Programmed Death 1 Expression on HIV-Specific CD4+ T Cells Is Driven by Viral Replication and Associated with T Cell Dysfunction

Michelle D'Souza, Andrew P. Fontenot, Doug G. Mack, Catherine Lozupone, Stephanie Dillon, Amie Meditz, Cara C. Wilson, Elizabeth Connick and Brent E. Palmer

*J Immunol* 2007; 179:1979-1987; doi: 10.4049/jimmunol.179.3.1979

http://www.jimmunol.org/content/179/3/1979
Programmed Death 1 Expression on HIV-Specific CD4⁺ T Cells Is Driven by Viral Replication and Associated with T Cell Dysfunction

Michelle D'Souza,* Andrew P. Fontenot,†‡ Doug G. Mack,* Catherine Lozupone,‡
Stephanie Dillon,* Amie Meditz,* Cara C. Wilson,*† Elizabeth Connick,* and Brent E. Palmer2*.

Functional impairment of HIV-specific CD4⁺ T cells during chronic HIV infection is closely linked to viral replication and thought to be due to T cell exhaustion. Programmed death 1 (PD-1) has been linked to T cell dysfunction in chronic viral infections, and blockade of the PD-1 pathway restores HIV-specific CD4⁺ and CD8⁺ T cell function in HIV infection. This study extends those findings by directly examining PD-1 expression on virus-specific CD4⁺ T cells. To investigate the role of PD-1 in HIV-associated CD4⁺ T cell dysfunction, we measured PD-1 expression on blood and lymph node T cells from HIV-infected subjects with chronic disease. PD-1 expression was significantly higher on IFN-γ-producing HIV-specific CD4⁺ T cells compared with total or CMV-specific CD4⁺ T cells in untreated HIV-infected subjects (p = 0.0001 and p < 0.0001, respectively). PD-1 expression on HIV-specific CD4⁺ T cells from subjects receiving antiretroviral therapy was significantly reduced (p = 0.007), and there was a direct correlation between PD-1 expression on HIV-specific CD4⁺ T cells and plasma viral load (r = 0.71; p = 0.005). PD-1 expression was significantly higher on HIV-specific T cells in the lymph node, the main site of HIV replication, compared with those in the blood (p = 0.0078). Thus, PD-1 expression on HIV-specific CD4⁺ T cells is driven by persistent HIV replication, providing a potential target for enhancing the functional capacity of HIV-specific CD4⁺ T cells. The Journal of Immunology, 2007, 179: 1979–1987.

The Journal of Immunology

Received for publication February 8, 2007. Accepted for publication May 21, 2007.

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

1 This work was supported by the Colorado Center for AIDS Research and by reagents provided through the National Institutes of Health AIDS Research and Reference Reagent Program.
2 Address correspondence and reprint requests to Dr. Brent E. Palmer, Department of Medicine, University of Colorado at Denver and Health Sciences Center, 4200 E 9th Ave Box B164, Denver, CO 80262. E-mail address: brent.palmer@UCHSC.edu.
3 Abbreviations used in this paper: TEm, effector memory T cell; PD-1, programmed death 1; LCMV, lymphocytic choriomeningitis virus; ART, antiretroviral therapy; CEF, CMV-EBV-influenza virus; SEB, staphylococcal enterotoxin B; MFI, mean fluorescence intensity; PD-L1, PD-1 ligand 1; PD-L2, PD-1 ligand 2; FMO, fluorescence 1; DC, dendritic cell; mDC, myeloid DC; pDC, plasmacytoid DC.

Copyright © 2007 by The American Association of Immunologists, Inc. 0022-1767/07/$2.00

www.jimmunol.org
expression on total CD4+ T cells directly correlates with HIV viral load and that blockade of the PD-1 pathway enhances HIV-specific CD4+ T cell proliferation (16). However, this is the first study that directly measures PD-1 expression on HIV-specific CD4+ T cells. In addition, little is known about the expression of PD-1 on T cells in lymphoid tissues, which are the target organ of HIV infection and where the immune response against HIV is generated (20, 21).

Using blood and lymph node samples from untreated and antiretroviral therapy (ART)-treated subjects with chronic infection, we examined PD-1 expression on HIV-specific CD4+ T cells to determine whether PD-1 expression is related to viral load and whether suppression of HIV replication by ART restores its expression to normal levels. Our data show that PD-1 expression is up-regulated on HIV-specific CD4+ T cells in chronically infected subjects and is associated with viral replication. When HIV replication is suppressed using ART, PD-1 expression on HIV-specific CD4+ T cells returns to the levels seen on CMV-specific CD4+ T cells. Most interestingly, we show that PD-1 expression is significantly increased on CD4+ and CD8+ T cells in the lymph node in comparison with the blood of HIV-infected subjects. This study directly demonstrates that PD-1 expression on HIV-specific CD4+ T cells is increased in chronic HIV infection and can be used as a phenotypic marker of disease. However, unlike other disease markers, blockade of the PD-1 pathway enhances HIV-specific CD4+ T cell function and could prove to be an important target for immune mediated treatment of HIV infection.

Materials and Methods

Study population

Thirty-one HIV-1-infected subjects enrolled into two clinical cohorts based on their treatment status: ART with virological suppression; and untreated. Inclusion criteria for the suppressed cohort (n = 17) included receiving a combination antiretroviral agents with suppression of plasma viral load to <20 copies of HIV-1 RNA per ml of plasma for ≥6 mo (median CD4+ T cell count, 664 cells/µl; range, 336–1400 cells/µl). Untreated subjects (n = 14) were either treatment naive or off treatment for ≥6 mo with a median viral load of 15,200 copies HIV-1 RNA per ml of plasma (range, 20–40,400 copies of HIV RNA per ml), and a median CD4+ T cell count of 493 cells/µl (range, 247–1269 cells/µl). Matched lymph node and PBMC samples were obtained from seven untreated HIV-infected subjects. The median CD4+ T cell count was 416 cells/µl (range, 256–932 cells/µl), and the median viral load was 23,100 copies HIV RNA per ml of plasma (range, 5670–181,000 copies HIV RNA per ml). HIV-1-seronegative subjects (n = 13) were normal healthy adult volunteers. All study subjects participated voluntarily and gave informed consent; the study was approved by the University of Colorado Health Sciences Center Institutional Review Board.

Collection and preparation of PBMC and lymph node cells

Blood was collected in Vacutainer tubes containing sodium heparin (BD Vacutainer). Within 4 h of venipuncture, PBMC were isolated from whole blood by density gradient centrifugation on Ficoll (Amersham Biosciences). Intravascular lymph node excisional biopsies were performed under local anesthesia and processed within 1 h. Excess fat was removed, and the lymph node tissue was minced in PBS (Life Technologies), cryopreserved immediately, and stored in liquid nitrogen.

T cell stimulations for Ag-specific cytokine production

PBMCs (2.5–5 × 10^6), isolated as described above, were resuspended in RPMI plus 10% human Ab serum, placed in 12- × 75-mm culture tubes, and incubated overnight at 37°C in a humidified 5% CO2 atmosphere. After the overnight incubation, 3 μg/ml anti-CD28 and -CD49d mAbs (BD Biosciences) were added, and the cells were stimulated under the following conditions: pooled HIV-1 Gag 15mers (2.5 μg/ml final concentration of each peptide; clade B HXB2 strain HIV-1 (National Institutes of Health AIDS Research and Reference Reagent Program), CMV lyse (1/10 dilution, derived from a G-lung cell line infected with CMV strain AD169, virus titer 2 × 10^7 PFU/ml, provided by A. Weinberg, University of Colorado at Denver Health Sciences Center), CMV-EBV-influenza (CEF) peptides used at 1 μg/ml final concentration (National Institutes of Health AIDS Research and Reference Reagent Program), staphylococcal enterotoxin B (SEB, 1 μg/ml; Toxin Technologies), or medium alone. The cultures were incubated at a 5-degree slant at 37°C in a humidified 5% CO2 atmosphere for 6 h with brefeldin A (BD Biosciences) added after the initial 2 h.

Immunofluorescence staining of stimulated T cells

Stimulated PBMCs were washed and surfaced stained with anti-CD4 (APC-Cy7; BD Biosciences), anti-CD3 (PE Texas Red; Beckman Coulter), anti-CD8 (Alexa Fluor 405; Caltag), anti-CD69 (Tricolor; Caltag) and anti-CD279 (FITC; BD Biosciences) for 30 min at 4°C. Cells were washed with PBS containing 1% BSA, fixed, permeabilized (Caltag), and stained with anti-IFN-γ (PECy7; BD Biosciences) and anti-IL-2 (APC; Caltag) mAbs for 30 min at 4°C, washed, and fixed with 1% formaldehyde. Fluorescein isothiocyanate (FITC) (FMO) or isotype controls were used in all staining.

Immunofluorescence staining of monocytes and dendritic cells (DC)

Eight-parameter flow cytometry was performed on PMBC or lymph node cells. DCs were identified by using the following mAb panel: FITC-labeled anti-lineage mixture (CD3-CD14-CD16-CD19-CD20-CD56; BD Biosciences), FITC-labeled anti-CD34 (BD Biosciences),
PD-1 expression (MFI) on CMV-specific and SEB-reactive CD4\(^+\) T cells in HIV\(^+\) (n = 28) and seronegative (n = 15) subjects. Statistical comparisons were made using the Mann-Whitney \(U\) test.
Despite this, a strong positive correlation existed between PD-1 expression on CD4$^+$ and CD8$^+$ T cells in HIV-infected patients ($r = 0.58, p = 0.0009$; Fig. 1C). The absolute number of PD-1-expressing CD4$^+$ T cells was lower in the viremic (84; range, 41–338 cells/μl) than in the suppressed (147; range, 48–337 cells/μl) subjects, although the difference was not statistically significant ($p = 0.1$).

**PD-1 expression is up-regulated on HIV-specific CD4$^+$ T cells**

To determine whether prolonged HIV replication induced changes in PD-1 expression exclusively on HIV-specific T cells, we measured the levels of PD-1 on Gag-specific, CMV-specific, and SEB-reactive IFN-γ-producing T cells in both viremic and ART-suppressed patients. IFN-γ-producing T cells were initially analyzed because HIV replication skews HIV-specific CD4$^+$ T cells toward poorly proliferating IFN-γ-producing T Effector cells. We used multiparametric flow cytometry to measure PD-1 expression on both Ag-specific CD4$^+$ (CD3$^+$CD4$^+$CD8$^-$) and CD8$^+$ (CD3$^+$CD8$^+$CD4$^-$) T cell populations. Representative dot plots clearly show that IFN-γ-producing Gag-specific CD4$^+$ T cells from viremic subjects expressed significantly higher levels of PD-1 than did suppressed subjects or IFN-γ-producing CMV-specific and SEB-reactive CD4$^+$ T cells (Fig. 2A). As shown in Fig. 2B, median PD-1 expression (MFI) on Gag-specific IFN-γ$^+$CD4$^+$ T cells (2844; range, 1236–6179) was significantly higher than on CMV-specific (823; range, 630–1981) and SEB-reactive (626; range, 0–1153) CD4$^+$ T cells ($p < 0.0001$ and 0.0001, respectively) in viremic subjects as well as Gag-specific IFN-γ$^+$CD4$^+$ T cells in suppressed subjects (1230; range, 871–2221; $p = 0.007$). There was no significant difference between median PD-1 expression (MFI) on Gag-specific IFN-γ$^+$CD4$^+$ T cells, CMV-specific (937; range, 630–1981) and SEB-reactive (706; range, 0–1153) CD4$^+$ T cells in suppressed subjects or in the expression level of PD-1 on CMV-specific or SEB-responsive CD4$^+$ T cells between viremic and suppressed subjects. The median PD-1 (MFI) on the Gag-specific IFN-γ$^+$CD8$^+$ T cells in viremic subjects was significantly higher than on CEF-specific and SEB-reactive CD8$^+$ T cells as well as on Gag-specific IFN-γ$^+$CD8$^+$ T cells in suppressed subjects. Although PD-1 expression (MFI) on HIV-specific CD8$^+$ T cells is decreased by suppression of HIV replication, it was not restored to the level of CEF-specific ($p = 0.006$) or SEB-reactive ($p = 0.0007$) CD8$^+$ T cells in suppressed subjects as seen in the HIV-specific CD4$^+$ T cell population (data not shown). Interestingly, in

---

**FIGURE 3.** Correlation between PD-1 expression on HIV-specific CD4$^+$ T cells and plasma HIV RNA concentration and PD-1 expression on HIV-specific CD8$^+$ T cells. A, There is a positive correlation between PD-1 expression (MFI) on HIV-specific CD4$^+$ T cells and plasma viral load (upper left). However, no correlation exists among PD-1 expression on total (upper right), CMV-specific (lower left), and SEB-reactive (lower right) CD4$^+$ T cells and plasma viral load in the same cohort of subjects. B, A positive correlation exists between PD-1 expressions (MFI) on Ag-specific CD4$^+$ and CD8$^+$ T cells. These relationships were evaluated using the Spearman correlation test.
viremic subjects the median PD-1 expression (MFI) on Gag-specific IFN-γ, IL-2, and dual cytokine-producing CD4+ T cells was 2.8 times greater than the median MFI observed on the Gag-specific IFN-γ, CD8+ T cells. These data were analyzed as percent positive rather than by MFI and all the same trends and statistical significance were still seen (data not shown).

PD-1 expression (MFI) on CMV-specific CD4+ T cells was significantly lower in the HIV-seronegative subjects (median, 577; range, 246–1089) than in HIV-infected subjects (median, 910; range, 111–1981; p < 0.04; Fig. 2C). In contrast, there was little difference in median PD-1 expression (MFI) on SEB-reactive CD4+ T cells in HIV-infected (684; range, 0–1238) and HIV-seronegative subjects (615; range, 237–779; p = 0.17).

A positive correlation exists between PD-1 expression on HIV-specific CD4+ T cells and viral load as well as PD-1 expression on HIV-specific CD8+ T cells

To determine whether an association exists between PD-1 expression on CD4+ T cells and markers of HIV disease progression, we evaluated the relationship between PD-1 expression on total and Ag-specific CD4+ T cells and plasma viral load (Fig. 3A). We found a strong positive correlation between HIV plasma viral load and PD-1 expression on Gag-specific CD4+ T cells (r = 0.71, p = 0.005) and a weaker but not statistically significant correlation on total CD4+ T cells (r = 0.34, p = 0.07) in HIV-infected subjects. There was no significant correlation between HIV viral load and PD-1 expression on CMV-specific (r = −0.14, p = 0.56) or SEB-reactive (r = 0.08, p = 0.67) CD4+ T cells in the same subject group. Similar correlations were also observed between PD-1 expression on CD8+ T cells in the same cohort of subjects tested and markers of disease progression (data not shown). A negative correlation between PD-1 expression on HIV-specific CD4+ T cells and CD4 count was seen, but it was not statistically significant (r = −0.43, p = 0.09; data not shown). We also found a strong positive correlation between PD-1 expression on Gag-specific CD4+ and CD8+ T cells in HIV-infected subjects (r = 0.73, p = 0.0065; Fig. 3B). These results suggest that viral load drives increased expression of PD-1 specifically on HIV-specific CD4+ T cells.

PD-1 is predominantly expressed on IFN-γ-producing CD4+ T cells

We have previously shown that IFN-γ-producing HIV-specific CD4+ T cells are more differentiated than IL-2-producing cells and that they express higher levels of the senescence marker, CD57. To determine whether PD-1 expression differs during the various stages of CD4+ T cell differentiation, we measured PD-1 expression on Gag, CMV, and SEB stimulated CD4+ T cells that produced IFN-γ alone, IL-2 alone, or both IFN-γ and IL-2 in HIV-infected subjects. Fig. 4A shows representative histograms. The median PD-1 expression (MFI) on Gag-specific CD4+ T cells was significantly higher on IFN-γ-producing CD4+ T cells (3164;
PD-1 expression on lymph node vs peripheral blood T cell populations

Lymphoid tissues are the primary site of HIV replication, so we hypothesized that PD-1 expression might be up-regulated on T cells in these tissues to an even greater extent than on T cells circulating in the blood. To examine this, we measured PD-1 expression on total and HIV-specific T cells on matched peripheral blood and lymph nodes cells from seven untreated subjects with chronic HIV infection. Fig. 5A shows representative plots of PD-1 staining of total (Fig. 5A) as well as on Gag-specific IFN-γ-producing CD4+ and CD8+ T cells (Fig. 5B) in the peripheral blood and lymph node. There are distinct populations of CD4+ and CD8+ T cells in the lymph node that express PD-1 at a much higher level than PBMC (Fig. 5A). Although this population is bimodal, we still express the data as MFI for consistency. PD-1 expression on total CD4+ cells was 4-fold higher in the lymph node (median, 855; range, 139–4671) than in the peripheral blood (median, 218; range, 139–4671) but was higher than IFN-γ-producing cells (911; range, 586–1544) or IL-2-only-producing cells (883; range, 449–1610; \( p < 0.0007 \)). Surprisingly, median PD-1 expression (MFI) on IFN-γ-producing CMV-specific CD4+ T cells in the lymph node and peripheral blood \( (n = 7) \). D, PD-1 expression (MFI) on Gag-specific IFN-γ-producing CD4+ \( (\square), n = 2 \) and CD8+ \( (\blacksquare), n = 6 \) T cells in lymph node and PBMC. E, PD-L1 and PD-L2 expression on CD4+ T cells in the lymph node and PBMCs \( (n = 5) \). Statistical significance was determined by using the Wilcoxon test.

**FIGURE 5.** PD-1 expression is elevated on T cells in the lymph node. Representative plots showing the expression of PD-1 on total T cells (A) and HIV-specific T cells (B) in peripheral blood and the lymph node (LN). C, PD-1 expression (MFI) on total CD4+ and CD8+ T cells in the lymph node and peripheral blood \( (n = 7) \). D, PD-1 expression (MFI) on Gag-specific IFN-γ-producing CD4+ \( (\square), n = 2 \) and CD8+ \( (\blacksquare), n = 6 \) T cells in lymph node and PBMC. E, PD-L1 and PD-L2 expression on CD4+ T cells in the lymph node and PBMCs \( (n = 5) \). Statistical significance was determined by using the Wilcoxon test.

range, 1411–4560) than on IFN-γ- and IL-2-producing cells (1713; range, 425–3073) or IL-2-only-producing cells (883; range, 449–1610; \( p = 0.0007 \); Fig. 4B). Surprisingly, median PD-1 expression (MFI) on IFN-γ-producing CMV-specific CD4+ T cells (911; range, 586–1544) was not higher than the double-positive IFN-γ- and IL-2-producing cells (942; range, 495–1537) but was higher than IL-2-only-producing cells (565; range, 434–708; \( p = 0.0004 \)). Like the Gag-specific CD4+ T cells, the median PD-1 expression (MFI) on SEB-reactive IFN-γ-producing cells was the highest (887; range, 397–1506), IFN-γ plus IL-2 was intermediate (683; range, 300–990), and IL-2-only-producing cells were the lowest (430; range, 0–11; \( p < 0.0001 \)).
between peripheral blood CD4 count and PD-1 expression on total viral load, and CD4 count. We did find a strong inverse correlation of PD-1 expression on CD4

levels of HIV disease progression, we evaluated the relationship between PD-1 expression on HIV-specific CD4+ T cell proliferation from the lymphoid tissues and plasma viral load as observed in the blood. These data suggest that PD-1 expression on CD4

expression of the PD-1 ligands was significantly up-regulated relative to peripheral blood. These data suggest that PD-1 expression on CD4

Expression of the PD-1 ligands was significantly up-regulated relative to peripheral blood. These data suggest that PD-1 expression on CD4

Discussion

Loss of CD4+ T cell function is central to the immunodeficiency of HIV infection. Recent studies have demonstrated the importance of the PD-1 pathway in HIV-induced T cell-proliferative dysfunction. These studies have mainly focused on virus-specific CD8+ T cells in the peripheral blood (15, 16); however, little is known about PD-1 expression on HIV-specific CD4+ T cells, particularly those in lymphoid tissues of HIV-infected individuals, where viral replication is primarily concentrated (20, 22). Our study corroborated previous studies of PD-1 expression in CD8+ T cell populations in HIV-infected individuals. Interestingly, however, the level of PD-1 expression seen in CD4+ T cells was not the same as that in the CD8+ T cell population, suggesting differential regulation of PD-1 within these two lymphocyte subsets. PD-1 expression was 2-fold higher on HIV-specific CD4+ T cells than on CD8+ T cells. PD-1 was also selectively up-regulated on HIV-specific CD4+ T cells, but not on total CD4+ T cells in the peripheral blood of HIV-infected individuals, unlike the global up-regulation of PD-1 seen in CD8+ T cells in HIV-1-infected individuals. However, this could be due to significantly greater frequencies of HIV-specific CD8+ than CD4+ T cells in the blood (23). PD-1 levels on HIV-specific CD4+ T cells exhibited a greater fold reduction than on HIV-specific CD8+ T cells in the setting of ART. Plasma HIV RNA concentrations correlated directly with PD-1 expression on HIV-specific CD4+ T cells. Furthermore, within lymphoid tissues where HIV-1 replication is known to be concentrated, PD-1 expression on virus-specific T cells as well as expression of the PD-1 ligands was significantly up-regulated relative to peripheral blood. These data suggest that PD-1 expression on CD4+ T cells is driven directly by HIV replication and that the greatest dysfunction related to PD-1 is within the HIV-1-specific population and within lymphoid tissues.

We examined PD-1 expression on HIV-specific IFN-γ-producing CD4+ T cells because we and others have previously shown these cells to be proliferation incompetent in untreated individuals (18, 19). IFN-γ-producing HIV-specific CD4+ T cells from subjects with untreated HIV-1 infection expressed significantly more PD-1 than HIV-1-infected subjects receiving ART with suppressed
viral loads. PD-1 expression on Gag-specific CD4+ T cells in untreated subjects with chronic infection was also 9-fold higher than on total CD4+ T cells and almost 3-fold higher than CMV-specific CD4+ T cells from the same subjects. We believe that the increase in PD-1 was not an artifact of general CD4+ T cell activation for several reasons. We measured the levels 6 h after induction, and it has been shown previously that although CD4+ T cell induction with anti-CD3/CD28 leads to maximal up-regulation of PD-1 at 48 h, it results in very little up-regulation at 24 h (24). Also, although, SEB-reactive IFN-γ-producing CD4+ T cells did have slightly higher median PD-1 expression (626) than unstimulated CD4+ T cells (308), the amount of PD-1 up-regulation was small in comparison with the median PD-1 MFI on HIV-specific CD4+ T cells (2844). These data demonstrate that PD-1 is exclusively up-regulated on HIV-specific CD4+ T cells as a result of persistent HIV replication.

PD-1 expression on HIV-specific CD4+ and CD8+ T cells was directly correlated, suggesting that viral replication is a common factor that regulates expression in these T cell compartments. PD-1 expression in viremic subjects was >2-fold higher on the HIV-specific CD4+ than CD8+ T cells (25). In subjects whose viral loads were suppressed using ART, PD-1 expression on HIV-specific CD4+ T cells decreased by 50% to similar levels as CMV-specific or SEB-reactive CD4+ T cells while it dropped by only 20% on Gag-specific CD8+ T cells. However, despite the greater drop in PD-1 expression, the absolute MFI was still higher on HIV-specific CD4+ T cells than on CD8+ T cells when viral load is suppressed. There was a strong correlation between PD-1 expression on HIV-specific CD4+ T cells and viral load but not between PD-1 expression on CMV-specific CD4+ T cells and viral load. This demonstrates that the association between PD-1 up-regulation and viral load is specific for HIV-specific CD4+ T cells, and PD-1 is therefore a good target for immunotherapy.

Because lymphoid tissues are the main site of HIV replication (20, 21), assessing T cell proliferative impairment in these important immune organs may better reflect the functional state of the T cells that are actively controlling HIV replication rather than those that are trafficking to or from the site of infection in the blood. Although previous studies have found little difference in the frequency, function, and phenotype of HIV-specific T cells in the lymph node in comparison with blood (26–28), we observed significantly greater PD-1 expression on both total and HIV-specific CD4+ and CD8+ T cells in the lymph node. Because we also saw a slight increase in PD-1 expression on non-HIV-specific T cells in lymphoid tissue (data not shown), it is likely that some of this increase is physiological. In addition, we found that the expression of the ligands for PD-1, PD-L1 and PD-L2, were also significantly increased on CD14+ and mDC but not pDC in the lymph node. The increase in PD-L1 and PD-L2 was greatest on CD14+ cells in the lymph node, suggesting that they may play an important role in the down-regulation of memory T cell function in lymphoid tissues. Furthermore, these results suggest that T cells in the lymph node may be even more functionally impaired than those in the blood, raising the concern that examining T cells in the blood alone may not adequately model their activity in the lymph node.

Blockade of PD-1 ligation has been previously shown to restore virus-specific CD8+ T cell proliferation. We investigated whether the same is true for CD4+ T cells by blocking the PD-1 pathway with anti-PD-L1 Abs. We observed increased HIV-specific proliferation in 8 of 10 subjects, although the number of subjects studied was small and these results did not achieve statistical significance. Using a larger cohort of subjects, Day et al. (16) were able to demonstrate that blocking the PD-1 pathway with anti-PD-L1 Abs did enhance Gag-specific proliferation in a statistically significant manner. It is likely that blocking of both PD-1 ligands would result in an even greater enhancement of HIV-specific CD4+ T cell proliferation than we or Day et al. documented. The increase in proliferation was most likely due to functional restoration of HIV-specific CD4+ T cells that produced IFN-γ. An increase in the frequency of cytokine-producing Gag-specific CD4+ T cells was not observed upon the addition of PD-L1 to the intracellular cytokine assays, suggesting that cytokine production during the first 6 h after stimulation is not enhanced by blockade of the PD-1 pathway. Petrovas et al. (29) were also unable to demonstrate increased cytokine production by HIV-specific CD8+ T cells using intracellular cytokine-staining assays. Examination of PD-1 expression on cytokine-producing subsets of CD4+ T cells demonstrated that expression levels were highest on IFN-γ-producing cells and that it was significantly reduced when the cells also produced IL-2. This result was not surprising given that HIV-specific CD4+ T cells that produce IL-2 are proliferation competent and of a central memory phenotype that is correlated with increased function and lower viral loads (2, 3). HIV-specific CD4+ T cells that produced IL-2 did have levels of PD-1 similar to those of IFN-γ-producing CMV-specific CD4+ T cells, suggesting that even the less mature IL-2-producing HIV-specific CD4+ T cells are somewhat impaired. In addition, we did see higher expression of PD-1 on CMV-specific CD4+ T cells in HIV-infected subjects than in HIV-seronegative subjects, which may reflect the higher level of CMV reactivation documented in subjects with active HIV disease (30, 31).

These results show for the first time using intracellular cytokine staining to detect Ag-specific CD4+ T cells that PD-1 expression is significantly up-regulated on HIV-specific CD4+ T cells from subjects with untreated chronic HIV infection and that this up-regulation is even more pronounced in both CD4+ and CD8+ T cells in the lymph node than in the blood. The substantial reduction of PD-1 expression on HIV-specific CD4+ T cells when viral replication is suppressed by ART suggests that it is chronic viral replication and repeated stimulation of HIV-specific CD4+ T cells induces PD-1 up-regulation. Because PD-1 up-regulation is associated with loss of T cell function, the exclusive increase in expression on HIV-specific T cells suggests that blockade of the PD-1 pathway might be used as a viable immunotherapy to specifically augment CD4+ T cell responses to HIV. A better understanding of the role of PD-1 in HIV-1 infection could provide important insight into the mechanisms of HIV-1-induced immune dysfunction and lead to immunotherapeutic strategies to reverse immune suppression in HIV-1-infected individuals.

Acknowledgments
We thank the participants in this study for their cooperation. We also thank Dr. Martin McCarter for performing the lymph node biopsies and Joy Folkvord for technical assistance.

Disclosures
The authors have no financial conflict of interest.

References
is associated with diminished interleukin-2 (IL-2) production and is recovered by