MAJOR ARTICLE

Cell-Based Measures of Viral Persistence Are Associated With Immune Activation and Programmed Cell Death Protein 1 (PD-1)–Expressing CD4$^+$ T cells

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Background. Studies aimed at defining the association between host immune responses and human immunodeficiency virus (HIV) persistence during therapy are necessary to develop new strategies for cure.

Methods. We performed a comprehensive assessment of ultrasensitive plasma HIV RNA levels, cell-associated HIV RNA levels, proviral HIV DNA levels, and T cell immunophenotyping in a cohort of 190 subjects in whom HIV levels were suppressed by highly active antiretroviral therapy.

Results. The median CD4$^+$ T cell count was 523 cells/mm^3, and the median duration of viral suppression was 31 months. Cell-associated RNA and proviral DNA levels (but not ultrasensitive plasma HIV RNA levels) were positively correlated with frequencies of CD4$^+$ and CD8$^+$ T cells expressing markers of T-cell activation/dysfunction (CD38, HLA-DR, CCR5, and/or programmed cell death protein 1 [PD-1]) ($P < .05$). Having a low CD4$^+$ T-cell count despite receipt of virologically suppressive therapy was associated with high cell-associated RNA and proviral DNA levels ($P < .01$) and higher frequencies of CD4$^+$ T cells expressing CD38, HLA-DR, CCR5, and/or PD-1 ($P < .0001$).

Conclusions. Cell-based measurements of viral persistence were consistently associated with markers of immune activation and the frequency of PD-1–expressing CD4$^+$ T cells. Treated patients with a low CD4$^+$ T-cell count had higher frequencies of PD-1–expressing CD4$^+$ T cells and cell-based measures of viral persistence, suggesting that HIV infection in these individuals may be more difficult to cure and may require unique interventions.

Keywords. HIV; raltegravir intensification; 2-LTR circles; ongoing viral replication; D-dimer.

Despite the effectiveness of long-term therapy, multiple studies have shown that human immunodeficiency virus (HIV) persists indefinitely in plasma [1, 2], peripheral blood mononuclear cells (PBMCs) [3, 4], and tissues [5–7]. Multiple mechanisms contribute to this phenomenon, including the presence of long-lived latently infected CD4$^+$ T cells [8], ongoing cryptic viral replication [9, 10], ineffective HIV-specific responses [11], and persistent immune activation [12–15]. The host immune environment is likely to be a strong determinant of each of these mechanisms. For example, a high density of activated CD4$^+$ T cells in lymphoid tissues could support isolated rounds of de novo infection [10, 16]. In addition, a chronic inflammatory environment might lead to dysfunctional HIV-specific T cells [13, 14] and, hence, to a relative inability to clear infected cells [15]. Finally, the upregulation of certain "negative regulators" of T-cell activation (eg, programmed cell death protein 1 [PD-1]) has been postulated as a mechanism that enables recently infected CD4$^+$ T cells to shift toward a state of persistence rather than one of activation-induced cell death [15]. Expression of PD-1 causes impaired HIV-specific immunity [14, 17], and PD-1^{high} CD4$^+$ T cells are highly

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enriched in integrated HIV DNA [15]. Given the potential role of the host immune environment in maintaining HIV persistence, we performed a comprehensive assessment of virologic and T-cell immunophenotyping in a large cohort of effectively treated individuals.

METHODS

Study Participants

A total of 190 HIV-infected individuals receiving highly active antiretroviral therapy (HAART) were identified from the University of California San Francisco (UCSF) SCOPE and OPTIONS cohorts. Subjects were required to have had at least 2 consecutive plasma HIV RNA levels below the level of detection by conventional tests (ie, <40–75 copies/mL) while taking antiretroviral therapy. Isolated blips were allowed, as long as they were <1000 copies/mL and preceded and followed by plasma HIV RNA levels <40–75 copies/mL. All subjects provided written informed consent. This study was approved by the UCSF Committee on Human Research.

Virologic Measurements

Ultrasensitive plasma HIV RNA levels were determined from 1 mL of cryopreserved plasma samples, using the COBAS AmpliPrep/COBAS TaqMan HIV-1 test, version 2.0 (Roche Molecular Diagnostics). The US Food and Drug Administration (FDA)–approved lower reporting level for this assay is 20 copies/mL, with a lower limit of detection (95% hit rate) of 17 copies/mL by probit analysis. For this study, plasma HIV RNA values below the lower reporting range of 20 copies/mL were calculated on the basis of an instrument-derived change in cycle threshold values. The lower limit of detection of this method is 9 copies/mL, using an analogous hit rate used to derive the single-copy assay detection limit reported by others [18].

Cell-associated RNA and proviral DNA levels were measured from cryopreserved PBMCs. The transcription-mediated amplification (TMA) assay (Aptima, Gen-Probe) was used to measure cell-associated RNA levels. The TMA assay is a nucleic acid–amplification test that has been FDA-approved for the early detection of HIV infection in plasma for screening blood donors and has also been validated for clinical diagnostic use [19]. A modified approach of previously published methods for PBMC extraction and TMA amplification of cell-associated hepatitis C virus was used [20, 21]. The output is a signal-to-cutoff ratio (S/Co; range, 0–30), with an S/Co <1.0 considered HIV RNA "negative" and an S/Co ≥1.0 considered "positive." All S/Co ratios were normalized to 1 million $CD4^+$ T cells (derived from the quantitation of human genomic DNA from a parallel real-time polymerase chain reaction amplification targeting a highly conserved region of the DQ-alpha locus, multiplied by the proportion of PBMCs that were $CD4^+$ T cells at each time point).

Total proviral DNA was extracted from PBMCs, using modifications of previously described methods [21, 22]. This assay has an overall lower limit of detection of 1 copy per 3 μg of input DNA, which is equivalent to approximately 450 000 PBMCs [23, 24]. All proviral DNA levels were normalized to 1 million $CD4^+$ T cells, as described above.

T-Cell Immunophenotyping

PBMCs were isolated from whole blood, cryopreserved, and stored at the UCSF AIDS Specimen Bank. Markers of T-cell activation were measured using flow cytometry at the UCSF Core Immunology Laboratory, using previously described methods that have been optimized and validated for cryopreserved PBMCs [25]. Briefly, cryopreserved PBMCs were rapidly thawed in warm media, counted on an Accuri C6 (BD Biosciences) with the Viacount assay (Millipore), washed, and stained with Aqua Amine Reactive Dye (AARD, Invitrogen) to discriminate dead cells. The average viability of thawed cells was 93% (range, 61%–98%; 80% of samples had a viability >90%). Cells were then stained with the following fluorescently conjugated monoclonal antibodies: CD3-Pacific Blue, CCR5-PECY5 (BD Pharmingen), CD38-PE, HLA-DR-FITC, PD-1-Alexa647 (BD Biosciences), CD4-PE Texas Red, and CD8-QDot 605 (Invitrogen). In each experiment a fluorescence minus one control was included for CD38, HLA-DR, CCR5, and PD-1 to help determine the cutoff for positive staining. Stained cells were washed, fixed in 0.5% formaldehyde (Polyscience), and held at 4°C until analysis. Stained cells were run on a customized BD LSR II (BD Bioscience). A total of 100 000 lymphocytes were collected for each sample. Data were compensated and analyzed using FlowJo (Tree Star) to determine the proportion of $CD4^+$ and $CD8^+$ T cells expressing each of the T-cell markers (CD38, HLA-DR, CCR5, and/or PD-1). Combinations of markers were calculated in FlowJo, using the Boolean gate function.

Statistical Methods

All statistical analyses were conducted with Stata, version 11.1 (StataCorp). Spearman rank correlation coefficients were calculated between virologic and immunologic measurements. Virologic and immunologic parameters were compared between subjects with a low (<350) and high (≥350) $CD4^+$ T-cell count, using the Wilcoxon rank sum test or Fisher exact test.

RESULTS

The median $CD4^+$ T-cell count was 523 cells/mm^3 (interquartile range [IQR], 249–728 cells/mm^3) (Table 1). The median duration of viral suppression during HAART was 31 months

Table 1. Baseline Characteristics Among 190 Study Subjects

Characteristic	Value
Age, years	51 (44–57)
Male sex, % of patients	92
$CD4^+$ T-cell count, cells/mm^3	523 (249–728)
T cells expressing CD4, %	23 (16–32)
$CD8^+$ T-cell count, cells/mm^3	844 (606–1185)
T cells expressing CD8, %	48 (37–57)
Nadir $CD4^+$ T-cell count, cells/mm^3	113 (29–227)
Duration of HAART suppression, months	31 (14–66)
Agent included in HAART, no. of patients	
Protease inhibitor	124
NNRTI	73
Raltegravir	14
Maraviroc	1
Enfuvirtide	3

Data are median (interquartile range), unless otherwise indicated.

Abbreviations: HAART, highly active antiretroviral therapy; NNRTI, nonnucleoside reverse transcriptase inhibitor.

(IQR, 14–66 months). Approximately half (53%) of the cohort had undetectable plasma HIV RNA levels, using the ultrasensitive Roche assay. The median plasma HIV RNA level for the entire cohort was 0 copies/mL; of those with detectable HIV RNA, the median value was 14 copies/mL.

T-Cell Activation and Viral Persistence

We found no associations between ultrasensitive plasma HIV RNA levels and any of our measures of T-cell activation (defined by coexpression of CD38 and HLA-DR, HLA-DR alone, or CCR5 alone, in either $CD4^+$ or $CD8^+$ T cells) (all $P > .40$) (Figure 1). In addition, when we compared individuals with an undetectable plasma HIV RNA level to those with low but detectable levels, we found no significant differences in any of these immunologic measurements between the 2 groups (data not shown).

In contrast, cell-associated RNA and proviral DNA levels were positively correlated with frequencies of T cells expressing these activation markers (Figure 2). However, these relationships were modest, suggesting that there are other virologic and immunologic factors contributing to this relationship.

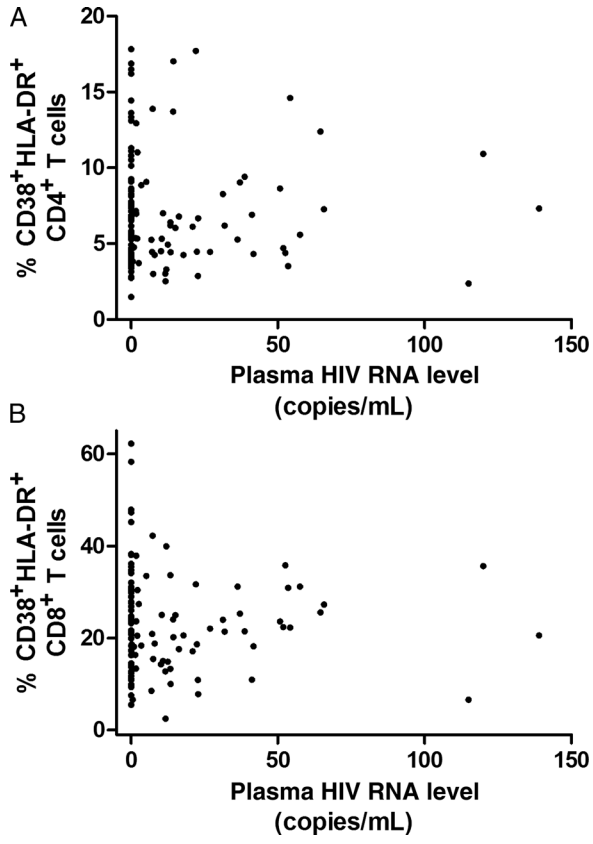

Figure 1. No associations between ultrasensitive plasma human immunodeficiency virus (HIV) RNA levels and T-cell activation. Ultrasensitive plasma HIV RNA levels were measured using the COBAS AmpliPrep/COBAS TaqMan HIV-1 test, version 2.0. All *P* values are >.40.

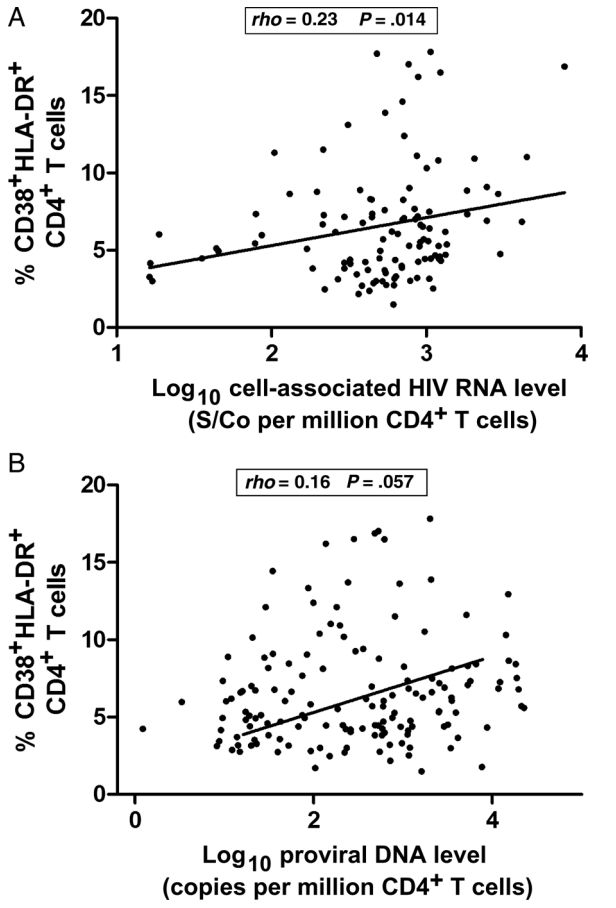

Figure 2. Cell-based measures of viral persistence are modestly associated with immune activation.

PD-1 Expression and Viral Persistence

We observed a statistically significant association between proviral DNA levels and the frequency of PD-1–expressing $CD4^+$ T cells ($\rho = 0.28$, $P = .0005$) (Figure 3*A*). We also observed an association between proviral DNA levels and the frequency of $CD4^+$ T cells expressing all markers of activation (CD38, HLA-DR, CCR5, and PD-1) ($\rho = 0.22$, $P = .008$) (Figure 3*B*). On the other hand, there were no consistent associations between ultrasensitive plasma HIV RNA levels and the frequency of PD-1–expressing $CD4^+$ or $CD8^+$ T cells (data not shown).

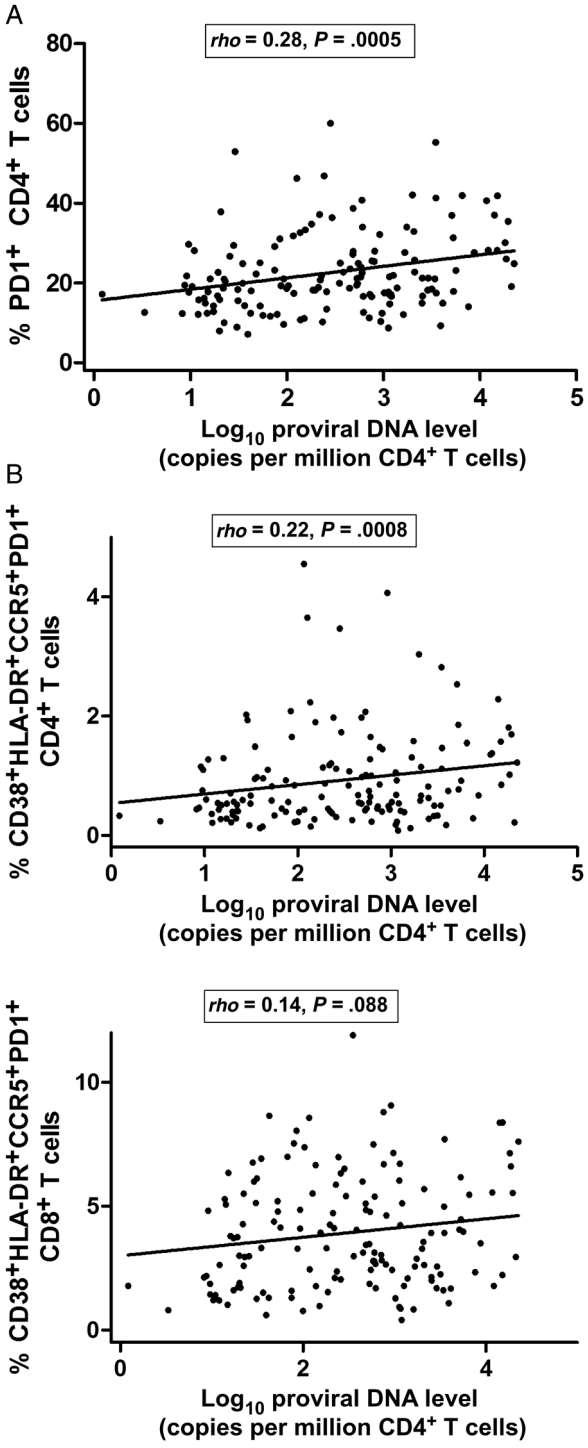

Figure 3. *A*, Highly significant association between proviral DNA levels and frequency of programmed cell death protein 1 (PD-1)–expressing $CD4^+$ T cells. *B*, Associations between proviral DNA levels and the frequency of $CD4^+$ and $CD8^+$ T cells expressing all markers of activation (CD38, HLA-DR, CCR5, and PD-1).

T-Cell Activation, T-Cell Dysfunction, and Viral Persistence in Low and High $CD4^+$ T-Cell States

There is growing recognition that the peripheral $CD4^+$ T-cell count during long-term antiretroviral therapy is a major predictor of morbidity and mortality [26, 27]. The detrimental effects of having a low $CD4^+$ T-cell count despite receipt of suppressive HAART have been most readily seen among individuals with a $CD4^+$ T-cell count <350 cells/mm^3 [26]; indeed, this threshold has often been used by our group and others to define "immunologic failure," or "incomplete $CD4^+$ T cell recovery" [11, 28, 29]. To explore host-virus interactions in individuals with low versus high $CD4^+$ T-cell counts [29], we performed an analysis comparing measures of viral persistence and immune activation/dysfunction in subjects with low (<350 cells/mm^3) and those with high (≥350 cells/mm^3) $CD4^+$ T-cell counts. Although ultrasensitive plasma HIV RNA levels were similar between low and high $CD4^+$ T-cell count groups ($P > .50$) (Figure 4), cell-associated RNA levels (878 vs 620 S/Co per million $CD4^+$ T cells) and proviral DNA levels (600 vs 204 copies per million $CD4^+$ T cells) were higher in the low $CD4^+$ T-cell count group ($P < .01$) (Figure 5). As expected, the low $CD4^+$ T-cell count group had lower frequencies of naive $CD4^+$ T cells and higher frequencies of $CD4^+$ T cells expressing CD38, HLA-DR, and/or CCR5 ($P < .0001$) (Figure 6*A*). The low $CD4^+$ T-cell count group also had remarkably higher frequencies of PD-1–expressing $CD4^+$ T cells ($P < .0001$)

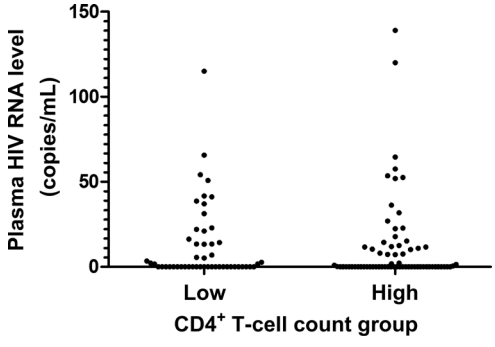

Figure 4. Ultrasensitive plasma human immunodeficiency virus (HIV) RNA levels were similar between low (<350 cells/mm^3) and high (≥350 cells/mm^3) $CD4^+$ T-cell count groups. Ultrasensitive plasma HIV RNA levels were measured using the COBAS AmpliPrep/COBAS TaqMan HIV-1 test, version 2.0 All p-values are >.50.

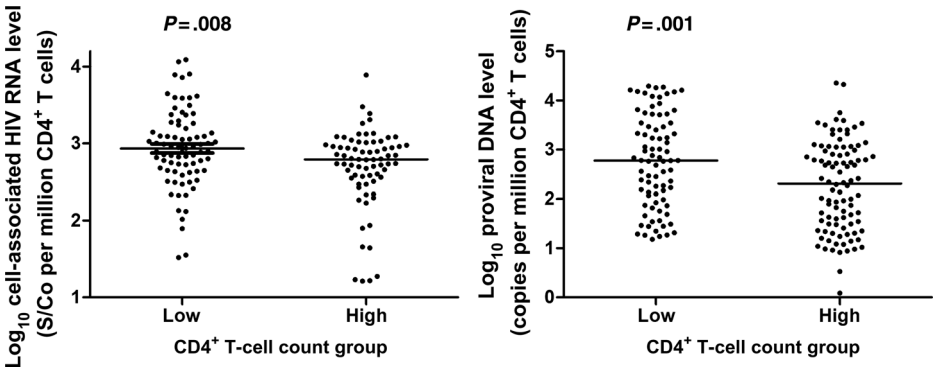

Figure 5. Cell-associated RNA and proviral DNA levels were significantly higher in the low (<350 cells/mm^3) CD4$^+$ T-cell count group. Abbreviation: S/Co, signal-to-cutoff ratio.

(Figure 6*A*); this was most consistently observed in the central memory compartment ($P < .0001$) (Figure 6*B*). These relationships held true even after adjustment for duration of viral load suppression and nadir CD4$^+$ T-cell count.

DISCUSSION

Given the widely assumed role of the host environment in determining the size and persistence of the HIV reservoir during effective antiretroviral therapy, we conducted a comprehensive cross-sectional study of virologic and immunologic measurements in a large cohort of long-term–treated individuals. We observed no associations between ultrasensitive plasma HIV RNA levels and measures of immune activation/dysfunction. The lack of correlation between ultrasensitive plasma HIV RNA levels and cellular markers of immune activation has been observed by other groups, using more-sensitive assays [30]. This is in contrast to the correlations between these measurements in untreated individuals [13]. We did, however, observe modest but consistent associations between our cell-based measures of viral persistence and T-cell activation, suggesting a common mechanistic pathway linking these measurements. We also found strong associations between proviral HIV DNA levels and the frequency of PD-1–expressing CD4$^+$ T cells, a finding that is consistent with prior studies suggesting that PD-1–expressing CD4$^+$ T cells are a preferential reservoir of HIV during effective HAART [15]. Collectively, these data suggest that the host inflammatory environment might be a cause or consequence of HIV persistence. Work aimed at defining the mechanisms for these associations could lead to

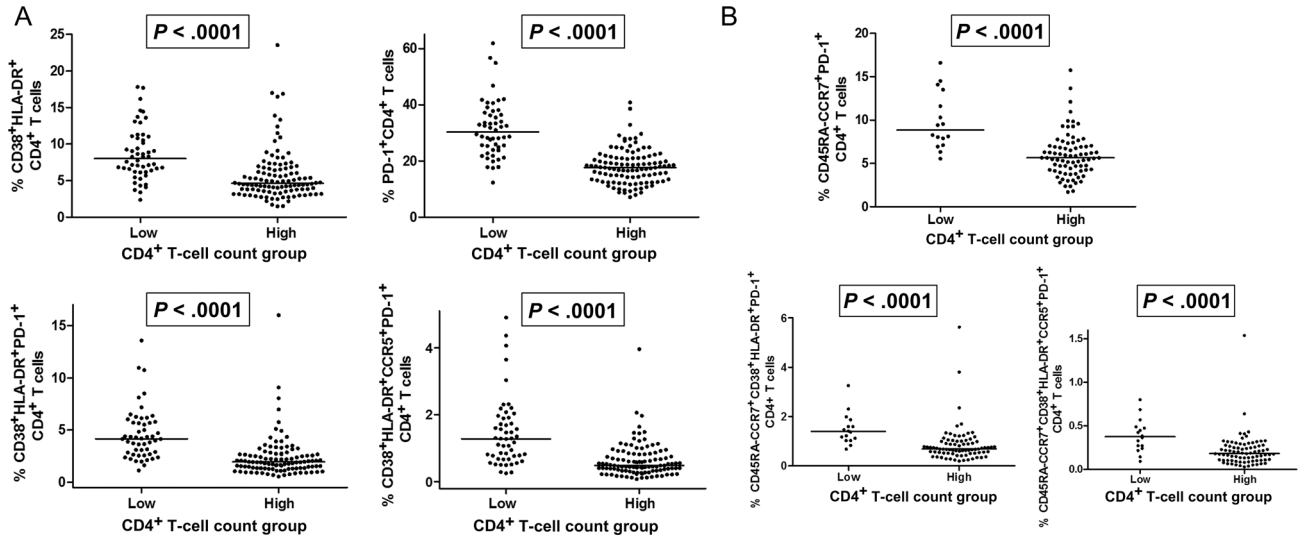

Figure 6. *A*, The low (<350 cells/mm^3) CD4$^+$ T-cell count group had significantly higher frequencies of CD4$^+$ T cells expressing CD38, HLA-DR, CCR5, and/or programmed cell death protein 1 (PD-1). *B*, The low (<350 cells/mm^3) CD4$^+$ T-cell count group had significantly higher frequencies of central memory CD4$^+$ T cells expressing CD38, HLA-DR, CCR5, and/or PD-1.

novel ways to reduce the size of the viral reservoir and/or the degree of persistent immune activation/dysfunction. If, as we predict, immune activation enables a greater degree of viral persistence (through a number of possible mechanisms, including increased target cell availability, loss of T-cell function, and upregulation of PD-1), then almost any intervention that directly affects the inflammatory process might also have secondary effects on HIV persistence. Our extended group is now pursuing this hypothesis through a number of studies that directly affect immune activation in antiretroviral-treated individuals.

Our secondary objective was to assess the relationship between $CD4^+$ T-cell treatment response and T-cell activation/dysfunction. A significant proportion of individuals are unable to achieve a normal $CD4^+$ T-cell count despite prolonged viral suppression with effective HAART ("immunologic nonresponders") [31]. Moreover, having a suboptimal $CD4^+$ T-cell response has been associated with significant clinical consequences, including increased AIDS-related and non–AIDS-related morbidity and mortality [26, 27, 32, 33]. We therefore investigated the relationship between $CD4^+$ T-cell treatment response and immune activation/dysfunction. We found that treated subjects with a low $CD4^+$ T-cell count (<350 cells/mm^3) during therapy not only had higher measures of viral persistence as compared to those with a high $CD4^+$ T-cell count (≥350 cells/mm^3), but they also had an expansion of $CD4^+$ T cells expressing CD38, HLA-DR, CCR5, and/or PD-1; this effect was most consistently observed in the central memory subset ($P < .0001$). This is consistent with findings from a study by Lederman et al, which showed that immunologic failure despite suppressive HAART was associated with increased immune activation and turnover of memory $CD4^+$ T cells [29].

The association between HIV persistence, chronic immune activation, T-cell dysfunction, and suboptimal $CD4^+$ T-cell gains is expected to be complex. Given the growing recognition that inflammation and immune dysfunction predict and presumably cause excess morbidity and mortality during otherwise effective therapy, detailed mechanistic studies in humans are clearly needed to untangle these complex associations. Perhaps the only way to truly understand how these factors interact is to intervene directly with either antiretroviral drugs (to reduce any residual replication) or immune-based therapies. Such studies are ongoing. On the basis of the general inability of intensification studies to affect systemic inflammation [11, 34], we generally favor a model in which residual immune dysfunction is the most proximal cause of our findings. Theoretically, persistent T-cell activation may be causally related to the inability to reconstitute normal $CD4^+$ T-cell counts due to its deleterious effects on lymphoid tissue architecture [35]. The degree of collagen deposition in lymphoid tissues has been shown to prevent access to T-cell survival factors such as interleukin 7 [36, 37] and has also been shown to predict the degree of treatment-mediated $CD4^+$ T-cell recovery [38, 39]. Collectively, these data suggest that suboptimal $CD4^+$ T-cell recovery despite prolonged and effective HAART may be a consequence of delayed initiation of effective antiretroviral therapy, and they argue for very early initiation of antiretroviral therapy [40–42].

Understanding the causes of viral persistence and immune activation/dysfunction in the setting of otherwise effective HAART is also necessary to develop new strategies for cure. Future studies aimed at eradication of HIV should focus on effects on cell-based measures of viral persistence, rather than on plasma-based measurements of HIV RNA load. The frequency of PD-1–expressing $CD4^+$ T cells and cell-based measures of viral persistence were elevated in treated patients with low $CD4^+$ T-cell counts. This suggests that when cure strategies are being studied, these individuals may be more difficult to cure and may require unique interventions.

Notes

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All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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