CD62L$ negative (Fig. 3C). We observed a similar subset of $CCR5^+CD127^+$ $CD4^+$ T cells in subjects with PHI, but within this subset, there were significantly fewer $\beta7^+$ $CD45RO^+CD62L$-negative cells (Fig. 3D).

$CD25^+$ $CD4^+$ T regulatory cells during primary HIV-1 infection. Another possible reason for the loss of antigen-specific $CD4^+$ T cells during the resolution of PHI may be the devel-

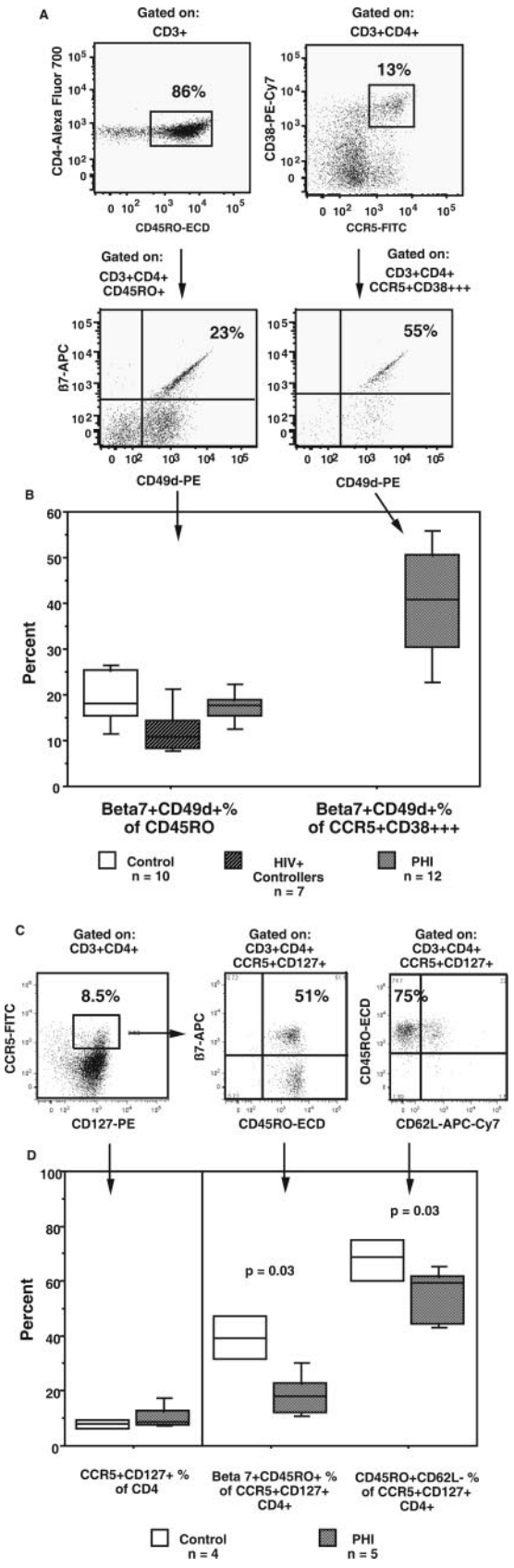

FIG. 3. Gut-homing of CCR5$^+$ CD4$^+$ T cells during PHI. Fresh whole-blood samples were stained for CD3, CD4, CD45RO, CD62L, integrin β7, CD49d (integrin α4), CCR5, and CD38. Gut-homing CD4$^+$ T cells were identified by coexpression of integrin β7 and CD49d. (A) The presence of integrin β7$^+$CD49d$^+$ cells within CD45RO$^+$ and CCR5$^+$CD38^{+++} CD4$^+$ T-cell subsets is shown for 1 representative subject out of 12 subjects studied during PHI. The percentages of integrin β7$^+$CD49d$^+$ cells within their respective CD45RO$^+$ and CCR5$^+$CD38^{+++} CD4$^+$ T-cell subsets are shown. (B) Box plots of integrin β7$^+$CD49d$^+$ cells as a percentage of CD45RO$^+$CD4$^+$ T cell for the three subject groups (left) and as a percentage of CCR5$^+$CD38^{+++} CD4$^+$ T cells for subjects with PHI only (right). Fresh whole-blood samples were also stained for CD3, CD4, CD45RO, CD62L, integrin β7, CCR5, and CD127. The CCR5$^+$CD127$^+$ subset present in a representative healthy adult control subject is shown in panel C, together with its expression of CD45RO, integrin β7, and CD62L. Box plots of CCR5$^+$CD127$^+$ cells as a percentage of CD4$^+$ are shown at the left side of panel D, and β7$^+$CD45RO$^+$ and CD45RO$^+$CD62L-negative cells as percentages of CCR5$^+$CD127$^+$ CD4$^+$ T cells are also shown at the right side of the panel. Box plots depict the 90th, 75th, median, 25th, and 10th percentiles for each subject group, and the number of subjects in each group is shown. The *P* values shown are for subjects with PHI compared to those for healthy adult controls by a Mann-Whitney nonparametric test.

opment of CD25$^+$ CD4$^+$ T regulatory cells, which were recently reported in chronic HIV-1 infection (1, 33). We determined whether there was an elevation in the proportion or number of circulating CD25$^+$ CD4$^+$ T regulatory cells in subjects with PHI, compared to that in healthy adult controls. T regulatory cells were identified by an improved phenotypic method, combining increased expression of CD25 with reduced CD127 expression (Fig. 4A) (65a), and validated by the confirmation of the expression of Foxp3 selectively within these cells (Fig. 4B).

In subjects with PHI, there was a slight elevation in the proportion of CD45RO$^+$ CD4$^+$ T reg cells (Fig. 4C) compared to that in healthy adult controls. This phenotypic method also readily identifies CD45RO-negative CD4$^+$ T reg cells, but these were not different between subjects with PHI and healthy adult controls (Fig. 4C). In some subjects, a small proportion, around 10%, of activated CCR5$^+$CD38^{+++} CD4$^+$ T cells during PHI also had the CD25$^+$CD127low T reg phenotype (data not shown).

However, commensurate with the decrease in total CD4 T-cell counts in subjects with PHI, there was a decrease in the absolute numbers of T reg cells in subjects with PHI, compared to that in healthy adult controls (data not shown).

CTLA-4 expression by HIV-specific CD4$^+$ T cells. CTLA-4 may be involved in T reg activity (61), and we previously observed that there was increased expression of CTLA-4 by polyclonally stimulated T cells during PHI (J. Zaunders et al., unpublished). Therefore, we used the intracellular cytokine assay to assess intracellular expression of CTLA-4 by HIV-specific CD4$^+$ T cells in response to antigen stimulation in vitro. The results show that a very large proportion of IFN-γ$^+$ Gag-specific CD4$^+$ T cells coexpressed CTLA-4 during PHI (Fig. 5A).

The expression of CTLA-4 by antigen-specific CD4$^+$ T cells from the different subject groups is summarized in Fig. 5B. CMV-specific CD4$^+$ T cells from healthy adult controls, HIV$^+$ controllers, and subjects with PHI were all predominantly negative for CTLA-4 (Fig. 5B).

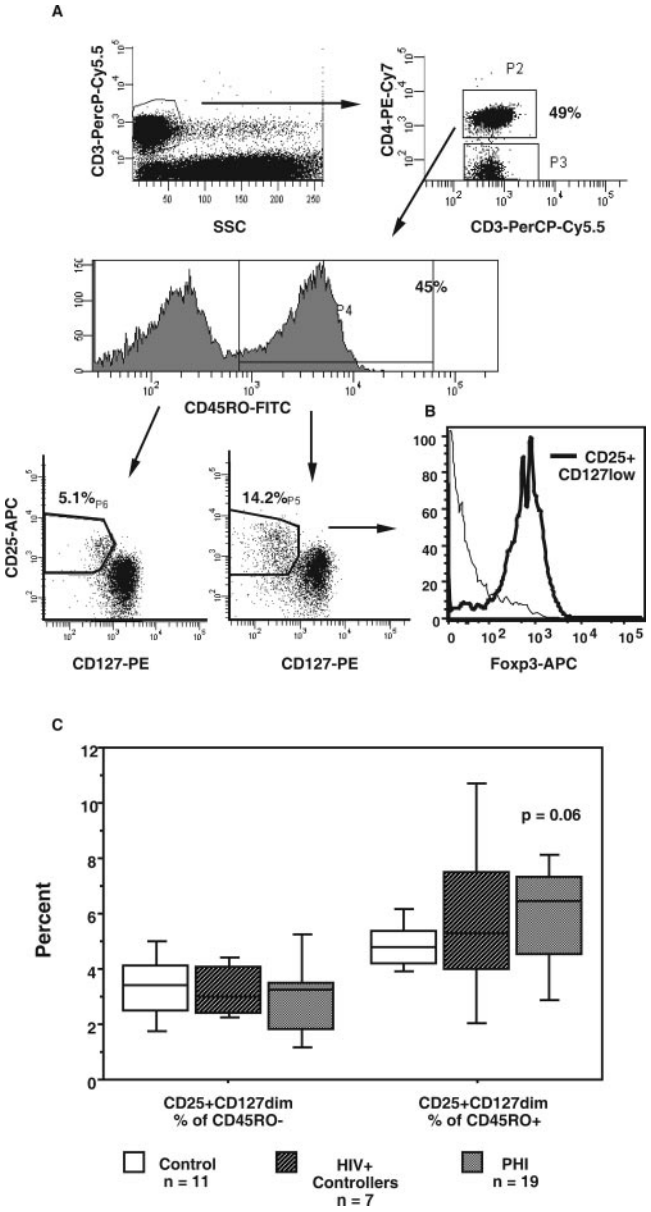

FIG. 4. T regulatory cells during PHI. T reg $CD3^+CD4^+$ cells were identified within $CD45RO^+$ and $CD45RO^-$ populations by high expression of CD25 and dim expression of CD127 (A). Representative histograms for a subject during PHI are shown. Identical gating was used for all cohorts. The selective expression of Foxp3 within $CD25^+CD127low$ $CD4^+$ T cells, compared with that for the remaining $CD4^+$ T cells, is shown in panel B. Overall results for all cohorts with the numbers of subjects in each cohort are shown in panel C. The results for each subpopulation are expressed as percentages of total $CD4^+$ T cells. Box plots depict 10th, 25th, median, 75th, and 90th percentiles. The *P* value shown is for subjects with PHI compared to that for healthy adult controls, by a Mann-Whitney nonparametric test.

Importantly, Gag-specific $CD4^+$ T cells from HIV^+ controllers also expressed a very low level of CTLA-4 in response to antigen-specific restimulation in vitro in contrast to the high level of CTLA-4 expression by HIV-1 Gag-specific $CD4^+$ T cells in subjects with PHI (Fig. 5B).

DISCUSSION

We embarked on the current studies with the hypothesis that the loss of HIV-specific $CD4^+$ T cells during the resolution of primary HIV-1 infection was the result of preferential infection of highly activated $CD38^{+++}$ CD127-negative $CCR5^+$ effector $CD4^+$ T cells, which we recently identified (94). In vitro and in vivo observations (14, 70, 72, 96) suggested that such activated $CD38^{+++}$ CD127-negative antigen-specific $CD4^+$ T cells carrying the coreceptor for transmitted strains of HIV-1 should have been a target for cytopathic HIV-1 infection during PHI.

While the initial results showed the presence of HIV-1 DNA in highly activated $CD38^{+++}$ $CD4^+$ T cells within the first weeks of symptomatic primary HIV-1 infection, the extent of infection of the $CD38^{+++}$ $CD4^+$ T cells in vivo appeared to be restricted. It was likely that only approximately 10% of these cells were infected, based on an average infection rate of 1.5 copies per infected cell (45). Therefore, the rapid decline in $CCR5^+CD38^{+++}$ HIV-specific $CD4^+$ T cells in the circulation that we previously observed (94) was probably only partly due to cytopathic infection. This conclusion is supported by a parallel study of vaccinia virus-specific $CD4^+$ T cells, where we observed a similar peak of antigen-specific $CCR5^+$ $CD38^{+++}$ $CD4^+$ T cells, followed by a decline coincident with the resolution of the vaccinia virus infection (95).

Nevertheless, cytopathic infection probably remains a significant factor in the decline of $CCR5^+$ HIV-specific $CD4^+$ T cells. Previously, we found that proliferating $CCR5^+$ $Ki\text{-}67^+$ $CD4^+$ T cells did not accumulate in the circulation during PHI, in contrast to a marked accumulation of $CCR5^+$ $Ki\text{-}67^+$ $CD4^+$ T cells in subjects with acute Epstein-Barr virus infection (92). A dramatic loss of $CCR5^+$ $CD4^+$ T cells, particularly in the gut, has been reported in acute SIV infection (40, 45, 82) and a similar loss of gut $CD4^+$ T cells may occur in primary HIV infection (7, 22, 48). It is therefore notable that during PHI, just under half of the circulating $CCR5^+CD38^{+++}$ $CD4^+$ T cells expressed the gut-homing markers CD49d and integrin β7.

Since the vast majority of HIV-1 DNA was found in $CD127^+$ $CD4^+$ T cells, we investigated the expression of CCR5 on these cells both in healthy adults and during PHI. Around 10% of $CD4^+$ T cells in peripheral blood are $CCR5^+CD127^+$, but in healthy adults, nearly half also express integrin β7, suggesting trafficking to GALT. It is possible that the $CCR5^+CD127^+\beta7^+$ $CD4^+$ T cells subset is a significant target for HIV-1 infection due to direct infection of resting cells in GALT, as seen in the acute SIV infection model (40). In our current study, the overall proportion of gut-homing $CD45RO^+$ memory cells in the circulation was not apparently reduced but the $CCR5^+CD127^+\beta7^+CD62L$-negative subset of $CD4^+$ T cells appeared to be selectively depleted during PHI.

Our findings are consistent with those of an earlier report of reduced circulating $\alpha4\beta7^+$ $CCR5^+$ $CD4^+$ T cells during PHI (34) as well as those of a previous study of acute SIV infection which reported a loss of the small subset of $CD4^+$ T cells expressing CD103 (integrins $\alpha E\beta7$) (46), although this may direct localization around E-cadherin$^+$ epithelial cells within GALT rather than direct trafficking to GALT (32). Detailed

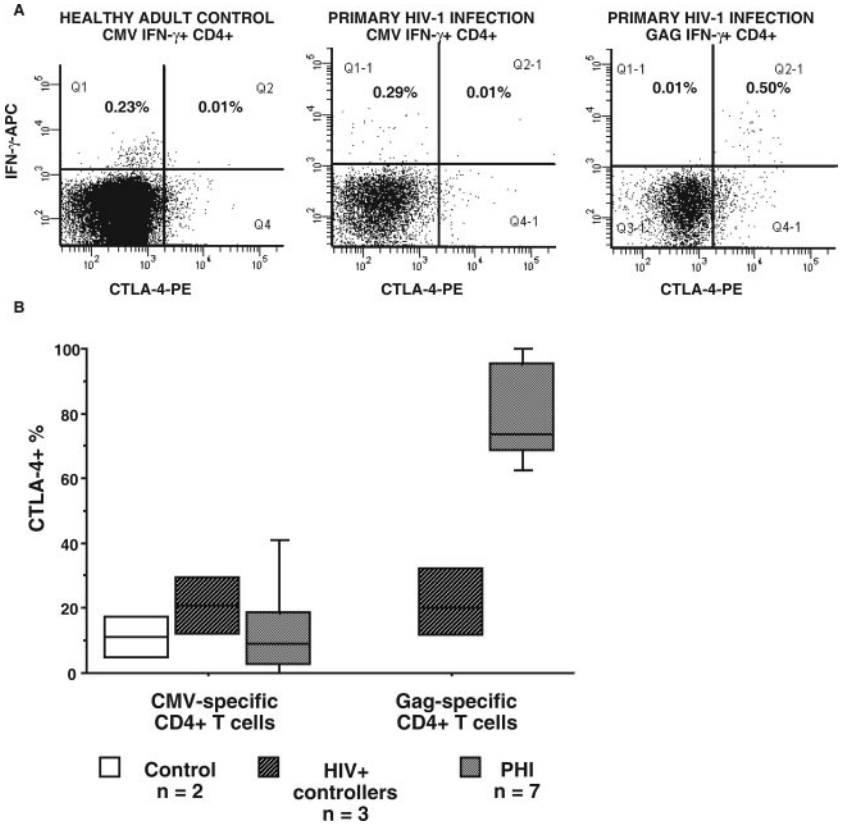

FIG. 5. CTLA-4 expression by *gag*-specific $CD4^+$ T cells during PHI. (A) Following the stimulation of whole blood from a healthy adult control with CMV lysate or from a subject during PHI with either CMV lysate or Gag peptides, in the intracellular cytokine assay, $CD4^+$ T cells were simultaneously stained with monoclonal antibodies to IFN-γ and CTLA-4. Representative histograms are shown. (B) Overall results for all cohorts are shown with the number of subjects in each cohort. The results for CTLA-4^+ cells are expressed as percentages of IFN-γ^+ $CD4^+$ T cells. Box plots depict the 10th, 25th, median, 75th, and 90th percentiles.

studies of cells from biopsies of GALT are therefore required to clarify the fate of HIV-specific $CD4^+$ T cells.

The level of HIV-1 DNA within $CD127^+$ $CD4^+$ T cells was much higher than that in their CD127-negative counterparts. We had previously shown that the CD127-negative subpopulation of T cells contained most of the HIV-specific $CD4^+$ T cells (94) but also contained highly activated $CD38^+$, Ki-67^+, and Bcl-2low cells prone to apoptosis during PHI (93). The observation that $CD127^+$ $CD4^+$ T cells are highly infected has two important implications for HIV-1 pathogenesis.

First, murine studies suggest that the small $CD127^+$ subset of $CCR5^+CD38^{+++}$ HIV-specific effector $CD4^+$ T cells that we previously observed (94) represents precursors of long-term memory $CD4^+$ T cells (30, 39, 63). Therefore, HIV-1 infection may preferentially target these nascent HIV-specific memory $CD4^+$ T cells. Future large-scale cell sorting experiments are required to purify sufficient $CD127^+CCR5^+CD38^{+++}$ effector $CD4^+$ T cells as well as $CD127^+\beta7^+$ $CD4^+$ T cells to confirm that these cells are highly infected as implied by our current results.

Second, there is an essential role for frequent signaling by IL-7 in memory T-cell survival (38, 63, 66, 77). However, several studies have shown that the culture of PBMCs from subjects with chronic HIV-1 infection in the presence of IL-7 efficiently leads to the production of HIV-1 (9, 49, 69), specifically from latently infected resting cells (83), probably involving the induction of NF-kB (8, 17).

Other studies have shown that treatment of PBMCs with IL-7 makes resting $CD4^+$ T cells permissive for productive HIV-1 infection (17, 64, 71, 81) without necessarily entering the cell cycle (81). Signaling by the IL-7 receptor leads to the up-regulation of NF-kB (reviewed in reference 28), which is a major transcription factor for the initiation of HIV-1 replication (reviewed in reference 57). We have found that plasma levels of IL-7 were elevated during PHI, and normal expression of the γ chain of the IL-7 receptor was perturbed (62). Taking these results together, it is therefore possible that increased IL-7 signaling in vivo can contribute significantly to promoting the infection of otherwise apparently resting $CCR5^+$ $CD127^+$ $CD4^+$ T cells during PHI. High levels of infection of resting $CD4^+$ T cells have been reported in lymphoid tissue during acute SIV infection (97), particularly in GALT (40), where IL-7 production is well described (28, 84). Subsequently, the homeostasis of resting $CD4^+$ T cells, involving intermittent delivery of the IL-7 survival signal (38, 63, 66, 77), may lead to the depletion of $CD127^+$ $CD4^+$ T cells containing HIV-1 DNA.

Further studies are required to determine whether the HIV-1 DNA in resting $CD4^+$ T cells was fully integrated or remained in a relatively labile unintegrated form (54) and to

what extent infectious virus can be recovered from either activated or resting subsets, particularly when incubated with exogenous IL-7.

However, the major mechanism believed to mediate the decrease in effector T cells during the resolution of a primary immune response is apoptosis (43). Central to the apoptosis of $CD8^+$ effector T cells is the balance between antiapoptotic Bcl-2 and proapoptotic Bim (52), while the reexpression of Bcl-2 mediated by IL-7R signaling is reportedly involved in the transition from effector to long-term memory cells (21, 63). We had directly observed a decrease in Bcl-2 expression in HIV-specific $CD4^+$ T cells during PHI (94), so we expected to observe spontaneous apoptosis of these cells in vitro, as has been reported with chronic HIV-1 infection (89). However, this was not observed in our intracellular cytokine assays, even though elevated levels of apoptotic $CD4^+$ and $CD8^+$ T cells were clearly seen, consistent with our previous results (93).

This discrepancy may be due to a difference between chronic and acute HIV-1 infection. Cells committed to apoptosis in acute infection may be unable to produce cytokines in vitro. HLA class II tetramers (65, 67) would provide a more direct means to detect apoptotic antigen-specific $CD4^+$ T cells.

Another possible mechanism of the down-regulation of the HIV-specific response is the suppression by $CD25^+$ $CD4^+$ T regulatory cells (60). While it has recently been reported that T reg cells can be infected by HIV-1 (50), most reports suggest that T reg cells (1, 4, 33, 86) are increased in chronic HIV-1 infection. We observed a slight increase in T reg cells, identified phenotypically in samples from subjects with PHI. It is possible that an effect of cytopathic infection simultaneously limited a potentially greater expansion of T reg cells, but our cell sorting experiments showed few copies of HIV-1 DNA in CD127-negative cells, which include T reg cells, suggesting that they were not preferentially infected.

A major finding is the high level of expression of CTLA-4 by HIV-specific CD4 T cells during PHI, and it is probable that this is a significant factor in their decline. Our results are consistent with reports of increased expression of CTLA-4 in $CD4^+$ T cells in peripheral blood and lymphoid tissue in HIV-1 infection (4, 73), including $CCR5^+$ and Ki-67^+ $CD4^+$ T cells (37). Importantly, murine genetic studies have shown that CTLA-4 expression is a potent constraint on $CD4^+$ T-cell expansion in vivo (78, 85). However, it is not clear whether the expression of CTLA-4 by antigen-specific $CD4^+$ T cells themselves is suppressive (16, 26) or whether there is an indirect suppressive effect mediated by other cells, such as T regulatory $CD4^+$ T cells expressing CTLA-4 (5, 61, 79), and we had expected to find an increased expression of CTLA-4 associated with an increase in T reg cells.

The rapid expression of CTLA-4 in response to HIV-1 Gag antigen would be predicted to interfere with CD28 signaling (11, 36), reducing the synthesis and stability of IL-2 mRNA (2). Therefore, the overexpression of CTLA-4 may provide a mechanistic explanation for the lack of IL-2 production and in vitro proliferation by HIV-specific $CD4^+$ effector T cells, which we and others have previously observed in PHI (24, 94) and which is a hallmark of untreated chronic HIV-1 infection (23, 27, 88). An exception may be those rare patients controlling viral replication without therapy who exhibit HIV-specific IL-2 production by $CD4^+$ T cells and proliferation (6, 25, 58, 91). Therefore, it is significant that in the HIV-specific $CD4^+$ T cells from HIV^+ controllers included in this study, there was little CTLA-4 production in response to antigenic stimulation. Therefore, one avenue of investigation may be the possible beneficial effect of selectively interfering with the binding of CTLA-4 to B7 molecules on antigen-presenting cells (53).

In our parallel study of vaccinia virus-specific $CD4^+$ T effector cells, less than half coexpressed CTLA-4 with IFN-γ (95), suggesting an important difference in cell fate decisions between early HIV- and vaccinia virus-specific $CD4^+$ T cells. The cause of the altered differentiation of HIV-specific $CD4^+$ T cells is currently unknown but could be due to differences in dendritic cells (35) between the two infections. HIV-1 infection reportedly leads to decreases in dendritic cell subsets in the circulation (13, 51) and alterations of the phenotype of these cells in lymphoid tissue (41). Therefore, there may be alterations in signaling by antigen-presenting cells in the very early stages of $CD4^+$ memory T-cell differentiation following HIV-1 infection compared with that following vaccinia virus inoculation.

Altogether, the overall results of the current study argue against cytopathic infection and apoptosis of activated $CCR5^+$ $CD4^+$ effector T cells or an overabundance of T regulatory cells as major reasons for the loss of HIV-specific $CD4^+$ T cells during the resolution of PHI. Instead our results indicate that the infection of $CCR5^+$ $CD127^+$ $CD4^+$ T cells, including early HIV-specific memory $CD4^+$ T cells that traffic through GALT, may be an important step in pathogenesis. Furthermore, the high level of CTLA-4 expression associated with limited IL-2 production probably plays a role in blocking the proliferative potential and differentiation of HIV-specific $CD4^+$ T cells which escape infection.

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