Published in final edited form as:

J Acquir Immune Defic Syndr. 2010 August 15; 54(5): 447–454. doi:10.1097/QAI.0b013e3181e0c7d0.

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

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Abstract

Background—Chronic HIV-1 infection is associated with excessive immune activation as well as immune exhaustion. We investigated the relationship of these two 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+CD25^{bright}FoxP3+ 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 multi-color flow cytometry.

Results—Activated and PD-1 expressing T cells were increased and Tregs were decreased in HIV-1 infected patients as compared to controls, and alterations were greatest in viremic patients. The proportion of activated CD8+ T cells exceeded activated CD4+ 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 following potent antiretroviral therapy (ART).

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 ART.

Keywords

HIV-1; ART; immune exhaustion; immune activation; CD8+ T cells

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Meetings at which data or part of data presented: Partly presented at the 15th Conference on Retroviruses and Opportunistic Infections (CROI), Feb 3–6, 2008, Boston, USA.

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.¹

Infection with the human immunodeficiency virus (HIV) results in a state of chronic immune activation which has deleterious consequences on immunologic competence.² For example, the aberrant immune activation contributes to the progressive loss of CD4⁺ and CD8⁺ T cells because of increased sensitivity to apoptosis, and activated CD4⁺ T cells are prime targets for HIV-1 infection and replication.^{3, 4} 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⁺ T cells. Immune exhaustion is characterized by deficiency of positive costimulatory molecules such as CD28 and BB-15^{5, 6}, and over-expression of the negative costimulatory molecules such as PD-17 and CTLA-4.⁸ 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.^{9,10} 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.¹¹

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 to HIV-1 uninfected healthy controls. Further, we investigated changes in the expression of these markers in HIV-1 infected individuals upon control of plasma HIV-1 viral load after initiation of potent antiretroviral therapy.

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 antiretroviral therapy (ART) in treatment naïve patients with plasma virus loads of >1000 HIV-1 RNA copies/mL. Patients received potent combination antiretroviral therapy 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 prior to initiation of ART (week 0) and at weeks 16, 24 and 48 post treatment initiation. The immunologic assays reported herein were the results of cross sectional evaluations performed while patients were enrolled in the study. Data is 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 viral load (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/mL. 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/mL at 48 weeks. The viremic patients had mean ($\pm$ SE) VL of 26,323 $\pm$ 7,442 copies/mL and CD4+ T cell counts of 362 $\pm$ 55/mm³. The aviremic patients had mean ($\pm$ SE) CD4+ T cell counts 613 $\pm$ 314/mm³ 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 Biosciences, San Jose, CA, USA) and used within 4 hours for the immunologic studies.

Monoclonal Antibodies

The following antibodies were purchased from BD Biosciences (San Jose, CA, USA): Anti-CD3 (fluorescein isothiocyanate [FITC]), 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 [PerCP]), anti-CD38 (PE-Cy7), anti-CD25 (APC-Cy7). Anti-Foxp3 antibody (APC) reagents were purchased from e-Bioscience (San Diego, CA, USA).

Analysis of Phenotypic markers

100 μ l of fresh whole blood per tube was incubated for 30 minutes with antibodies to different cell surface markers in dark at room temperature. Following incubation, RBCs were lysed with FACS lysing solution (BD Bioscience, San Jose, CA, USA) 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% para formaldehyde solution. For intracellular staining cells were fixed following 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. Following staining, the cells were acquired on a BD LSRII Flow cytometer (BD Bioscience, San Jose, CA, USA). All data was analyzed using FlowJo software (version 4.6.2, Tree Star, Inc., Ashland, OR, USA). Tregs were defined as: CD4+CD25^{bright}FoxP3⁺. 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's correlation coefficient was used to determine the correlation between two variables. All data were analyzed using SAS software (version 9.1, SAS Institute Inc., Cary, NC, USA). 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, USA).

RESULTS

Immune activation markers are increased during chronic HIV-1 infection and are related to plasma viral load

As expected the peripheral blood CD4+ T cell count was inversely correlated with plasma viral load ($r = -0.46$, $P < 0.05$, data not shown). Our patient cohort exhibited an increase in immune activation markers, HLA-DR and CD38 on CD8+ T cells and to a lesser extent on CD4+ T cells (data not shown). We observed that the aviremic patients had lower percentage of activated CD8+ T cells indicated by decreased coexpression of HLA-DR and CD38 as compared to 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+ T cells ($3.0 \pm 1.8\%$) than either of the patient groups. In contrast to CD8+ T cells, the percentage of activated CD4+ 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+HLA-DR+CD38+ cells showed a direct correlation with plasma viral load and an inverse correlation with CD4+ T cell counts (Fig. 1A). In the 8 patients analyzed longitudinally, the percentage of CD8+HLA-DR+CD38+ T cells decreased through 48 weeks of ART (Fig. 1B).

PD-1 expression is increased on CD8+ and CD4+ T cells which correlate with viral load and immune activation

The expression of PD-1 on CD4+ and CD8+ T cells was determined as depicted in representative Fig. 2A in a healthy control subject. Since the cells expressing high PD-1 are considered to be exhausted, the expression of PD-1 on CD8+ and CD4+ T cells was determined in terms of MFI in addition to their percentage. Healthy controls had lower percentage and MFI of CD8+ T cells expressing PD-1 ($7.4 \pm 1.9\%$, MFI=277 $\pm$ 50) than either of the HIV-1 infected patient groups ($p < 0.05$). Viremic patients had significantly higher percentage as well as higher MFI of CD8+PD-1+ cells ($24.27 \pm 3.3\%$, MFI=643 $\pm$ 103) than aviremic patients ($16.27 \pm 1.5\%$, MFI=373 $\pm$ 42), suggesting that percentage and intensity of PD-1 expression on CD8+ T cells was increased with viremia (Fig. 2B). PD-1 expression on CD8+ T cells had direct correlation with plasma viral load (Fig. 2C), and with HLA-DR+CD38+CD8+ 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+ T cells at week 48 as compared to week 0 (Fig. 2E). PD-1 expression on CD4+ 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+ T cells had direct correlation with activated CD4+ T cells and showed a negative trend with CD4+ T cell counts and a positive trend with plasma viral load though the relationships were statistically insignificant (data not shown). The expression of PD-1 on CD8+ T cells ($28.3 \pm 3.8\%$, MFI=642 $\pm$ 103) was higher ($P < 0.05$) than that on CD4+ T cells ($15.34 \pm 2.6\%$, MFI=598 $\pm$ 96) amongst the viremic patients suggesting that there is a differential regulation of PD-1 on the two 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+ 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 $35.18 \pm 5.86\%$ in viremic patients, $14.87 \pm 4.0\%$ in aviremic and $6.0 \pm 2.4\%$ in healthy controls (Fig. 3A). Following 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⁺ cells after 48 weeks of therapy (Fig. 3B). Since 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⁺ T cells (Fig. 3C) or on CD8⁺ T cells (Fig. 3D). Healthy controls had significantly lower expression of PD-L1 on both CD4⁺ and CD8⁺ T cells as compared to HIV-1 infected patients. Interestingly, a small percentage of CD4⁺ and CD8⁺ T cells coexpressed PD-1 and PD-L1 (Figs. 3E, 3F), and CD8⁺ T cells coexpressing PD-1 and PD-L1 were significantly higher in viremic patients as compared to healthy controls.

Regulatory T cells are decreased in patients and bear a negative correlation with markers of immune activation and exhaustion

Regulatory T cells are defined as CD4⁺, CD25^{bright} and FoxP3⁺, with CD25^{bright} cells constituting < 5% of CD3⁺CD4⁺ 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⁺CD25^{bright} population. An example of a viremic patient's flow histogram and gating strategy is depicted in Fig 4A. In order to distinguish Tregs from non Treg activated CD25^{bright} T cells, we calculated the relative proportion of FoxP3⁺ and FoxP3⁻ negative cells within the CD25^{bright} cell population. In the CD4⁺ CD25^{bright} T cell population, the percentage of FoxP3⁺ cells in viremic patients was significantly lower (21%) as compared to the aviremic patients (30%), shown in Fig. 4B, and both the groups had significantly lower FoxP3⁺ cells in the CD25^{bright} T cell population than the healthy controls (57%). The percentage of Tregs in the total CD4⁺ T cell population of the viremic patients ($0.16 \pm 0.013\%$) was significantly lower ($p < 0.05$) as compared to aviremic patients ($0.3 \pm 0.04\%$) and healthy controls ($2.0 \pm 0.5\%$) (data not shown). These findings are indicative of reduced regulatory T cells and increased activated CD4⁺ T cells, both findings being most prominent in the viremic patients. In contrast to correlation of markers of immune activation and exhaustion, the CD25^{bright}FoxP3⁺ T cells exhibited a negative correlation with the frequency of CD8⁺PD-1⁺ and CD8⁺HLA-DR⁺CD38⁺ 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⁺CD25^{bright}FoxP3⁺ Treg cells relative to the CD4⁺CD25^{bright}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 factors implicated in immune activation are microbial translocation in the gut leading to increased LPS, and HIV-1 viral products driving the immune activation.^{12,13} The role of regulatory T cells in chronic HIV-1 infection is controversial¹⁴⁻¹⁷ 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 regulatory T cells. 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⁺HLA-DR⁺CD38⁺ cells, indicative of ongoing immune activation. Persistent immune activation is associated with disease progression in HIV-1 infection.¹⁸ Viral suppression by ART has been associated with a reduction in T cell activation¹⁹, and in our study a stronger effect

was observed on CD8+ T cells as compared to CD4+ T cell activation. Activated CD8+ T cells have been shown to express higher levels of PD-1, as compared to resting cells, and a positive correlation between PD-1 and expression of HLA-DR and CD38 has been reported.²⁰ In the patients studied herein, PD-1 expression was elevated on both CD4+ and CD8+ T cells and was more pronounced on CD8+ T cells. The observation that the expression of PD-1 correlated with markers of immune activation and viral load is supported by previous report showing that PD-1 is expressed by activated, but not naïve, CD4+ and CD8+ T cells, B cells and myeloid cells.²¹ The expression of PD-1 on T cells is known also 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+ T cells in treatment naïve patients at study entry, when they had the highest viral load and maximal immune activation. Two previous studies have found a higher PD-1 expression on both tetramer+ and total CD8+ T cell population of viremic HIV-1 infected patients than aviremic individuals and it was correlated with antigenemia.^{9, 10} In a recent report, PD-1 has been considered as a preapoptotic factor for CD8+ T cells in HIV-1 infection; PD-1 expression correlated with increased *ex vivo* spontaneous and CD95/Fas induced apoptosis.²² Thus the immune activation- 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.²³ In contrast, PD-L2 is inducible only on macrophages and dendritic cells after cytokine stimulation.²⁴ PD-L1 expressing unstimulated and mitogen stimulated CD14+ cells have been shown to be significantly increased in HIV-1 patients as compared to control subjects²⁵ and also to be correlated directly with viral load. The mechanism of this upregulation of PD-L1 on APCs has been attributed to signaling by HIV-1 derived Toll-like receptor (TLR) 7/8 ligands which can induce MyD88-dependent upregulation of PD-L1 on plasmacytoid, myeloid dendritic cells and monocytes.²⁶ In our study we have found that the PD-L1 expression was increased not only on monocytes but also on subpopulations of CD4+ and CD8+ 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+ and CD8+ T cells has previously been reported in chronic LCMV infection, a classical model of viral persistence in its natural host just like HIV-1.²⁷ The authors confirmed these findings both at protein and RNA levels, and the coexpression has also been demonstrated by Laser Confocal Microscopy. 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+ T cells. Although a positive correlation between PD-1 expression on CD4+ T cells and viral load 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+ T cells correlates with viral load, ART fails to decrease PD-1 on CD4+ T cells in HIV-1-infected children.²⁹ 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 following ART, even though the expression of PD-L1 on T cells does not decrease with therapy.²⁵ 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+ 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.³⁰⁻³¹

The mechanisms underlying the regulation of immune activation and immune exhaustion of T cells are unclear. Regulatory T cells have been investigated in HIV-1 infected subjects with conflicting results. Our data suggests that exhausted T cells are not only associated with hyper-activated T cells but also with reduced numbers of regulatory T cells. When we determined the CD4+CD25^{bright}FoxP3⁺ Treg population in proportion to CD4+CD25^{bright}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+ T cell frequency. On the other hand, the percentage of Tregs in the total CD4+ T cell population of the viremic patients was also significantly lower as compared to 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+CD25^{bright}FoxP3⁺ T cells in viremic patients are associated with a hyper-activated state of T cells. Several factors contribute to the state of immune activation in chronic HIV-1 infection.¹²⁻¹³ 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.¹⁴⁻¹⁶⁻¹⁸ A beneficial role has been ascribed to Tregs based on findings that the levels of CD4+CD25⁺FoxP3⁺ Treg cells are decreased in untreated HIV-1 infected persons as compared to HIV-1-seronegative controls, and lower numbers of Treg cells are associated with higher levels of T cell activation and lower CD4+ T cell counts. In this study, Tregs have been shown to be normal in individuals receiving potent ART with full viral suppression.¹⁷ 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.¹⁵⁻³²⁻³³ We favor the former viewpoint and contend that cellular immune activation in treatment naïve viremic chronically HIV-1 infected subjects are associated with decreased regulatory T cells 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.³⁴ HIV-1 infected women had depleted frequencies of Treg cells 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 replication²⁷ 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.³³⁻³⁵

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+ 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 naïve 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.

Sources of Support: This study was supported in part by the “The International Maternal Pediatric Adolescent AIDS Clinical Trials Network of the National Institute of Allergy and Infectious Diseases” (U01-A141089 and N01-HD-3-3345) and University of Miami Development Center for AIDS Research Grant (D-CFAR: 1P30AI073961-01).

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FIGURE 1. Expression of activation markers, HLA-DR and CD38 and their relationship with viral load and CD4+ T cell counts

Percentage of CD8+HLA-DR+CD38+ cells show a significant positive correlation with viral load ($r = 0.76$, $P < 0.05$, dotted line) and a negative correlation with CD4+ T cell count ($r = -0.27$, $P = \text{ns}$, bold line) (A); Longitudinal analysis of eight patients show a significant decrease in the expression of activation markers on CD8+ T cells at week 48 in comparison to week 0 (B); ns, not significant

FIGURE 2. Expression of PD-1 on CD8+ T cells in HIV-1 infected patients and healthy controls (HC) in relation to viral load, CD4+ 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+ T cells and PD-L1 expression on monocytes; B. Expression of PD-1 (MFI) on CD8+ T cells in viremic and aviremic patients and healthy controls (HC); C. PD-1 expressing CD8+ T cells had a positive correlation with viral load ($r = 0.366$, $P < 0.05$, dotted line) and negative correlation with CD4+ T cell counts ($r = -0.11$, ns, bold line); D. PD-1 expressing CD8+ T cells had a positive correlation with HLA-DR and CD38 on CD8+ T cells ($r = 0.5$, $P < 0.05$); E. PD-1 expression on CD8+ T cells decreased after 48 weeks on ART; ns, not significant; *, $P < 0.05$.

FIGURE 3. Expression of PD-L1 and of both PD-L1 and PD-1 on monocytes, CD8+ T cells and CD4+ 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 on CD4+ T cells (C) and on CD8+ T cells (D) in viremic and aviremic patients and healthy controls. Coexpression of PD-1 and PD-L1 on CD4+ T cells (E) and CD8+ T cells (F); *, $P < 0.05$, **, $P < 0.01$.

FIGURE 4. Regulatory CD4⁺ T cells and their relationship to activated T cells and to CD8⁺ PD-1 T cells

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