

# Immune Exhaustion Occurs Concomitantly With Immune Activation and Decrease in Regulatory T Cells in Viremic Chronically HIV-1–Infected Patients

Meenakshi Sachdeva, MS,\* Margaret A. Fischl, MD,† Rajendra Pahwa, MD,‡  
Naresh Sachdeva, PhD,§ and Savita Pahwa, MD\*

## INTRODUCTION

T-cell activation is initiated by a complex receptor–ligand interaction leading to downstream signaling events. Antigen recognition by T-cell receptors is accompanied by antigen-independent positive costimulatory signals resulting in sustained T-cell proliferation and effector/memory cell generation. Additional negative costimulatory signals curtail the T-cell response, and an intricate balance between positive and negative signals is required to maintain a healthy state.<sup>1</sup>

Infection with the HIV results in a state of chronic immune activation which has deleterious consequences on immunologic competence.<sup>2</sup> For example, the aberrant immune activation contributes to the progressive loss of CD4<sup>+</sup> and CD8<sup>+</sup> T cells because of increased sensitivity to apoptosis, and activated CD4<sup>+</sup> T cells are prime targets for HIV-1 infection and replication.<sup>3,4</sup> Another characteristic feature of chronic HIV-1 infection is termed “immune exhaustion” which is implicated in impairment of effector T-cell functions, especially HIV-1–specific effector CD8<sup>+</sup> T cells. Immune exhaustion is characterized by deficiency of positive costimulatory molecules such as CD28 and BB-1<sup>5,6</sup> and over expression of the negative costimulatory molecules such as programmed death-1 (PD-1)<sup>7</sup> and cytotoxic T lymphocyte-associated antigen 4 (CTLA-4).<sup>8</sup> Among these, PD-1 has received considerable attention because of the potential to block the interaction of this molecule with its ligands to rejuvenate exhausted T cells.<sup>9,10</sup> Besides regulation through negative costimulatory molecules on activated T cells, another important mechanism to curtail the immune response is through the activity of regulatory T cells (Tregs) that play an important role in preventing autoimmune diseases by suppressing self-reactive lymphocytes and activation of the effector cells. In HIV-1 infection, Tregs have also been implicated in impairment of antiviral activity of T cells, thereby facilitating viral persistence.<sup>11</sup>

The relationship of Tregs in influencing HIV-1–associated detrimental immune activation or immune exhaustion is not well understood. We hypothesized that PD-1 is upregulated concurrently with HIV-1–mediated chronic immune activation and that Tregs play a role to keep them in check. In this study, we investigated PD-1 expression on T cells in conjunction with its ligand PD-L1, with markers of immune activation and in relation to Tregs in HIV-1–infected patients in comparison with HIV-1–uninfected healthy controls. Further, we investigated changes in the expression of

**Background:** Chronic HIV-1 infection is associated with excessive immune activation and immune exhaustion. We investigated the relationship of these 2 phenotypes and frequency of regulatory T cells (Tregs) in controlled and uncontrolled chronic HIV-1 infection.

**Methods:** Immune exhaustion marker PD-1, its ligand PD-L1, CD4<sup>+</sup>CD25<sup>bright</sup> FoxP3<sup>+</sup> Tregs, HLA-DR, and CD38 coexpression as activation markers were investigated in peripheral blood lymphocytes of 44 HIV-1–infected patients and 11 HIV-1–uninfected controls by multicolor flow cytometry.

**Results:** Activated and PD-1 expressing T cells were increased, and Tregs were decreased in HIV-1–infected patients as compared with controls, and alterations were greatest in viremic patients. The proportion of activated CD8<sup>+</sup> T cells exceeded activated CD4<sup>+</sup> T cells. Tregs had an inverse correlation with activated T cells and PD-1 expressing T cells. PD-L1 was highly expressed on monocytes and to a lesser extent on T lymphocytes of patients. These abnormalities partially reversed with virologic control after potent antiretroviral therapy.

**Conclusions:** Immune exhaustion is a component of aberrant immune activation in chronic HIV-1 infection and is associated with loss of Tregs and ongoing virus replication. These defects are corrected partially with effective virologic control by potent antiretroviral therapy.

**Key Words:** ART, CD8<sup>+</sup>, T cells, HIV-1, immune activation, immune exhaustion

(*J Acquir Immune Defic Syndr* 2010;54:447–454)

Received for publication December 5, 2009; accepted March 30, 2010.

From the \*Department of Microbiology and Immunology, Developmental Center for AIDS Research, University of Miami-Miller School of Medicine, Miami, FL; †Department of Medicine, AIDS Clinical research Unit, University of Miami-Miller School of Medicine, Miami, FL; ‡Diabetes Research Institute, University of Miami Miller School of Medicine, Miami, FL; and §Department of Endocrinology, Post Graduate Institute of Medical Education and Research, Chandigarh, India.

Presented partly at the 15th Conference on Retroviruses and Opportunistic Infections (CROI), February 3–6, 2008, Boston, MA.

Correspondence to: Dr Savita Pahwa, MD, 1580 NW 10th Avenue, BCRI 712, University of Miami-Miller School of Medicine, Miami, FL 33136 (e-mail: spahwa@med.miami.edu).

Copyright © 2010 by Lippincott Williams & Wilkins

these markers in HIV-1-infected individuals upon control of plasma HIV-1 viral load (VL) after initiation of potent antiretroviral therapy (ART).

## METHODS

### Patient Population

The current study was performed in 44 patients (male = 31, female = 13, mean age = 35.8 years), with chronic HIV-1 infection, who were enrolled in a protocol for initiating highly potent ART in treatment-naïve patients with plasma virus loads of >1000 HIV-1 RNA copies per milliliter. Patients received potent combination ART consisting of efavirenz, atazanavir sulfate with ritonavir, or lopinavir/ritonavir in combination with fixed-dose lamivudine/zidovudine, lamivudine/abacavir, or emtricitabine/tenofovir disoproxil fumarate. Patients were evaluated clinically and for CD4 counts and virus load determinations at study entry before initiation of ART (week 0) and at weeks 16, 24, and 48 posttreatment initiation. The immunologic assays reported herein were the results of cross-sectional evaluations performed while patients were enrolled in the study. Data are also presented for 8 patients in this cohort who were studied prospectively from the time of study entry till 48 weeks post-treatment initiation. Based on the plasma VL levels at time of the study, the patients were arbitrarily designated as viremic (VL > 50 copies/mL) or aviremic (VL < 50 copies/mL), although we could not rule out virus replication which could still be ongoing at a plasma VL of <50 copies per milliliter. At the time of immunologic evaluation, 18 patients were viremic and 26 patients were aviremic. All 8 patients followed longitudinally also had plasma VL <50 copies per milliliter at 48 weeks. The viremic patients had mean ( $\pm$ SE) VL of  $26,323 \pm 7442$  copies per milliliter and CD4<sup>+</sup> T-cell counts of  $362 \pm 55$  cells per cubic millimeter. The aviremic patients had mean ( $\pm$ SE) CD4<sup>+</sup> T-cell counts  $613 \pm 314$  cells per cubic millimeter at the time of immunologic evaluations. Eleven healthy HIV-1-negative volunteers were also included in the study as controls. All patients and controls were recruited at the University of Miami Miller School of Medicine, and the study was approved by the institutional review board. After obtaining informed consent from the study participants, peripheral venous blood was collected in heparin-coated vacutainer tubes (BD Diagnostics, Franklin Lakes, NJ) and used within 4 hours for the immunologic studies.

### Monoclonal Antibodies

The following antibodies were purchased from BD Biosciences: Anti-CD3 (fluorescein isothiocyanate), anti-CD4 (Pacific blue), anti-CD8 [allophycocyanin (APC)], anti-CD14 (APC-Cy7), anti-PD-1 [phycoerythrin (PE)], anti-PD-L1 (PE-Cy7), anti-HLA-DR (peridinin chlorophyll protein), anti-CD38 (PE-Cy7), and anti-CD25 (APC-Cy7). Anti-Foxp3 antibody (APC) reagents were purchased from e-Bioscience (San Diego, CA).

### Analysis of Phenotypic Markers

One-hundred microliters of fresh whole blood per tube was incubated for 30 minutes with antibodies to different cell

surface markers in dark at room temperature. After incubation, red blood cells were lysed with FACS lysing solution (BD Biosciences, San Jose, CA) for 10 minutes. Cells were then washed with wash buffer (2% fetal bovine serum and 0.02% sodium azide in phosphate buffer saline).

The stained cells were suspended in equal volumes of wash buffer and 1% paraformaldehyde solution. For intracellular staining, cells were fixed after extracellular staining, permeabilized, and finally stained for intracellular markers for 30 minutes. The first panel included markers of exhaustion (PD-1 and PD-L1 and their isotype controls with CD3, CD4, CD8, and CD14). The second panel included CD3, CD4 with Treg markers (CD25, FoxP3) and activation markers (HLA-DR and CD38), and isotype controls. After staining, the cells were acquired on a BD LSR II Flow Cytometer System (BD Biosciences, San Jose, CA). All data were analyzed using FlowJo software (version 4.6.2, Tree Star Inc, Ashland, OR). Tregs were defined as CD4<sup>+</sup>CD25<sup>bright</sup>FoxP3<sup>+</sup>. The gating for all other markers was based on isotype controls. PD-1 and PD-L1 single- and double-positive T cells were also analyzed.

### Statistical Analyses

The study groups were compared using Kruskal–Wallis test, and pair-wise comparisons were done using Wilcoxon signed rank test. Spearman correlation coefficient was used to determine the correlation between 2 variables. All data were analyzed using SAS software (version 9.1, SAS Institute Inc, Cary, NC). The *P* value less than 0.05 was considered significant. The comparative graphs between different groups were plotted using Graph Pad Prism software (version 4.0, Graph Pad Software Inc, La Jolla, CA).

## RESULTS

### Immune Activation Markers are Increased During Chronic HIV-1 Infection and are Related to Plasma VL

As expected, the peripheral blood CD4<sup>+</sup> T-cell count was inversely correlated with plasma VL ( $r = -0.46$ ,  $P < 0.05$ , data not shown). Our patient cohort exhibited an increase in immune activation markers, HLA-DR and CD38 on CD8<sup>+</sup> T cells, and to a lesser extent on CD4<sup>+</sup> T cells (data not shown). We observed that the aviremic patients had lower percentage of activated CD8<sup>+</sup> T cells indicated by decreased coexpression of HLA-DR and CD38 as compared with viremic patients (Mean  $\pm$  SE,  $10 \pm 3.8\%$  versus  $25.7 \pm 2.2\%$ , respectively). The healthy controls had a lower percentage of activated CD8<sup>+</sup> T cells ( $3.0\% \pm 1.8\%$ ) than either of the patient groups. In contrast to CD8<sup>+</sup> T cells, the percentage of activated CD4<sup>+</sup> T cells were lower and did not differ between viremic and aviremic patients (Mean  $\pm$  SE,  $8.23\% \pm 1.4\%$  versus  $7.2\% \pm 1.0\%$ , respectively). CD8<sup>+</sup>HLA-DR<sup>+</sup>CD38<sup>+</sup> cells showed a direct correlation with plasma VL and an inverse correlation with CD4<sup>+</sup> T-cell counts (Fig. 1A). In the 8 patients analyzed longitudinally, the percentage of CD8<sup>+</sup>HLA-DR<sup>+</sup>CD38<sup>+</sup> T cells decreased through 48 weeks of ART (Fig. 1B).

### PD-1 Expression is Increased on CD8<sup>+</sup> and CD4<sup>+</sup> T Cells Which Correlate With VL and Immune Activation

The expression of PD-1 on CD4<sup>+</sup> and CD8<sup>+</sup> T cells was determined as depicted in representative Figure 2A in a healthy control subject. Because the cells expressing high PD-1 are considered to be exhausted, the expression of PD-1 on CD8<sup>+</sup> and CD4<sup>+</sup> T cells was determined in terms of median fluorescence intensity (MFI) in addition to their percentage. Healthy controls had lower percentage and MFI of CD8<sup>+</sup> T cells expressing PD-1 (7.4% ± 1.9%, MFI = 277 ± 50) than either of the HIV-1-infected patient groups (*P* < 0.05). Viremic patients had significantly higher percentage and higher MFI of CD8<sup>+</sup>PD-1<sup>+</sup> cells (24.27% ± 3.3%, MFI = 643 ± 103) than aviremic patients (16.27% ± 1.5%, MFI = 373 ± 42), suggesting that percentage and intensity of PD-1 expression on CD8<sup>+</sup> T cells was increased with viremia (Fig. 2B). PD-1 expression on CD8<sup>+</sup> T cells had direct correlation with plasma VL (Fig. 2C), and with CD8<sup>+</sup>HLA-DR<sup>+</sup>CD38<sup>+</sup> T cells (Fig. 2D). In the patients followed longitudinally from week 0 through week 48, there was a significant decrease in PD-1 expression on CD8<sup>+</sup> T cells at week 48 as compared with week 0 (Fig. 2E). PD-1 expression on CD4<sup>+</sup> T cells was also higher in HIV-1-infected patients than the healthy controls but did not show any significant difference between the viremic and aviremic patients. However, PD-1 expressing CD4<sup>+</sup> T cells had direct correlation with activated CD4<sup>+</sup> T cells and showed a negative trend with CD4<sup>+</sup> T-cell counts and a positive trend with plasma VL though the relationships were statistically insignificant (data not shown). The expression of PD-1 on CD8<sup>+</sup> T cells (28.3% ± 3.8%, MFI = 642 ± 103) was higher (*P* < 0.05) than that on CD4<sup>+</sup> T cells (15.34% ± 2.6%, MFI = 598 ± 96) amongst the viremic patients suggesting that there is a differential regulation of PD-1 on the 2 T-cell subsets.

### PD-L1 Expression is Increased on Monocytes and T Cells

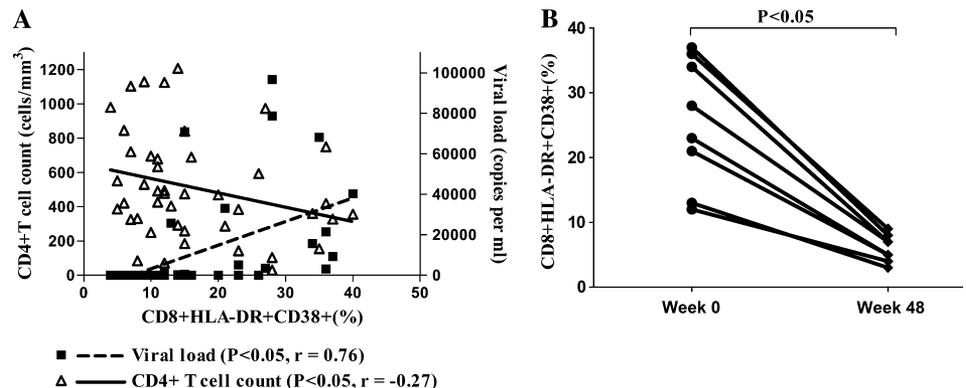
Engagement of PD-1 with its ligands, PD-L1 and PD-L2, is important for conferring a state of immune exhaustion on T cells. As PD-L1 is known to be maximally expressed on antigen presenting cells, we therefore investigated its expression on peripheral blood monocytes (CD14<sup>+</sup> cells). A representative flow cytogram showing PD-L1 expression on monocytes from

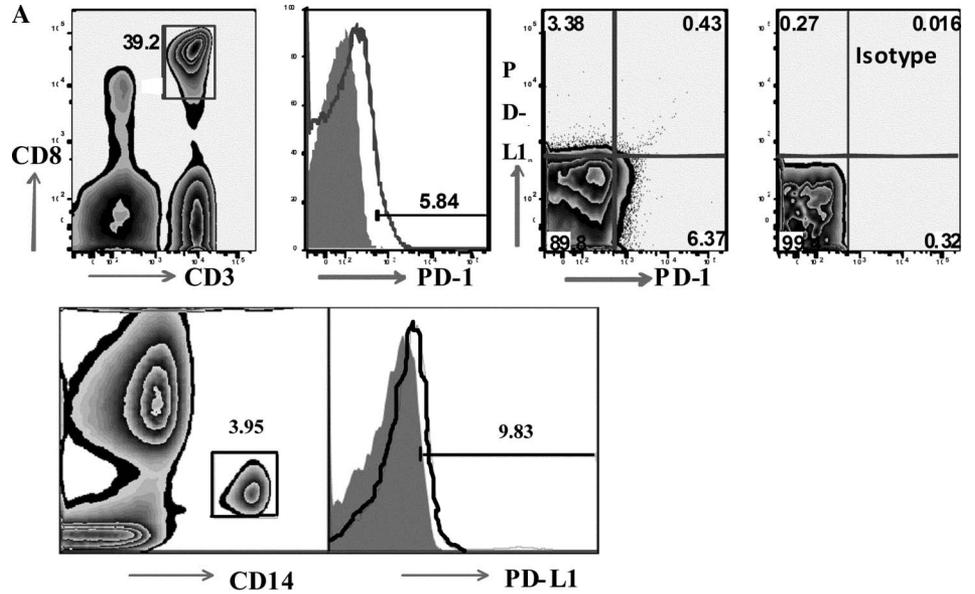
a healthy control is shown in Fig. 2A. PD-L1 expression on monocytes was 35.18% ± 5.86% in viremic patients, 14.87% ± 4.0% in aviremic, and 6.0 ± 2.4 % in healthy controls (Fig. 3A). After ART, there was a significant decline in the expression of PD-L1 on monocytes (*P* < 0.05) to the extent that the difference between aviremic patients and healthy controls became insignificant. In the longitudinal analysis, we found a significant decrease in the expression of PD-L1 on CD14<sup>+</sup> cells after 48 weeks of therapy (Fig. 3B). Because PD-L1 is also known to be expressed by T lymphocytes, we examined the expression of PD-L1 on T cells. The expression of PD-L1 was not different among the viremic and aviremic patient groups either on CD4<sup>+</sup> T cells (Fig. 3C) or on CD8<sup>+</sup> T cells (Fig. 3D). Healthy controls had significantly lower expression of PD-L1 on both CD4<sup>+</sup> and CD8<sup>+</sup> T cells as compared with HIV-1-infected patients. Interestingly, a small percentage of CD4<sup>+</sup> and CD8<sup>+</sup> T cells coexpressed PD-1 and PD-L1 (Figs. 3E, 3F), and CD8<sup>+</sup> T cells coexpressing PD-1 and PD-L1 were significantly higher in viremic patients as compared with healthy controls.

### Regulatory T Cells are Decreased in Patients and Bear a Negative Correlation With Markers of Immune Activation and Exhaustion

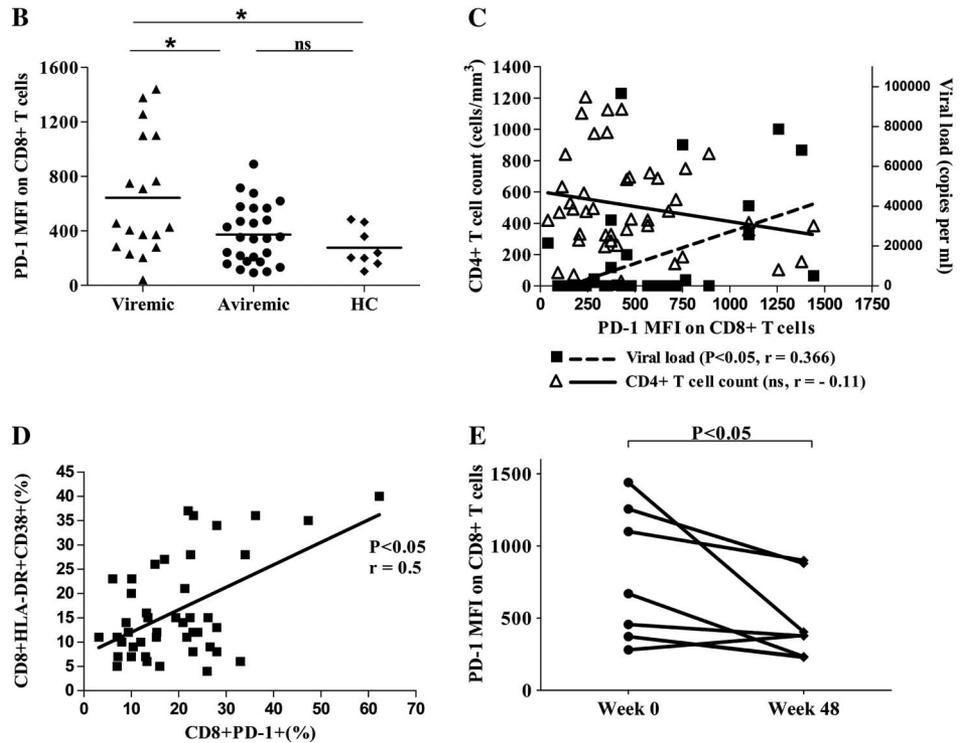
Tregs are defined as CD4<sup>+</sup>, CD25<sup>bright</sup>, and FoxP3<sup>+</sup>, with CD25<sup>bright</sup> cells constituting <5% of CD3<sup>+</sup>CD4<sup>+</sup> T cells in healthy subjects. As CD4-negative cells do not express FoxP3, this subset was used as a negative control for gating FoxP3 in CD4<sup>+</sup>CD25<sup>bright</sup> population. An example of a viremic patient's flow histogram and gating strategy is depicted in Figure 4A. To distinguish Tregs from non-Treg-activated CD25<sup>bright</sup> T cells, we calculated the relative proportion of FoxP3<sup>+</sup> and FoxP3-negative cells within the CD25<sup>bright</sup> cell population. In the CD4<sup>+</sup> CD25<sup>bright</sup> T-cell population, the percentage of FoxP3<sup>+</sup> cells in viremic patients was significantly lower (21%) as compared with the aviremic patients (30%), shown in Figure 4B, and both the groups had significantly lower FoxP3<sup>+</sup> cells in the CD25<sup>bright</sup> T-cell population than the healthy controls (57%). The percentage of Tregs in the total CD4<sup>+</sup> T-cell population of the viremic patients (0.16% ± 0.013%) was significantly lower (*P* < 0.05) as compared with aviremic patients (0.3% ± 0.04%) and healthy controls (2.0% ± 0.5%) (data not shown). These

**FIGURE 1.** Expression of activation markers, HLA-DR and CD38 and their relationship with VL and CD4<sup>+</sup> T-cell counts. A, Percentage of CD8<sup>+</sup>HLA-DR<sup>+</sup>CD38<sup>+</sup> cells show a significant positive correlation with VL (*r* = 0.76, *P* < 0.05, dotted line) and a negative correlation with CD4<sup>+</sup> T-cell count (*r* = -0.27, *P* < 0.05, bold line); B, Longitudinal analysis of eight patients show a significant decrease in the expression of activation markers on CD8<sup>+</sup> T cells at week 48 in comparison with week 0.





**FIGURE 2.** Expression of PD-1 on CD8<sup>+</sup> T cells in HIV-1–infected patients and healthy controls in relation to VL, CD4<sup>+</sup> T cells, and markers of immune activation. A, Representative flow cytogram from a healthy control showing the gating scheme for PD-1, coexpression of PD-1 and PD-L1 on CD4<sup>+</sup> T cells and PD-L1 expression on monocytes; B, Expression of PD-1 (MFI) on CD8<sup>+</sup> T cells in viremic and aviremic patients and healthy controls; C, PD-1 expressing CD8<sup>+</sup> T cells had a positive correlation with VL ( $r = 0.366$ ,  $P < 0.05$ , dotted line) and negative correlation with CD4<sup>+</sup> T-cell counts ( $r = -0.11$ , ns, bold line); D, PD-1 expressing CD8<sup>+</sup> T cells had a positive correlation with HLA-DR and CD38 on CD8<sup>+</sup> T cells ( $r = 0.5$ ,  $P < 0.05$ ); E, PD-1 expression on CD8<sup>+</sup> T cells decreased after 48 weeks on ART; ns, not significant; \* $P < 0.05$ .

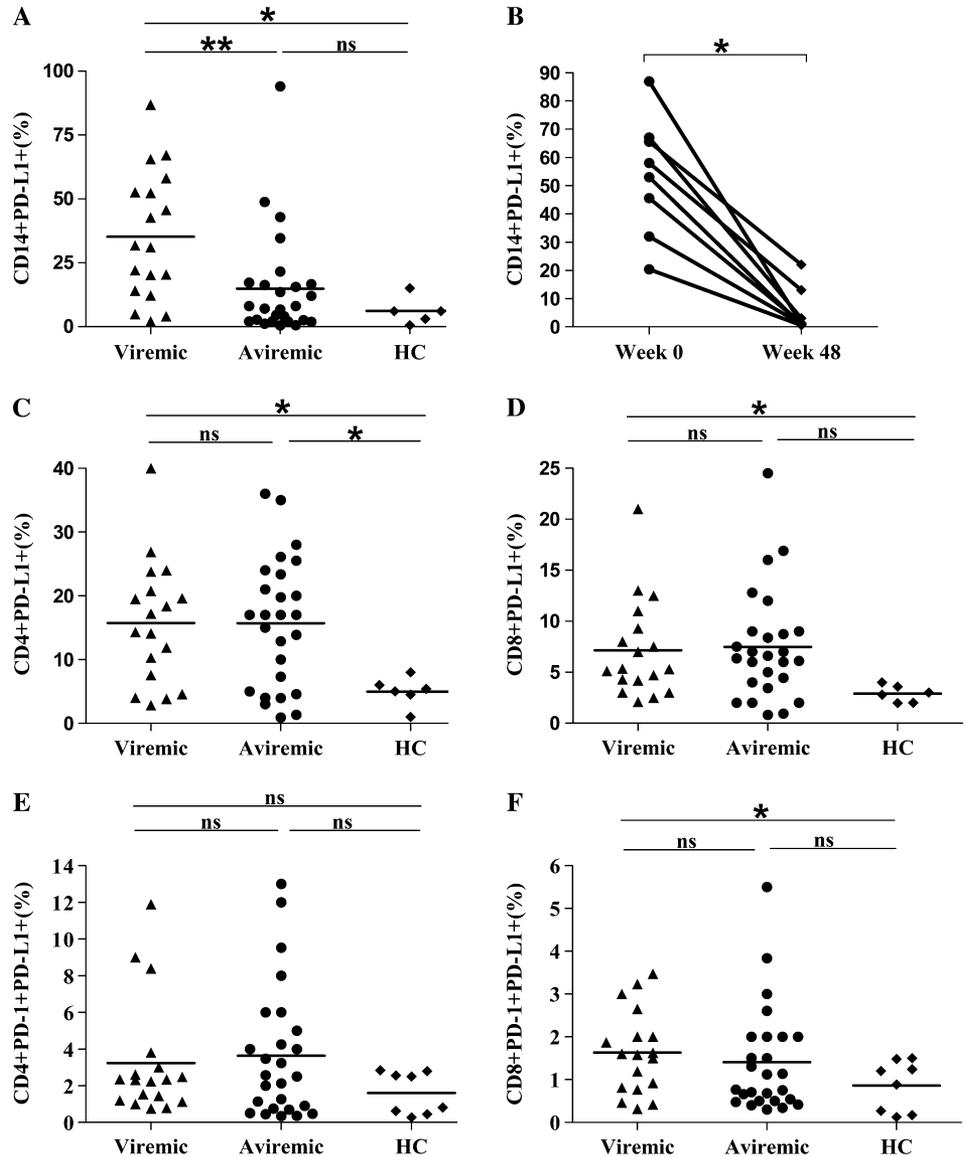


findings are indicative of reduced Tregs and increased activated CD4<sup>+</sup> T cells, both findings being most prominent in the viremic patients. In contrast to correlation of markers of immune activation and exhaustion, the CD25<sup>bright</sup> FoxP3<sup>+</sup> T cells exhibited a negative correlation with the frequency of CD8<sup>+</sup>PD-1<sup>+</sup> and CD8<sup>+</sup>HLA-DR<sup>+</sup>CD38<sup>+</sup> cells in HIV-1–infected patients including those who were viremic and aviremic (Figs. 4C, 4D, respectively). In patients studied longitudinally, ART resulted in an increase in the percentage

of CD4<sup>+</sup>CD25<sup>bright</sup>FoxP3<sup>+</sup> Treg cells relative to the CD4<sup>+</sup>CD25<sup>bright</sup>FoxP3–negative cells (Fig. 4E).

**DISCUSSION**

Aberrant immune activation is a hallmark of chronic HIV-1 infection, as is immune exhaustion characterized by expression of markers such as PD-1, among others, but the underlying mechanisms remain controversial. The major



**FIGURE 3.** Expression of PD-L1 and of both PD-L1 and PD-1 on monocytes, CD8<sup>+</sup> T cells and CD4<sup>+</sup> T cells. A, Expression of PD-L1 on monocytes of viremic and aviremic patients and healthy controls; B, Decrease in PD-L1 expression on monocytes at week 48 compared to week 0 in 8 patients followed longitudinally; Expression of PD-L1, (C), on CD4<sup>+</sup> T cells and (D), on CD8<sup>+</sup> T cells of viremic and aviremic patients and healthy controls; Coexpression of PD-1 and PD-L1, (E), on CD4<sup>+</sup> T cells and (F), on CD8<sup>+</sup> T cells; \**P* < 0.05, \*\**P* < 0.01.

factors implicated in immune activation are microbial translocation in the gut leading to increased lipopolysaccharide (LPS) and HIV-1 viral products driving the immune activation.<sup>12,13</sup> The role of Tregs in chronic HIV-1 infection is controversial,<sup>14–17</sup> and their role in modulating the persistent immune activation that accompanies HIV-1 is not established. This study investigated PD-1 expression in peripheral blood lymphocytes and of its ligand PD-L1 on monocytes and T cells of chronically HIV-1–infected patients in conjunction with markers of immune activation and Tregs. Our findings indicate that PD-1 and immune activation are closely linked, that paucity of Tregs could be a contributor to immune activation, and PD-L1 expression on monocytes is increased in viremic patients. Further, we show that virologic control associated with ART partially reverses the observed abnormalities.

The HIV-1–infected patients in this study manifested increased proportions of CD8<sup>+</sup>HLA-DR<sup>+</sup>CD38<sup>+</sup> cells, indicative of ongoing immune activation. Persistent immune activation is associated with disease progression in HIV-1 infection.<sup>18</sup> Viral suppression by ART has been associated with a reduction in T-cell activation,<sup>19</sup> and in our study, a stronger effect was observed on CD8<sup>+</sup> T cells as compared with CD4<sup>+</sup> T-cell activation. Activated CD8<sup>+</sup> T cells have been shown to express higher levels of PD-1, as compared with resting cells, and a positive correlation between PD-1 and expression of HLA-DR and CD38 has been reported.<sup>20</sup> In the patients studied herein, PD-1 expression was elevated on both CD4<sup>+</sup> and CD8<sup>+</sup> T cells and was more pronounced on CD8<sup>+</sup> T cells. The observation that the expression of PD-1 correlated with markers of immune activation and VL is supported by previous report showing that PD-1 is expressed by activated,

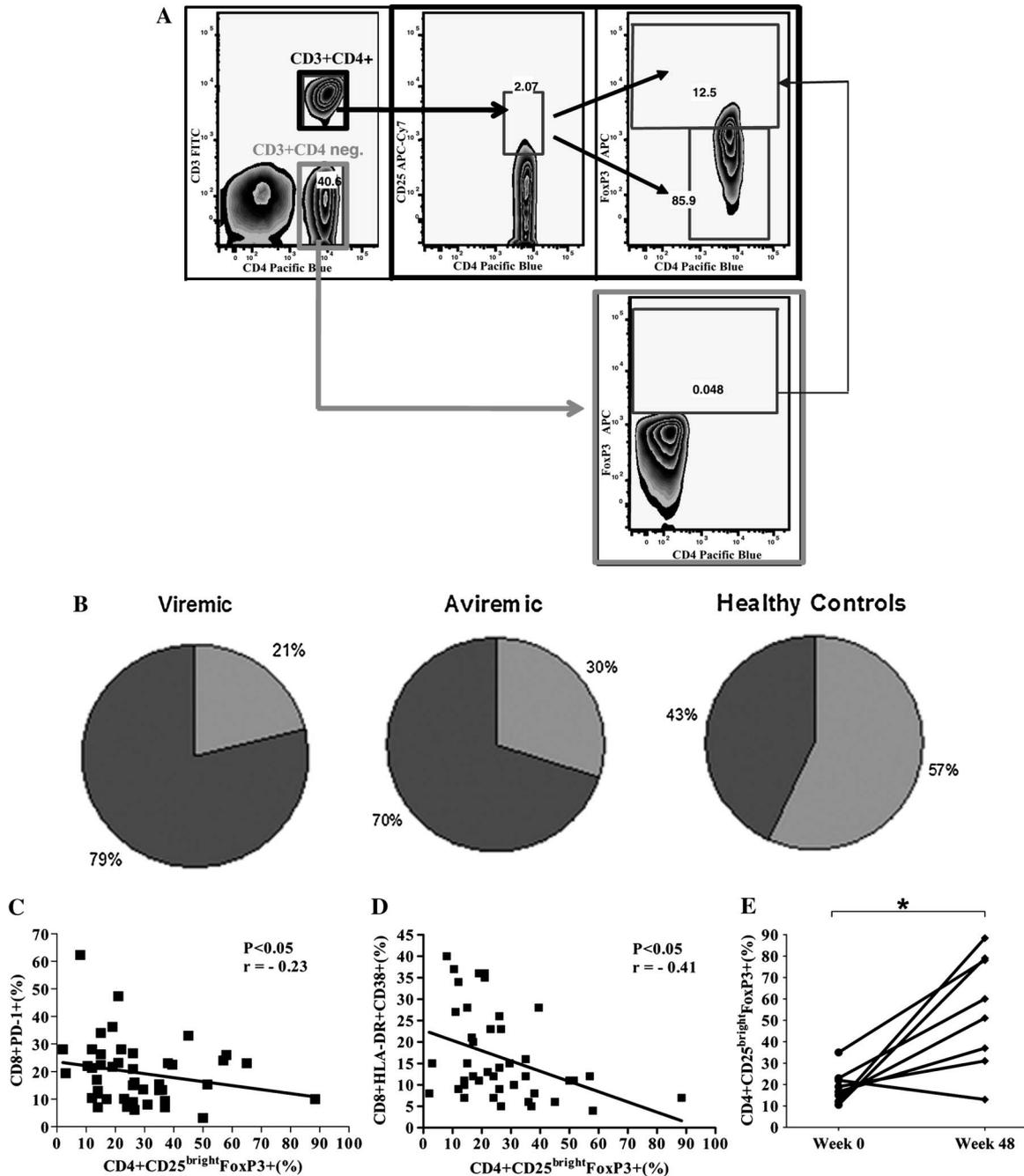
but not naive, CD4<sup>+</sup> and CD8<sup>+</sup> T cells, B cells, and myeloid cells.<sup>21</sup> The expression of PD-1 on T cells is also known to negatively downregulate T-cell function, and thus leads to immune exhaustion of these cells, especially during chronic infections like HIV-1. We observed that PD-1 was highest on CD8<sup>+</sup> T cells in treatment-naive patients at study entry, when they had the highest VL and maximal immune activation. Two previous studies have found a higher PD-1 expression on both tetramer<sup>+</sup> and total CD8<sup>+</sup> T-cell population of viremic HIV-1–infected patients than aviremic individuals and it was correlated with antigenemia.<sup>9,10</sup> In a recent report, PD-1 has been considered as a preapoptotic factor for CD8<sup>+</sup> T cells in HIV-1 infection; PD-1 expression correlated with increased ex vivo spontaneous and CD95/Fas-induced apoptosis.<sup>22</sup> Thus the immune activation-associated and PD-1 expression–associated increased lymphocyte apoptosis may be causally linked, with the highest PD-1 expression marking exhausted cells.

The ligands for PD-1 are PD-L1 and PD-L2. PD-L1 is constitutively expressed on freshly isolated splenic T cells, B cells, macrophages, and pancreas, and its expression is upregulated after activation.<sup>23</sup> In contrast, PD-L2 is inducible only on macrophages and dendritic cells after cytokine stimulation.<sup>24</sup> PD-L1 expressing unstimulated and mitogen-stimulated CD14<sup>+</sup> cells have been shown to be significantly increased in HIV-1 patients as compared with control subjects<sup>25</sup> and also to be correlated directly with VL. The mechanism of this upregulation of PD-L1 on APCs has been attributed to signaling by HIV-1–derived Toll-like receptor 7/8 ligands which can induce MyD88-dependent upregulation of PD-L1 on plasmacytoid, myeloid dendritic cells, and monocytes.<sup>26</sup> In our study, we have found that the PD-L1 expression was increased not only on monocytes but also on subpopulations of CD4<sup>+</sup> and CD8<sup>+</sup> T cells of patients and that a small subset of T cells expressed both PD-1 and PD-L1. Coexpression of PD-1 and PD-L1 on a small subset of CD4<sup>+</sup> and CD8<sup>+</sup> T cells has previously been reported in chronic lymphocytic choriomeningitis virus (LCMV) infection, a classical model of viral persistence in its natural host just like HIV-1.<sup>27</sup> The authors confirmed these findings both at protein and RNA levels, and the coexpression has also been demonstrated by Laser Confocal Microscopy.<sup>28</sup> These results suggest that engagement of PD-1 on activated T lymphocytes by PD-L1 expressing monocytes and T cells contributes to the immune dysfunction in HIV-1 infection.

Advances in ART have improved the clinical outcome in many HIV-1–infected patients. With potent ART, suppression of HIV-1 RNA to undetectable levels is achievable in the vast majority of patients, especially in those who are initiating ART for the first time. In the patient cohort under investigation in this study, viral control was associated with decrease in immune activation and decrease in PD-1 on CD8<sup>+</sup> T cells. Although a positive correlation between PD-1 expression on CD4<sup>+</sup> T cells and VL was observed, the effect of ART on downregulation of PD-1 on these cells was not so striking. These findings are in agreement with a prior study showing that although PD-1 expression on HIV-1–specific CD4<sup>+</sup> T cells correlates with VL, ART fails to decrease PD-1 on CD4<sup>+</sup> T cells in HIV-1–infected children.<sup>29</sup> However, despite this finding, a decrease in engagement of PD-1 is expected as

a consequence of reduced expression of its ligand PD-L1 on monocytes in association with viral control after ART, even though the expression of PD-L1 on T cells does not decrease with therapy.<sup>25</sup> This is the first report to show that the expression of PD-L1 on monocytes decreases with ART, coupled with reduction of PD-1 on CD8<sup>+</sup> T cells. The decrease in PD-L1 expression on monocytes after ART is important because the interaction of PD-1 with PD-L1 on APCs can be deleterious for T cells. Moreover, PD-L1 can also interact with B7-1 (CD80), although with a lesser affinity than with PD-1, and signals through this interaction result in reduced T-cell activation, decreased T-cell proliferation, and reduced cytokine production.<sup>30,31</sup>

The mechanisms underlying the regulation of immune activation and immune exhaustion of T cells are unclear. Tregs have been investigated in HIV-1–infected subjects with conflicting results. Our data suggest that exhausted T cells are not only associated with hyperactivated T cells but also with reduced numbers of Tregs. When we determined the CD4<sup>+</sup>CD25<sup>bright</sup> FoxP3<sup>+</sup> Treg population in proportion to CD4<sup>+</sup>CD25<sup>bright</sup> FoxP3 negative non-Treg activated CD4 T cells, we noted that the proportions were altered in favor of the non-Treg–activated CD4 T cells in HIV-positive subjects. In this analysis, however, the changes observed in Treg frequency could have simply been a consequence of changes in activated CD4<sup>+</sup> T-cell frequency. On the other hand, the percentage of Tregs in the total CD4<sup>+</sup> T-cell population of the viremic patients was also significantly lower as compared with aviremic patients and to healthy controls. As activated CD8 T cells were also clearly higher in viremic patients, this provides support to the contention that lower percentage of CD4<sup>+</sup>CD25<sup>bright</sup> FoxP3<sup>+</sup> T cells in viremic patients are associated with a hyperactivated state of T cells. Several factors contribute to the state of immune activation in chronic HIV-1 infection.<sup>12,13</sup> We contend that a lack of Tregs could also play a role in failure to subdue or prevent hyperactivation in chronic HIV-1 infection. The role of Tregs in HIV-1 infection is controversial, with arguments in favor or against them. In favor of Tregs is a potential protective role in HIV-1 pathogenesis by limiting T-cell dysfunction and depletion.<sup>14,16,18</sup> A beneficial role has been ascribed to Tregs based on findings that the levels of CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> Treg cells are decreased in untreated HIV-1–infected persons as compared with HIV-1–seronegative controls, and lower numbers of Treg cells are associated with higher levels of T-cell activation and lower CD4<sup>+</sup> T-cell counts. In this study, Tregs have been shown to be normal in individuals receiving potent ART with full viral suppression.<sup>17</sup> The contrasting view is that Tregs may contribute to HIV-1 pathogenesis by altering the function of HIV-1–specific effector T-cell responses in HIV-1–infected patients.<sup>15,32,33</sup> We favor the former viewpoint and contend that cellular immune activation in treatment-naive viremic chronically HIV-1–infected subjects are associated with decreased Tregs concomitant with excessive immune exhaustion, which predominantly affects CD8 T cells. Additional support for our viewpoint comes from the observation that in HIV-1–resistant women, chronic activation markers HLA-DR and CD38 were not upregulated, and levels of Tregs relative to HIV-1–negative controls were greater.<sup>34</sup> HIV-1–infected women had depleted frequencies of Treg cells



**FIGURE 4.** Regulatory CD4<sup>+</sup> T cells and their relationship to activated T cells and to CD8<sup>+</sup> PD-1<sup>+</sup> T cells. **A**, Identification of Tregs defined as CD4<sup>+</sup>CD25<sup>bright</sup>FoxP3<sup>+</sup> cells in a viremic patient. First, upper bright portion of CD25 expressing cells in the CD3<sup>+</sup>CD4<sup>+</sup> subset were gated to identify CD4<sup>+</sup>CD25<sup>bright</sup> cells. Next the CD3<sup>+</sup>CD4<sup>+</sup> negative subset (which does not express FoxP3) was used to set gates for identifying FoxP3<sup>+</sup> cells in CD4<sup>+</sup>CD25<sup>bright</sup> population as shown. **B**, Pie charts depicting the proportion of Tregs (CD3<sup>+</sup> CD4<sup>+</sup>CD25<sup>bright</sup> FoxP3<sup>+</sup>, shown in gray) and activated non-Treg cells (CD3<sup>+</sup> CD4<sup>+</sup>CD25<sup>bright</sup> FoxP3 negative, shown in black) in viremic and aviremic patients and healthy controls. Treg cells show a negative correlation (**C**), with CD8<sup>+</sup>PD-1<sup>+</sup> T cells ( $r = -0.23$ ,  $P < 0.05$ ), and (**D**), with CD8<sup>+</sup>HLA-DR<sup>+</sup>CD38<sup>+</sup> cells ( $r = -0.41$ ,  $P < 0.05$ ); **E**, Increase in Tregs at week 48 as compared with week 0 in patients followed longitudinally; \* $P < 0.05$ .

when expressed as a percentage of total T cells. Loss of Treg cells in the periphery may be partially due to compartmentalization in sites of viral replication<sup>27</sup> and to migration of Tregs from blood and their accumulation in lymphoid tissues. Evidence in this regard has been generated by in situ phenotypic

and mRNA studies that Tregs are not necessarily lost over the course of HIV disease.<sup>33,35</sup>

Our findings suggest that the state of immune activation in viremic HIV-1-infected persons leads to upregulation of PD-1, and of its ligand PD-L1 not only on antigen presenting

cells but also on T cells, engagement of which leads to immunologic unresponsiveness, termed as exhaustion. Another important observation of our study is the inverse relationship of activation and exhaustion markers with regulatory CD4<sup>+</sup> T cells. Additionally, virologic control with ART partially reversed the observed abnormalities. A shortcoming of our study is that our sample size is limited, and we performed cross-sectional evaluations of patients who were on ART. The strengths of the study were that all patients were on the same treatment regimen and all started out as treatment naive with detectable viremia. Moreover, we did have the opportunity to validate our observations in a small subset of patients who were evaluated longitudinally over 48 weeks and came to the same conclusions. These preliminary data suggest mechanisms by which potent ART can partially overcome immune dysfunction in patients on their first ART regimen. These observations need to be corroborated in larger cohorts, with supporting functional data.

### ACKNOWLEDGMENTS

We thank Dr. Kristopher L. Arheart, Department of Epidemiology and Public Health, University of Miami Miller School of Medicine, for his help in statistical analysis. Laboratory Support was provided in part by the International Maternal Pediatric Adolescent AIDS Clinical Trials Group (IMPAACT). Overall support for IMPAACT was provided by the National Institute of Allergy and Infectious Diseases (NIAID) [U01 AI068632], the Eunice Kennedy Shriver National Institute of Child Health and Human Development (NICHD), and the National Institute of Mental Health (NIMH) [AI068632]. The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIH. We thank the University of Miami Developmental Center for AIDS Research (DCFAR, P30AI073961) for assistance with this study.

### REFERENCES

1. Rothenstein D, Sayegh MH. T-cell costimulatory pathways in allograft rejection and tolerance. *Immunol Rev*. 2003;196:185–108.
2. Appay V, Sauce D. Immune activation and inflammation in HIV-1 infection: causes and consequences. *J Pathol*. 2008;214:231–241.
3. Levaucher M, Hulstaert F, Tallet S, et al. The significance of activation markers on CD8 lymphocytes in human immunodeficiency syndrome: staging and prognostic value. *Clin Exp Immunol*. 1992;90:376–382.
4. Phillips AN, Sabin CA, Elford J, et al. CD8 lymphocyte counts and serum immunoglobulin A levels early in HIV infection as predictors of CD4 lymphocyte depletion during 8 years of follow-up. *AIDS*. 1993;7:975–980.
5. Wen T, Bukczynski J, Watts TH. 4-1BB ligand-mediated costimulation of human T cells induces CD4 and CD8 T cell expansion, cytokine production, and the development of cytolytic effector function. *J Immunol*. 2002;168:4897–4906.
6. Greenfield EA, Nguyen KA, Kuchroo VK. CD28/B7 costimulation: a review. *Crit Rev Immunol*. 1998;18:389–418.
7. Sharpe AH, Freeman GJ. The B7-CD28 superfamily. *Nat Rev Immunol*. 2002;2:116–126.
8. Chambers CA, Allison JP. Costimulatory regulation of T cell function. *Curr Opin Cell Biol*. 1999;11:203–210.
9. Day CL, Kaufman DE, Kiepiela P, et al. PD-1 expression on HIV-specific T cells is associated with T-cell exhaustion and disease progression. *Nature*. 2006;443:350–354.
10. Trautmann L, Janbazian L, Chomont N, et al. Upregulation of PD-1 expression on HIV-specific CD8<sup>+</sup> T cells leads to reversible immune dysfunction. *Nat Med*. 2006;12:1198–1202.
11. Sempere JM, Soriano V, Benito JM. T regulatory cells and HIV infection. *AIDS Rev*. 2007;9:54–60.
12. Marchetti G, Bellistri GM, Borghi E, et al. Microbial translocation is associated with sustained failure in CD4<sup>+</sup> T-cell reconstitution in HIV-infected patients on long-term highly active antiretroviral therapy. *AIDS*. 2008;22:2035–2038.
13. Brenchley JM, Price DA, Schacker TW, et al. Microbial translocation is a cause of systemic immune activation in chronic HIV infection. *Nat Med*. 2006;12:1365–1371.
14. Eggena MP, Barugahare B, Jones N, et al. Depletion of regulatory T cells in HIV infection is associated with immune activation. *J Immunol*. 2005;174:4407–4414.
15. Lim A, Tan D, Price P, et al. Proportions of circulating T cells with a regulatory cell phenotype increase with HIV-associated immune activation and remain high on antiretroviral therapy. *AIDS*. 2007;21:1525–1534.
16. Oswald-Richter K, Grill SM, Shariat N, et al. HIV infection of naturally occurring and genetically reprogrammed human regulatory T-cells. *PLoS Biol*. 2004;2:E198.
17. Baker CA, Clark R, Ventura F, et al. Peripheral CD4 loss of regulatory T cells is associated with persistent viraemia in chronic HIV infection. *Clin Exp Immunol*. 2007;147:533–539.
18. Eggena MP, Barugahare B, Okello M, et al. T cell activation in HIV-seropositive Ugandans: differential associations with viral load, CD4<sup>+</sup> T cell depletion, and coinfection. *J Infect Dis*. 2005;191:694–701.
19. Zhang ZN, Shang H, Jiang YJ, et al. Activation and coreceptor expression of T lymphocytes induced by highly active antiretroviral therapy in Chinese HIV/AIDS patients. *Chin Med J (Engl)*. 2006;119:1966–1971.
20. Sauce D, Almeida JR, Larsen M, et al. PD-1 expression on human CD8 T cells depends on both state of differentiation and activation status. *AIDS*. 2007;21:2005–2013.
21. Agata Y, Kawasaki A, Nishimura H, et al. Expression of the PD-1 antigen on the surface of stimulated mouse T and B lymphocytes. *Int Immunol*. 1996;8:765–772.
22. Petrovas C, Chaon B, Ambrozak DR, et al. Differential association of programmed death-1 and CD57 with ex vivo survival of CD8<sup>+</sup> T cells in HIV infection. *J Immunol*. 2009;183:1120–1132.
23. Liang SC, Latchman YE, Buhlmann JE, et al. Regulation of PD-1, PD-L1, and PD-L2 expression during normal and autoimmune responses. *Eur J Immunol*. 2003;33:2706–2716.
24. Yamazaki T, Akiba H, Iwai H, et al. Expression of programmed death-1 ligands by murine T cells and APC. *J Immunol*. 2002;169:5538–5545.
25. Trabattini D, Saresella M, Biasin M, et al. B7-H1 is up-regulated in HIV infection and is a novel surrogate marker of disease progression. *Blood*. 2003;101:2514–2520.
26. Meier A, Bagchi A, Sidhu HK, et al. Upregulation of PD-L1 on monocytes and dendritic cells by HIV-1 derived TLR ligands. *AIDS*. 2008;22:655–658.
27. Barber DL, Wherry EJ, Masopust D, et al. Restoring function in exhausted CD8 T cells during chronic viral infection. *Nature*. 2006;439:682–687.
28. Sun ZW, Qiu YH, Shi YJ, et al. Time courses of B7 family molecules expressed on activated T-cells and their biological significance. *Cell Immunol*. 2005;236:146–153.
29. D'Souza M, Fontenot AP, Mack DG, et al. Programmed death-1 expression on HIV-specific CD4<sup>+</sup> T cells is driven by viral replication and associated with T cell dysfunction. *J Immunol*. 2007;179:1979–1987.
30. Butte MJ, Pena-Cruz V, Kim MJ, et al. Interaction of human PD-L1 and B7-1. *Mol Immunol*. 2008;45:3567–3572.
31. Butte MJ, Keir ME, Phamduy TB, et al. Programmed death-1 ligand 1 interacts specifically with the B7-1 costimulatory molecule to inhibit T cell responses. *Immunity*. 2007;27:111–122.
32. Weiss L, Donkova-Petrini V, Caccavelli L, et al. Human immunodeficiency virus-driven expansion of CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells, which suppress HIV-specific CD4 T-cell responses in HIV-infected patients. *Blood*. 2004;104:3249–3256.
33. Nilsson J, Boasso A, Velilla PA, et al. HIV-1-driven regulatory T-cell accumulation in lymphoid tissues is associated with disease progression in HIV/AIDS. *Blood*. 2006;108:3808–3817.
34. Card CM, McLaren PJ, Wachihi C, et al. Decreased immune activation in resistance to HIV-1 infection is associated with an elevated frequency of CD4(+)CD25(+)FOXP3(+) regulatory T cells. *J Infect Dis*. 2009;193:1318–1322.
35. Andersson J, Boasso A, Nilsson J, et al. The prevalence of regulatory T cells in lymphoid tissue is correlated with viral load in HIV-infected patients. *J Immunol*. 2005;174:3143–3147.