Effect of CMV and HIV Transcription on CD57 and PD-1 T-Cell Expression During Suppressive ART

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Abstract: HIV-infected men who have sex with men are nearly universally coinfected with cytomegalovirus (CMV). In this study of 45 HIV-infected men who have sex with men virologically suppressed on ART, we found that presence of seminal CMV DNA shedding and higher levels of systemic cellular HIV RNA transcription were both independently associated with increased PD-1 expression on circulating $CD4^+$ T cells, but not with higher levels of senescent ($CD57^+$) T cells. In addition, greater HIV RNA transcription was associated with lower CD57 expression on CD8 T cells. Although causality cannot be inferred from this retrospective study, these results suggest that asymptomatic CMV replication and residual cellular HIV transcription may contribute to persistent immune dysregulation during suppressive ART.

Key Words: CMV, immune exhaustion, immune senescence, PD-1, CD57

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INTRODUCTION

Antiretroviral therapy (ART) can suppress HIV replication in most HIV-infected individuals who adhere to their medications.[1] Nevertheless, HIV-infected individuals still have greater morbidity and mortality than uninfected controls, including non-AIDS defining disorders such as cardiovascular disease and neurocognitive impairment.[2] This increased morbidity and mortality has been associated with residual immune dysfunction, which persists in some individuals, despite long-term ART and suppressed viral loads in blood.[3] The exact mechanism of chronic immune dysregulation during ART is not completely understood and most likely multifactorial.

Persistent coinfections with other pathogens are common in HIV-infected individuals, and likely contribute to the overall immune dysfunction during HIV disease.[4,5] For example, HIV-infected individuals are nearly universally coinfected with cytomegalovirus (CMV) and both viruses have been independently associated with increased inflammation and inflammation-related morbidities.[6–8]

In addition, chronic antigen stimulation is associated with accelerated T-cell immunosenescence (characterized by increased $CD57^+$ expression)[3] and immune exhaustion [characterized by increased expression of programmed death 1 (PD-1)].[9] These phenotypes are both associated with reduced T-cell functionality, reflected by decreased proliferative capacity and inflammatory cytokine production.[10] In the setting of HIV infection, $CD4^+$ T cells expressing PD-1 are enriched with integrated HIV DNA[11,12] and might contribute significantly to the HIV DNA reservoir.

In this article, we investigate whether asymptomatic seminal CMV shedding and residual cellular HIV RNA transcription might be associated with markers of T-cell exhaustion ($PD\text{-}1^+$) and senescence ($CD57^+$) in virologically suppressed HIV-infected individuals.

METHODS

Participants and Samples

Paired semen and blood samples were collected from HIV-infected men who have sex with men (MSM) prospectively enrolled in the California Collaborative Treatment Group (CCTG) 592 trial.[6] This trial was an internet-based behavioral intervention study of 180 HIV-infected MSM with risk factors for HIV transmission (NCT01198418). For this substudy, we included baseline samples from 45 CMV-seropositive participants who were receiving effective ART (blood HIV RNA <50 copies/mL) at the time of enrollment.[13] Blood $CD4^+$ T-cell counts were measured by flow cytometry

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and blood HIV RNA levels were quantified by Amplicor HIV Monitor Test (Roche Molecular Systems Inc., Pleasanton, CA). The study was conducted with appropriate written subject consent and was approved by the Human Research Protections Program at the University of California, San Diego, the Los Angeles Biomedical Research Institute at Harbor-UCLA Medical Center, and the University of Southern California.

Flow Cytometry

Cryopreserved aliquots of 5 million peripheral blood mononuclear cells (PBMCs) were quickly thawed at 37°C and cells were stained using the following combination of antibodies from BD Biosciences: CD57–FITC, CD45RA–PE, CD28–ECD, CD4–PerCP-Cy5.5, PD-1–PE-Cy7, CD27–APC, CD3–APC-Cy7, CD8–V450, and LIVE/DEAD Fixable Aqua Dead Cell Stain Kit (Life Technologies, Carlsbad, CA). $CD4^+$ T-cell subsets were defined as naive ($CD45RA^+CD27^+$), central memory ($CD45RA^-CD27^+$), effector memory ($CD45RA^{+/-}CD27^-CD28^+$), and terminally differentiated ($CD45RA^{+/-}CD27^-CD28^-$). $CD8^+$ T-cell subsets were defined as naive ($CD45RA^+CD27^+CD28^+$), central memory ($CD45RA^-CD27^+CD28^+$), effector memory ($CD45RA^{+/-}CD27^+CD28^-$), and terminally differentiated ($CD45RA^{+/-}CD27^-CD28^-$). Levels of T-cell immune activation (as determined by coexpression of HLA-DR $+CD38^+$) were measured as part of a previous study.[6]

Specimens were run on a FACS Canto Flow Cytometer (BD Biosciences, Franklin Lakes, NJ) and analyzed using FlowJo software, version 9.8.

DNA Quantification

DNA was extracted from paired 5 million PBMCs and 200 μL of seminal plasma (QIAmp DNA Mini Kit, Qiagen, Valencia, CA). Total HIV DNA (pol) and 2-long terminal repeat (2-LTR) junction were quantified by droplet digital PCR (ddPCR) from extracted DNA for PBMCs,[14] and normalized to 1×10^6 $CD4^+$ T cells, as determined by RPP30 (total cell count) and flow cytometry (percentage of $CD4^+$ T cells). CMV DNA was measured by real-time PCR in both PBMC and seminal plasma.[15]

Cellular HIV RNA Quantification

Similarly, 2 types of cell-associated HIV RNA transcripts [ie, unspliced encoding for gag (HIV usRNA) and multiply spliced encoding for tat-rev (HIV msRNA)] were measured for a subset of 41 individuals.[15]

CMV IgG Quantification

Anti-CMV IgG antibody levels were measured in blood plasma, using an enzyme-linked immunosorbent assay, as previously described.[13]

Statistical Analyses

Statistical analyses were performed using SAS (version 9.2) and GraphPad Prism (version 6). Each continuous variable was assessed for normal distribution. Viral load variables were transformed to $\log_{10}$ values. CMV shedding and presence of 2-LTR were dichotomized (undetectable/detectable) by estimated level of detection. Comparisons were performed using the Fisher exact test (for sparse data), *t* test (for continuous normally distributed variables) or Mann–Whitney *U* test (for continuous, non–normally distributed variables). Correlation analyses were performed using nonparametric correlation coefficients. A multivariate linear regression model was performed for the association of PD-1 expression including factors that were significant at $P < 0.05$ at the univariate level.

RESULTS

Study Population

Matched seminal and blood specimens were obtained from 45 HIV-infected MSM, who were receiving ART and with ≤50 HIV RNA copies/mL in blood plasma. Eighteen individuals (40%) were asymptomatically shedding CMV in seminal plasma, whereas the remaining 27 individuals (60%) had no detectable CMV DNA in their semen. There were no differences in age, $CD4^+$ and $CD8^+$ T-cell counts, $CD4^+$ nadir, and frequency of detectable CMV in PBMC between groups (ie, CMV shedder versus non-CMV shedder). Individuals who were shedding CMV in semen had a trend toward a lower CD4/CD8 ratio compared with that of nonshedders (0.78 versus 0.96, $P = 0.098$). Subjects' characteristics are summarized in Table S1, Supplemental Digital Content, http://links.lww.com/QAI/A784.

Association of Seminal CMV Shedding With PD-1 and CD57 Expression on Circulating T Cells

We first assessed whether the presence of seminal CMV was associated with increased PD-1 expression on total peripheral $CD4^+$ and $CD8^+$ T cells. Individuals with seminal CMV had higher PD-1 expression on circulating total $CD4^+$ T cells ($P = 0.017$, Fig. 1A) compared with that of nonshedders. Further analyses of the $CD4^+$ T-cell subsets revealed higher PD-1 expression on terminally differentiated ($P = 0.039$) and effector memory $CD4^+$ T cells ($P = 0.030$, Fig. 1B) in CMV shedders compared with that of nonshedders. Greater PD-1 expression on $CD4^+$ T cells was also associated with higher levels of $CD4^+$ T-cell immune activation ($HLA\text{-}DR^+CD38^+$) ($r = 0.194$, $P = 0.02$).

There was no difference in PD-1 expression on circulating $CD8^+$ T cells or any $CD8^+$ T-cell subset between groups (Figs. 1C, D, respectively). We next assessed levels of $CD57^+$ expression on total $CD4^+$ and $CD8^+$ T cells and we found no differences between CMV shedders and nonshedders (Table S2, Supplemental Digital Content, http://links.lww.com/QAI/A784).

Association of Cellular HIV RNA Transcription With PD-1 and CD57 Expression on Circulating T Cells

As $PD\text{-}1^+$ expression is enriched on $CD4^+$ T-cell populations with integrated HIV DNA,[12] we investigated

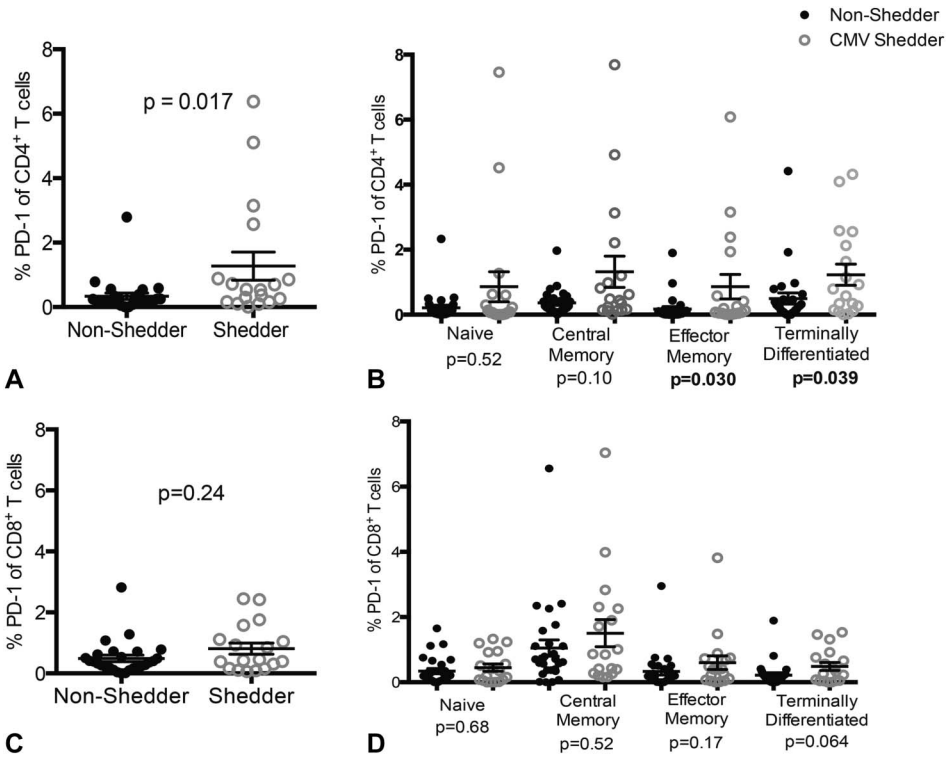

FIGURE 1. Percent of PD-1 expression on $CD4^+$ and $CD8^+$ T-cell subsets from individuals with and without detectable CMV DNA in seminal plasma. CMV shedders (based on detection of CMV in seminal plasma) had higher PD-1 expression on total $CD4^+$ T cells (A). CMV shedders had higher PD-1 expression in the effector memory and terminally differentiated subsets but not naïve or central memory subsets (B). CMV shedders did not have higher PD-1 expression on total $CD8^+$ T cells (C) or $CD8^+$ T-cell subsets (D).

the relationship of PD-1 expression with indicators of HIV persistence. Unlike previous reports,[11] there was no association between PD-1 expression on $CD4^+$ T cells and HIV DNA or 2-LTR circles in blood. However, PD-1 expression on total $CD4^+$ T cells was positively correlated with cell-associated HIV RNA [both HIV usRNA (encoding for gag), r = 0.1, P = 0.007, and HIV usRNA (encoding for tat-rev), r = 0.43, P = 0.005, Table 1]. Among T-cell subsets, higher expression of PD-1 positively correlated with increased HIV usRNA (gag) in central memory T cells (r = 0.47, P = 0.002) and with increased HIV msRNA (tat-rev) in effector memory T cells (r = 0.42, P = 0.006), terminally differentiated T cells (r = 0.42, P = 0.006), and naive T cells (r = 0.45, P = 0.003). There was no correlation between levels of any cell-associated HIV RNA transcripts and PD-1 expression on $CD8^+$ T cells, except for a borderline positive association between higher HIV msRNA (tat-rev) and increased PD-1 expression on terminally differentiated CD8 T cells (r = 0.33, P = 0.033) (Table 1).

Subsequently, we did perform a linear regression multivariate analysis for PD-1 expression on $CD4^+$ T cells including the presence of seminal CMV shedding, levels of HIV usRNA transcription, and $HLA\text{-}DR^+CD38^+CD4^+$ expression (all $P < 0.05$ at univariate level). Interestingly, presence of CMV shedding and greater HIV usRNA (gag) transcription remained significantly associated with higher PD-1 expression, whereas $HLA\text{-}DR^+$ $CD38^+$ CD4 lost significance (Table S3, Supplemental Digital Content, http://links.lww.com/QAI/A784).

Finally, we evaluated whether levels of cellular HIV RNA transcription were associated with greater CD57 expression on T cells. Although no correlation was observed between levels of HIV usRNA (gag) transcripts and CD57 expression on either $CD4^+$ or $CD8^+$ T cells, CD57 expression was positively associated with HIV msRNA (tat-rev) in the central memory (r = 0.44, P = 0.004) and naïve (r = 0.42, P = 0.006) $CD4^+$ T-cell populations. Regarding the $CD8^+$ T cells, there was a negative correlation between levels of HIV msRNA (tat-rev) and CD57 expression on total $CD8^+$ T cells (r = −0.35, P = 0.026) and this was particularly true in the terminally differentiated $CD8^+$ T-cell subpopulations (r = −0.43, P = 0.055). We also found a negative correlation between higher CD4/CD8 ratio and lower levels of HIV msRNA (tat-rev) (r = −0.34, P = 0.03), but not with HIV usRNA (gag).

DISCUSSION

To understand why HIV-infected individuals are at greater risk of age-related diseases during suppressive ART, it is important to elucidate the mechanisms underlying persistent T-cell dysfunction. In our study, presence of seminal CMV shedding was associated with a 4-fold increase in PD-1 expression on total circulating $CD4^+$ T cells, particularly in the effector and terminally differentiated subsets. Similarly, increased levels of cellular HIV RNA transcripts (usRNA and msRNA) were positively associated with higher PD-1 expression on total circulating $CD4^+$ T cells. Interestingly, these associations were independent from the previously described associations between HIV and CMV replication and T-cell immune activation.[6]

Although this cross-sectional study cannot distinguish whether CMV replication in semen or HIV transcription is the cause or the consequence of immune dysfunction, it does

TABLE 1. Correlation of Cell-Associated HIV RNA to %PD-1 and %CD57 Expression on $CD4^+$ and $CD8^+$ Subsets and CD4/CD8 Ratio

	Unspliced HIV RNA		Multiply Spliced HIV RNA	
	Spearman, R	*P*	Spearman, R	*P*
%PD-1 of total $CD4^+$	0.1	0.007	0.43	0.005
%PD-1 of naive $CD4^+$	0.15	0.34	0.45	0.003
%PD-1 of central memory $CD4^+$	0.47	0.002	0.30	0.058
%PD-1 of effector memory $CD4^+$	0.30	0.058	0.42	0.006
%PD-1 of terminally differentiated $CD4^+$	0.23	0.15	0.42	0.006
%CD57 of total $CD4^+$	0.04	0.78	0.03	0.86
%CD57 of naive $CD4^+$	−0.04	0.82	0.42	0.006
%CD57 of central memory $CD4^+$	0.01	0.94	0.44	0.004
%CD57 of effector memory $CD4^+$	0.15	0.35	−0.23	0.14
%CD57 of terminally differentiated $CD4^+$	−0.03	0.85	0.086	0.59
%PD-1 of total $CD8^+$	0.16	0.31	0.17	0.28
%PD-1 of naive $CD8^+$	0.057	0.72	−0.055	0.73
%PD-1 of central memory $CD8^+$	0.18	0.26	0.048	0.76
%PD-1 of effector memory $CD8^+$	0.14	0.37	0.19	0.24
%PD-1 of terminally differentiated $CD8^+$	0.020	0.90	0.33	0.033
%CD57 of total $CD8^+$	−0.069	0.67	−0.35	0.026
%CD57 of naive $CD8^+$	−0.029	0.86	0.23	0.15
%CD57 of central memory $CD8^+$	−0.046	0.78	−0.19	0.23
%CD57 of effector memory $CD8^+$	0.14	0.38	−0.17	0.29
%CD57 of terminally differentiated $CD8^+$	−0.051	0.75	−0.43	0.055
CD4/CD8 ratio	−0.17	0.28	−0.34	0.03

P values were determined using Spearman test.

demonstrate that asymptomatic CMV replication is associated with increased levels of circulating $CD4^+$ T cells carrying phenotypic markers of exhaustion (PD-1) during suppressive ART. PD-1 expression is upregulated on activated $CD4^+$ T cells and may represent a homeostatic response to total $CD4^+$ T-cell depletion in the setting of HIV infection.[16,17] It is conceivable that a more profound immunodeficiency, secondary to viral or genetic factors, could contribute to persistent genital CMV shedding, which, in turn, might increase $CD4^+$ T-cell activation and maintain high-level of PD-1 expression during ART. Although not statistically significant in our cohort, CMV shedders did have lower $CD4^+$ T-cell nadirs, compared with those of non-CMV shedders (199 vs 255 cells/μL) and lower CD4/CD8 ratio (0.78 versus 0.96).

Because PD-1 expressing cells are more frequently infected with HIV,[11,12] this mechanism could possibly link persistent CMV replication to increased markers of HIV persistence, inclusive of HIV DNA and cellular RNA.[6,18] In this study, we did find a positive correlation between higher levels of cell-associated HIV RNA and PD-1 expression on total $CD4^+$ T cells. Although persistent HIV transcription itself might just be an additional driver of PD-1 expression, it is possible that CMV-induced exhaustion and activation of T cells might be more permissive to cellular HIV transcription despite effective ART, but unfortunately the directionality cannot be inferred from this study design. Unlike previous reports, we did not observe a correlation between PD-1 expression and HIV DNA.[11]

Finally, we observed a significant correlation between lower CD57 expression on $CD8^+$ T cells and HIV msRNA (tat-rev). This is consistent with a previous study showing that untreated viremic HIV-infected individuals had lower CD57 expression on $CD28^-CD8^+$ cells compared to both healthy individuals and HIV-infected individuals suppressed on ART.[19] By inhibiting the T-cell maturation process, HIV replication might be promoting the expansion of a population of less differentiated $CD28^-CD8^+$ T cells (lacking the proliferative history marker, CD57). This population of cells was previously associated with a functional defect but this phenomenon and its connection to viral persistent (for both CMV and HIV) need further investigation.[19,20]

This study has a number of limitations. First, because this was a cross-sectional observational study, we cannot establish a causal relationship between asymptomatic CMV shedding, cellular HIV transcription, and markers of T-cell dysfunction. Second, we were not able to differentiate between people who are continuously shedding CMV versus those intermittently shedding CMV, and some of the observed associations might be transient. Thirdly, the small sample size limited our power to observe significant associations within the $CD4^+$ and $CD8^+$ T-cell subsets. Finally, it is not clear if the presence of seminal CMV DNA is a surrogate for active systemic infection or rather a localized sequestered genital infection. Unfortunately, we did not have enough specimens to evaluate markers of CMV-specific responses in this cohort.

Despite these limitations, this study provides interesting insights into possible connections among asymptomatic CMV replication, markers of T-cell dysfunction, and HIV persistence during suppressive ART. Confirming this hypothesis will require a randomized placebo-controlled trial of anti-CMV interventions. Suppression of subclinical CMV replication might decrease expression of PD-1 on circulating $CD4^+$ T cells, providing overall improved antigen-specific immune memory and might allow improved control on HIV replication and decrease in the HIV viral reservoir.

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