Title

Replication of Human Herpesviruses Is Associated with Higher HIV DNA Levels during Antiretroviral Therapy Started at Early Phases of HIV Infection

Journal

Journal of Virology, 90(8)

ISSN

0022-538X

Authors

Gianella, Sara

Anderson, Christy M

Var, Susanna R

et al.

Publication Date

2016-04-15

DOI

10.1128/jvi.02638-15

Peer reviewed

AMERICAN SOCIETY FOR MICROBIOLOGY | Journal of Virology

Replication of Human Herpesviruses Is Associated with Higher HIV DNA Levels during Antiretroviral Therapy Started at Early Phases of HIV Infection

Sara Gianella,[a] Christy M. Anderson,[a] Susanna R. Var,[a] Michelli F. Oliveira,[a] Steven M. Lada,[a] Milenka V. Vargas,[a] Marta Massanella,[a] Susan J. Little,[a] Douglas D. Richman,[a,b] Matthew C. Strain,[a] Josué Pérez-Santiago,[a] Davey M. Smith[a,b]

University of California, San Diego, La Jolla, California, USA[a]; Veterans Affairs San Diego Healthcare System, San Diego, California, USA[b]

ABSTRACT

Asymptomatic replication of human herpesviruses (HHV) is frequent in HIV-infected men and is associated with increased T-cell activation and HIV disease progression. We hypothesized that the presence of replication of cytomegalovirus (CMV) and Epstein-Barr virus (EBV) (the most frequently detected HHV) might influence HIV DNA decay during antiretroviral therapy (ART). We investigated 607 peripheral blood mononuclear cell (PBMC) samples from 107 CMV-seropositive, HIV-infected men who have sex with men, who started ART within a median of 3 months from their estimated date of infection (EDI) and were monitored for a median of 19 months thereafter. Levels of HIV, CMV, and EBV DNA and cellular HIV RNA were measured by droplet digital PCR (ddPCR) for each time point. Using a general linear mixed-effect regression model, we evaluated associations between the presence of detectable CMV DNA and EBV DNA levels and HIV DNA decay and cellular HIV RNA levels, while adjusting for peak HIV RNA, nadir CD4$^+$ count, CD4/CD8 ratio, CMV IgG levels, time from EDI to ART initiation, time from ART initiation to virologic suppression, detectable CMV DNA pre-ART, and age. The presence of intermittent CMV DNA in PBMC during ART was significantly associated with slower decay of HIV DNA ($P = 0.011$) but not with increased cellular HIV RNA transcription or more detectable 2-long terminal repeat circles. Higher levels of EBV DNA were also associated with higher levels of HIV DNA ($P < 0.001$) and increased unspliced cellular HIV RNA transcription ($P = 0.010$). These observations suggest that replication of HHV may help maintain a larger HIV DNA reservoir, but the underlying mechanisms remain unclear.

IMPORTANCE

Over three-fourths of HIV-infected men have at least one actively replicating human herpesvirus (HHV) in their mucosal secretions at any one time. Cytomegalovirus (CMV) and Epstein-Barr virus (EBV) are the most common, and although it is often asymptomatic, such CMV and EBV replication is associated with higher levels of immune activation and HIV disease progression. We hypothesized that HHV-associated activation of HIV-infected CD4$^+$ T cells might lead to increased HIV DNA. This study found that detectable CMV in blood cells of HIV-infected men was associated with slower decay of HIV DNA even during antiretroviral therapy (ART) that was started during early HIV infection. Similarly, levels of EBV DNA were associated with higher levels of HIV DNA during ART. If this observation points to a causal pathway, interventions that control CMV and EBV replication may be able to reduce the HIV reservoir, which might be relevant to current HIV cure efforts.

The long-lived latent HIV reservoir that persists during suppressive antiretroviral therapy (ART) is the major obstacle in achieving a cure (1). Two possible mechanisms underlying HIV persistence during suppressive ART include residual HIV replication (2, 3) and proliferation of latently infected CD4$^+$ T cells (4). Further, chronic inflammation and immune activation modulate both HIV replication and T-cell proliferation and contribute to the maintenance of the HIV DNA reservoir even during ART (5–7). Similar to the case with HIV, asymptomatic replication of human herpesviruses (HHV) also mediates immune activation (8–10). This can be important since subclinical bursts of cytomegalovirus (CMV) and Epstein-Barr virus (EBV) reactivation are common among HIV-infected individuals, even those with high CD4$^+$ T-cell counts and those receiving effective ART (11). Given that most HIV-infected individuals and almost all HIV-infected men who have sex with men (MSM) are coinfected with multiple HHV (12) and persistent viral replication is a known driver of immune activation, we hypothesized that CMV, and possibly EBV, drives activation and proliferation of latently infected CD4$^+$ T cells and plays a role in the maintenance of the HIV DNA reservoir during ART.

In a cross-sectional study of HIV-infected individuals who started ART during chronic infection, we previously observed that asymptomatic shedding of CMV in the male genital tract was associated with increased systemic T-cell immune activation and proliferation and with higher levels of HIV DNA in peripheral

Received 14 October 2015 **Accepted** 25 January 2016

Accepted manuscript posted online 3 February 2016

Citation Gianella S, Anderson CM, Var SR, Oliveira MF, Lada SM, Vargas MV, Massanella M, Little SJ, Richman DD, Strain MC, Pérez-Santiago J, Smith DM. 2016. Replication of human herpesviruses is associated with higher HIV DNA levels during antiretroviral therapy started at early phases of HIV infection. J Virol 90:3944–3952. doi:10.1128/JVI.02638-15.

Editor: G. Silvestri

Address correspondence to Sara Gianella, gianella@ucsd.edu.

J.P.-S. and D.M.S. contributed equally to this article.

$CD4^+$ T cells (10, 14). This cross-sectional study had a number of limitations which may have confounded the analysis, including the lack of longitudinal data and missing information about timing of HIV infection, ART history, and dynamics of viral suppression. Additionally, since the HIV DNA reservoir is established during the earliest stages of HIV infection, it may be particularly important to evaluate people who initiated ART during primary HIV infection. To address these issues, in this study we measured levels of CMV, EBV, and HIV DNA, as well as cellular HIV RNA transcripts, longitudinally in peripheral blood mononuclear cells (PBMC) of 107 HIV-infected individuals who started ART within the first months of HIV infection and achieved continuous HIV RNA suppression for up to 75 months.

MATERIALS AND METHODS

Participants, samples, and clinical laboratory tests. A total of 607 blood samples were collected longitudinally from 107 recently HIV-infected CMV-seropositive participants from the San Diego Primary Infection Resource Consortium (15) between October 1996 and October 2012. Most participants (81%) started ART within the first year of HIV infection, and all participants achieved complete suppression of HIV RNA (defined as <50 HIV RNA copies/ml) and maintained undetectable HIV RNA levels in blood plasma for a median of 19.2 months. The estimated date of infection (EDI) was calculated using established algorithms (16). Longitudinal blood samples were collected during suppressive ART at approximately 6-month intervals ($n = 515$), while pre-ART blood samples were available for a subset of 92 participants. In blood, percentage and absolute counts of $CD4^+$ T lymphocyte subsets were measured by flow cytometry (VA Flow Lab) and HIV-1 RNA was quantified (Amplicor HIV Monitor test; Roche Molecular Systems Inc.). All adult subjects provided their written informed consent. No children were included in this study. The Office of Human Research Protections Program of the University of California approved the study.

HIV DNA, 2-LTR circular HIV DNA, CMV DNA, and EBV DNA in PBMC. DNA was extracted from 5 million PBMC for each time point using the AllPrep DNA/RNA minikit (Qiagen, CA). Total HIV DNA (polymerase [Pol]) and the 2-long terminal repeat (2-LTR) junction were quantified by droplet digital PCR (ddPCR) from extracted DNA (17). Briefly, 1,000 ng of DNA per replicate was digested with BSAJ1 enzyme (New England BioLabs) prior to ddPCR quantification. Total HIV DNA (Pol) and 2-LTR PCRs were performed as a duplex assay with VIC (Pol) and 6-carboxyfluorescein (FAM) (2-LTR) probes, respectively, with the following cycling conditions: 10 min of initial enzyme activation at 95°C, 40 cycles consisting of a 30-s denaturation at 94°C followed by a 60°C extension for 60 s, and a final inactivation of 10 min at 98°C. Total CMV and EBV DNA PCRs were performed as a duplex with FAM (CMV) and HEX (EBV), respectively, with the following cycling conditions: 10 min of initial enzyme activation at 95°C, 40 cycles consisting of a 30-s denaturation at 94°C followed by a 54°C extension for 60 s, and a final inactivation of 10 min at 98°C. We used the host cell RNase P/MRP 30-kDa-subunit gene (RPP30) as our cellular normalizer, as it appears in 2 copies per cell. The digested DNA was diluted 10-fold, and 100 ng of the digested DNA per replicate was used for quantification of RPP30 by ddPCR (probe VIC) and cycled with the same parameters as for the Pol/2-LTR duplex. Copy numbers were calculated as the mean of replicate PCR measurements and normalized to one million $CD4^+$ T cells as determined by RPP30 (total cell count) and flow cytometry (percentage of $CD4^+$ T cells) (18).

Cellular HIV RNA in PBMC. Cellular HIV RNA was extracted from PBMC using the AllPrep DNA/RNA minikit (Qiagen, CA) by following the manufacturer's protocol, with addition of a DNase step to avoid DNA contamination (RNase-free DNase Set, Qiagen, CA). Extracted RNA (500 ng) was reverse transcribed into 20 μl of cDNA (iScript Advanced cDNA synthesis kit; Bio-Rad) using the manufacturer's protocol. The cDNA product (8 μl; approximately 300 ng) was added to the ddPCR mixture. Unspliced HIV RNA (usRNA) (Gag) and multiply spliced HIV RNA (msRNA) (Tat/Rev) PCRs were performed as a duplex with HEX (usGag) and FAM (msTat/Rev) probes, respectively, using primers and probes as previously described (20). Additionally, levels of all fully elongated and correctly processed HIV mRNA molecules (referred to as polyA) were measured as described previously (21). Cycling conditions for this PCR were 10 min at 95°C, 60 cycles consisting of a 30-s denaturation at 94°C followed by a 60°C extension for 60 s, and a final 10 min at 98°C. Copy numbers were calculated as the means of replicate PCR measurements and normalized to total RNA as determined by A_{260}/A_{280} absorptivity ratio using a NanoDrop 2000 spectrophotometer (Thermo Scientific) (10).

Anti-CMV IgG antibody levels. Anti-CMV IgG antibody levels were measured in blood plasma at baseline (pre-ART) and at week 48 after the initiation of ART. Units per milliliter were determined by interpolation from a standard curve of a known anti-CMV IgG solution, as previously described (22, 23).

Statistical analysis. Since levels of CMV DNA were undetectable in 65% ($n = 337$) of sampled time points, CMV DNA measurements were dichotomized as detectable or undetectable for this analysis. Additionally, since CMV shedding often occurs intermittently, participants who had at least one time point with a detectable level of CMV DNA during ART ($n = 69$; 64.5%) were compared to participants without detectable CMV DNA at any time point ($n = 38$; 35.5%). A common-log transformation of the longitudinal measures of HIV DNA was used to conform best to model assumptions of normality and homoscedasticity. Undetectable levels of HIV DNA ($n = 108$; 21%) were replaced with a value just below the minimum level detected for our primary analysis (minimum value was 1.48 copies per 10^6 cells; replacement value was 1.4 copies per 10^6 cells). Since the continuous variables of time, peak HIV RNA, nadir $CD4^+$, CD4/CD8 ratio, time from EDI to ART initiation, and time from ART initiation to virologic suppression lacked a linear relationship with HIV DNA levels, we transformed or dichotomized all of these covariates, including a log transformation of time and EBV DNA, classification of early ART initiation (<3 months of infection), and dichotomization of the remainder variables at their medians. Time points with missing CD4/CD8 ratios ($n = 24$; 4.7%) were imputed using the mean of the previous and subsequent known values or the last known value carried forward.

Baseline characteristics and demographics were compared using Fisher's exact tests and Wilcoxon-Mann-Whitney tests. To assess the decline of HIV DNA over time between participants with and without CMV, we used linear mixed-effects regression analysis with random-subject intercepts. The primary fixed effects were detectable CMV DNA, time, and the interaction of CMV and time. The interaction term in the model tested whether the change (or decay) in HIV DNA levels over time was significantly different for participants with intermittent CMV DNA replication compared to those without any detectable CMV. With the inclusion of this interaction term in the model, the group effect (or CMV term) tested the difference in HIV DNA levels at baseline (at initiation of ART). We used the same modeling strategy to test the association of EBV DNA levels on HIV DNA during ART. In secondary analysis, we investigated the effects of the following covariates: peak HIV RNA level, nadir $CD4^+$ count, CD4/CD8 ratio, CMV IgG, time from EDI to the initiation of ART, time from the initiation of ART to viral suppression, presence of detectable CMV DNA pre-ART, and age.

Before introducing other covariates into the model, we tested the association between individual covariates and HIV DNA levels by building separate mixed-effects models for each covariate. Except for CMV and early initiation of ART, none of the interactions between covariates and time were significantly associated with HIV DNA in the individual models or in the final multivariable model, so only the results for the main effects are presented.

To evaluate the effect of the censored data for HIV DNA, we performed multiple sensitivity analyses and repeated our regression analysis (i) using half the minimum level detected (0.7 copies per 10^6 cells), (ii)

TABLE 1 Characteristics at first sampled time point (post-ART)

Characteristic	Value for: Subjects without detectable CMV($n = 38$)	Subjects with intermittently detectable CMV($n = 69$)	Total subjects ($n = 107$)	*P* value
MSM, no. (%)	38 (100)	69 (100)	107 (100)	NA[a]
Race/ethnicity, no. (%), ($n = 107$)				
White, non-Hispanic	21 (55.3)	48 (70.6)	69 (65.1)	0.25
Hispanic	10 (26.3)	12 (17.7)	22 (20.8)	
Other	7 (18.4)	8 (11.8)	15 (14.2)	
Age (yrs), median (IQR)	35 (29–42)	35 (28–42)	35 (28–42)	1.00
Stage of HIV infection, no. (%)				
Acute infection (≤70 days)	28 (73.7)	58 (84.1)	86 (80.4)	0.21
Early infection (>70 days)	10 (26.3)	11 (15.9)	21 (19.6)	
$CD4^+$ cell count, cells/μl, median (IQR)	499 (333–587)	472 (360–649)	490 (337–618)	0.60
CD4/CD8 ratio, median (IQR)	0.9 (0.8–1.1)	0.7 (0.5–1.0)	0.8 (0.5–1.1)	0.18
HIV RNA $\log_{10}$ copies/ml, median (IQR)	5.3 (4.9–5.8)	5.4 (4.6–5.9)	5.4 (4.7–5.9)	0.93
Presence of CMV in PBMC pre-ART, no. (%)	12 (34.3)	24 (42.9)	36 (39.6)	0.51
Nadir $CD4^+$ cells, cells/μl, median (IQR)	398 (284–489)	432 (300–552)	405 (297–524)	0.20
Peak HIV RNA, $\log_{10}$ copies/ml, median (IQR)	5.6 (5.1–6.4)	5.8 (5.2–6.4)	5.7 (5.1–6.4)	0.80
HIV DNA, $\log_{10}$ copies/10^6 $CD4^+$, median (IQR), pre-ART ($n = 92$)	2.6 (1.7–3.1)	2.5 (1.6–3.2)	2.6 (1.7–3.2)	0.97
HIV DNA, $\log_{10}$ copies/10^6 $CD4^+$, median (IQR)	2.1 (1.0–2.5)	2.2 (1.4–3.0)	2.1 (1.3–2.7)	0.16
Months from EDI to ART start, median (IQR)	5 (3–12)	3 (2–5)	3 (2–7)	0.04
Months from ART to suppression, median (IQR)	3.0 (1.1–5.1)	3.9 (2.1–5.5)	3.6 (1.8–5.5)	0.08
EBV DNA ($\log_{10}$ copies/μl)	1.4 (0.8–1.8)	1.5 (0.8–2.0)	1.5 (0.8–1.9)	0.54
CMV IgG, UI/ml, median (IQR), pre-ART ($n = 85$)	30 (24–35)	24.5 (14–34)	26 (16–34)	0.08
CMV IgG, UI/ml, median (IQR) ($n = 57$)	31 (17–34)	25 (10–34)	27 (14–34)	0.54

[a] NA, not applicable.

performing a Tobit regression instead of a linear mixed-effects regression, and (iii) excluding time points with RPP30 levels below the 5th percentile (<13,440 copies/well). To evaluate for potential differences in HIV RNA transcription, we used a mixed-effects logistic regression model to investigate whether having detectable CMV DNA was associated with the presence of detectable cell-associated HIV usRNA (Gag), HIV msRNA (Tat/Rev), polyA HIV RNA, or detectable 2-LTR circles. The association between the HIV RNA transcription variables and possible covariates were also investigated.

RESULTS

Participants, samples, and clinical laboratory tests. Study participants ($n = 107$) were all HIV-infected MSM with recent HIV infection who were monitored as part of the San Diego Primary Infection Resource Consortium (16) for up to 75 months (median, 19.2 months; interquartile range [IQR], 11.4 to 38.5). The median time from EDI to the initiation of ART was 3 months (IQR, 2 to 8.7 months), and half of our participants achieved viral suppression within 3.6 months (IQR, 1.8 to 5.5 months). Most were white, non-Hispanic participants (65.1%) who had been infected less than 70 days before study enrollment (80.4%). At baseline (first post-ART time point), participants were a median of 35 years old, had a median $CD4^+$ T-cell count of 490 cells/μl, and had a median of 5.4 $\log_{10}$ HIV RNA copies/ml. In our analysis, we divided participants in two groups: those with intermittent CMV DNA in PBMC ($n = 69$) and those without any detectable CMV DNA ($n = 38$). Overall, characteristics were similar between the two groups (Table 1), although as previously reported (23), individuals with CMV replication had lower levels of CMV IgG than did people with no CMV replication (median of 24.5 versus 30 IU/ml blood plasma [$P = 0.08$]), and participants with CMV started ART a median of 2 months earlier than those without CMV ($P = 0.04$).

Herpesvirus and HIV characteristics in PBMC before and after the initiation of ART. CMV and EBV DNA were detectable in 35% and 83% of the PBMC samples, respectively; 64.5% of the study participants had at least one time point with detectable CMV DNA during the follow-up period. Total levels of HIV DNA (Pol) as measured by ddPCR were detectable in 85% of the 92 pre-ART PBMC samples, with a median level of 2.6 $\log_{10}$ copies per million $CD4^+$ T cells (IQR, 1.7 to 3.2), while 2-LTR circles were detectable in two-thirds of all pre-ART PBMC samples, with a median level of 1.7 $\log_{10}$ copies per million $CD4^+$ T cells among detectable samples (IQR, 1.4 to 2.3). After the initiation of ART, HIV DNA was detectable in 79% of all 515 PBMC samples, with a median level of 1.7 $\log_{10}$ copies per million $CD4^+$ T cells (IQR, 1.0 to 2.5), while 2-LTR circles were detectable in 53% of all PBMC samples, with a median level of 1.6 $\log_{10}$ copies per million $CD4^+$ T cells among detectable samples (IQR, 1.2 to 2.1). HIV usRNA (Gag) was detectable in 96% of samples before ART initiation and 82% of samples during ART, HIV msRNA encoding Tat/Rev was detectable in 89% of samples before ART and 52% during ART, and poly(A) HIV RNA was detectable in 93% of samples before ART and 65% of samples during ART.

Individual covariate associations with HIV DNA levels. The results from the individual regressions of HIV DNA on each co-

TABLE 2 Predictors of HIV DNA decay

Fixed effect (no. of TP[a] = 515; no. of subjects = 107[b])	Individual models[c]		Multivariable model	
	Unadjusted effect	*P* value	Adjusted effect	*P* value
Time (log_2 months)	−0.20 (−0.23 to −0.17)	**<0.001**	−0.23 (−0.32 to −0.13)	**<0.001**
Intermittent CMV replication	−0.40 (−0.86 to 0.07)	0.095	−0.41 (−0.87 to 0.04)	0.074
Intermittent CMV replication × time	0.13 (0.03 to 0.22)	**0.008**	0.12 (0.03 to 0.21)	**0.011**
Pre-ART detectable CMV DNA ($n = 92$)	0.29 (−0.05 to 0.62)	0.091		
EBV DNA (log_{10} copies/million cells)	0.24 (0.17 to 0.32)	**<0.001**	0.23 (0.14 to 0.32)	**<0.001**
High CD4/CD8 ratio (≥1)	−0.54 (−0.69 to −0.40)	**<0.001**	0.06 (−0.15 to 0.27)	0.586
Interaction of EBV and low CD4/CD8 ratio			−0.17 (−0.29 to −0.05)	**0.006**
Early ART initiation (<3 mo)	0.16 (−0.21 to 0.53)	0.403	0.25 (−0.11 to 0.62)	0.175
Early ART × time	−0.05 (−0.11 to 0.005)	0.071	−0.08 (−0.14 to −0.02)	**0.012**
Long time to suppression (>3.6 mo)	0.17 (−0.14 to 0.48)	0.284		
High peak HIV RNA (>5.7 log_{10} copies/ml)	0.02 (−0.29 to 0.34)	0.879		
Low nadir CD4 (<405 cells/μl)	−0.26 (−0.57 to 0.05)	0.100		
Older age (>35 yrs)	−0.01 (−0.32 to 0.31)	0.964		
Higher levels of CMV IgG (>2.8 units; $n = 102$)	−0.17 (−0.50 to 0.17)	0.324		

[a] TP, time point.
[b] Unless specified otherwise.
[c] One individual longitudinal model for each covariate.

variate are presented in Table 2. For each doubling of time on ART (i.e., months 1, 2, 4, 8, etc.), HIV DNA levels declined an average of 0.20 log_{10} copy/10^6 $CD4^+$ T cells without adjusting for other effects. At baseline, there was no significant difference in the mean levels of HIV DNA for participants with CMV and participants without detectable CMV ($P = 0.095$), but the mean level of HIV DNA for subjects with CMV declined significantly slower than for subjects with no detectable CMV ($P = 0.009$). Specifically, for each doubling of month on ART, HIV DNA declined 0.13 log_{10} copies/10^6 $CD4^+$ cells more slowly for people with detectable CMV DNA in their PBMC than for people without detectable CMV (Fig. 1).

Similarly, higher EBV DNA levels were significantly associated with higher levels of HIV DNA ($P < 0.001$). In a separate model of HIV DNA regressed on EBV, time, and the interaction of EBV and time (not shown), the interaction term was not significantly associated with HIV DNA ($P = 0.393$), indicating that EBV was not significantly associated with the change or decay in HIV DNA. Additionally, time points with CD4/CD8 ratios greater than the median (≥1) were more likely to exhibit mean levels of HIV DNA approximately 0.5 log_{10} copies/10^6 $CD4^+$ cells lower (<0.001). No other examined variable had a significant association with the decline in HIV DNA over time when considered individually. Also, pre-ART CMV, early initiation of ART (<3 months), nadir CD4, age, and CMV IgG did not have a significant association with the mean levels of HIV DNA during ART.

Multivariable model of HIV DNA levels. In our multivariable model, HIV DNA levels declined significantly more slowly (i) for participants with detectable CMV than for those with undetectable CMV DNA ($P < 0.011$) and (ii) for participants who did not initiate ART within 3 months of infection than for those with early initiation of ART ($P = 0.012$). Specifically, for each doubling of months on ART (2, 4, 8, 16, etc.), the adjusted level of HIV DNA declined 0.12 log_{10} copy slower for participants with detectable CMV DNA compared to those with no detectable CMV, with all other characteristics being equal. This represented about double the rate of decline in HIV DNA levels for participants with no detectable CMV (0.23 versus 0.11 log_{10} copy/10^6 $CD4^+$ T cells for each doubling of months on ART [Fig. 1 and 2]). In comparison, HIV DNA levels declined 0.08 log_{10} copies/10^6 $CD4^+$ T cells faster for each doubling of months on ART for participants who started ART within 3 months of infection than for people who started ART later. There was a significant interaction between low CD4/CD8 ratios and EBV levels ($P = 0.006$), meaning that EBV levels also affected HIV DNA levels differently in the presence of low or high CD4/CD8 ratios. Specifically in stratified analysis, higher EBV levels were significantly associated with higher levels of HIV DNA ($P < 0.001$) in the presence of low CD4/CD8 ratios (<1), but not when CD4/CD8 ratios were high (≥1) ($P = 0.145$).

In the subset of 92 participants with pre-ART measurements of HIV DNA, we investigated how adjusting for the pre-ART level of HIV DNA influenced the observed associations between detectable CMV and HIV DNA levels. In this analysis, participants with higher pre-ART levels of HIV DNA presented significantly higher HIV DNA levels at baseline ($P < 0.001$)and had a faster decrease in HIV DNA levels on ART ($P = 0.004$). When pre-ART levels of HIV DNA were included in the model, baseline HIV DNA levels were not different between the CMV and non-CMV groups ($P = 0.25$) and HIV DNA levels still declined significantly more slowly, by 0.10 log_{10} copy for participants with detectable CMV during ART ($P = 0.033$).

To evaluate the effect of the censored data for HIV DNA, we performed three sensitivity analyses (as described under "Statistical analysis" above), and in all cases we confirmed the significant associations between detectable CMV and decay rate of HIV DNA levels (*P* value using half the minimum level detected = 0.016; *P* values using a Tobit regression = 0.017; *P* value excluding time points with RPP30 levels below the 5th percentile = 0.021). Finally, we investigated whether frequency of detectable CMV had an effect on HIV DNA levels. In this analysis, the longitudinal change in HIV DNA levels was similar for participants who had detectable CMV in 1 to 50% or >50% of the included time points ($P = 0.598$ [Fig. 2]), and both groups presented slower HIV DNA decay than did the no-CMV group.

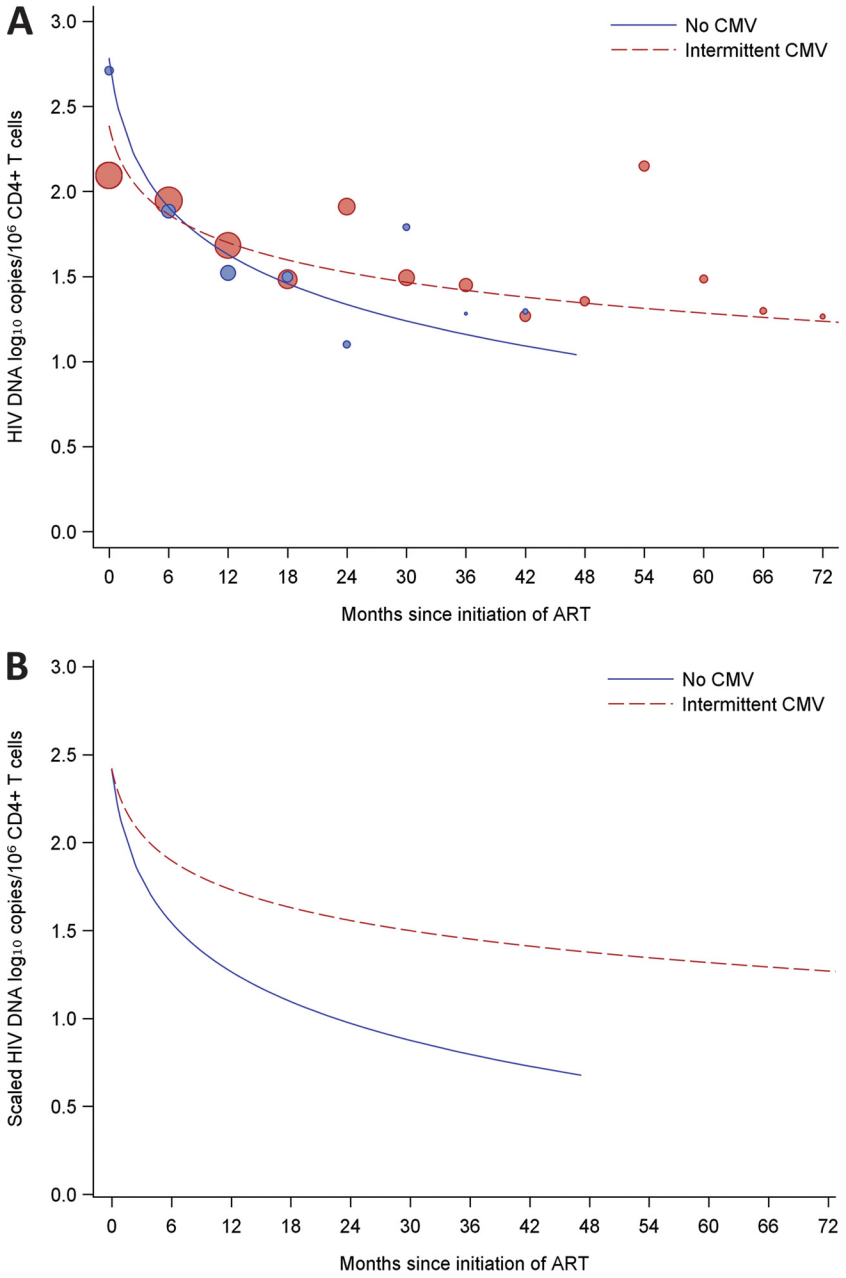

FIG 1 (A) Mean HIV DNA decay for the no-CMV group and intermittent-CMV group. The size of the bubbles corresponds to the number of time points used to calculate each mean. (B) To compare the differences in the decay of HIV DNA for each CMV group, the predicted values were scaled so that each HIV DNA curve starts at the predicted mean value at baseline.

Associations between detectable CMV and EBV DNA, cellular HIV RNA, and 2-LTR circles. To evaluate possible mechanisms of slower HIV DNA decay in the detectable-CMV group, mixed-effects logistic regression models were used to investigate the associations between detectable levels of CMV DNA during ART and cellular HIV msRNA (Tat/Rev), HIV usRNA (Gag), polyA HIV RNA, and 2-LTR HIV DNA circles. In this analysis, detectable CMV during ART was not significantly associated with levels of HIV msRNA ($P = 0.934$), usRNA ($P = 0.755$), polyA HIV RNA ($P = 0.064$), or 2-LTR circles ($P = 0.809$). In a similar model, higher levels of EBV during ART were significantly associated with higher levels of HIV usRNA ($P = 0.010$) but not with msRNA ($P = 0.138$), polyA HIV RNA ($P = 0.070$), or 2-LTR circles ($P = 0.448$). Independent of viral infections, lower CD4/CD8 ratios (≤ 1) and longer time to suppression were significantly associated with higher levels of polyA HIV RNA ($P = 0.001$ and $P = 0.041$, respectively). No other tested variable (CD4 count, peak HIV RNA level, or time on ART) was associated with increased levels of cellular HIV RNA transcription or 2-LTR circles.

DISCUSSION

The immunologic and virologic connections between HIV and HHV are intertwined and complicated, especially with the very prevalent HHV CMV and EBV (9, 24). To better understand how

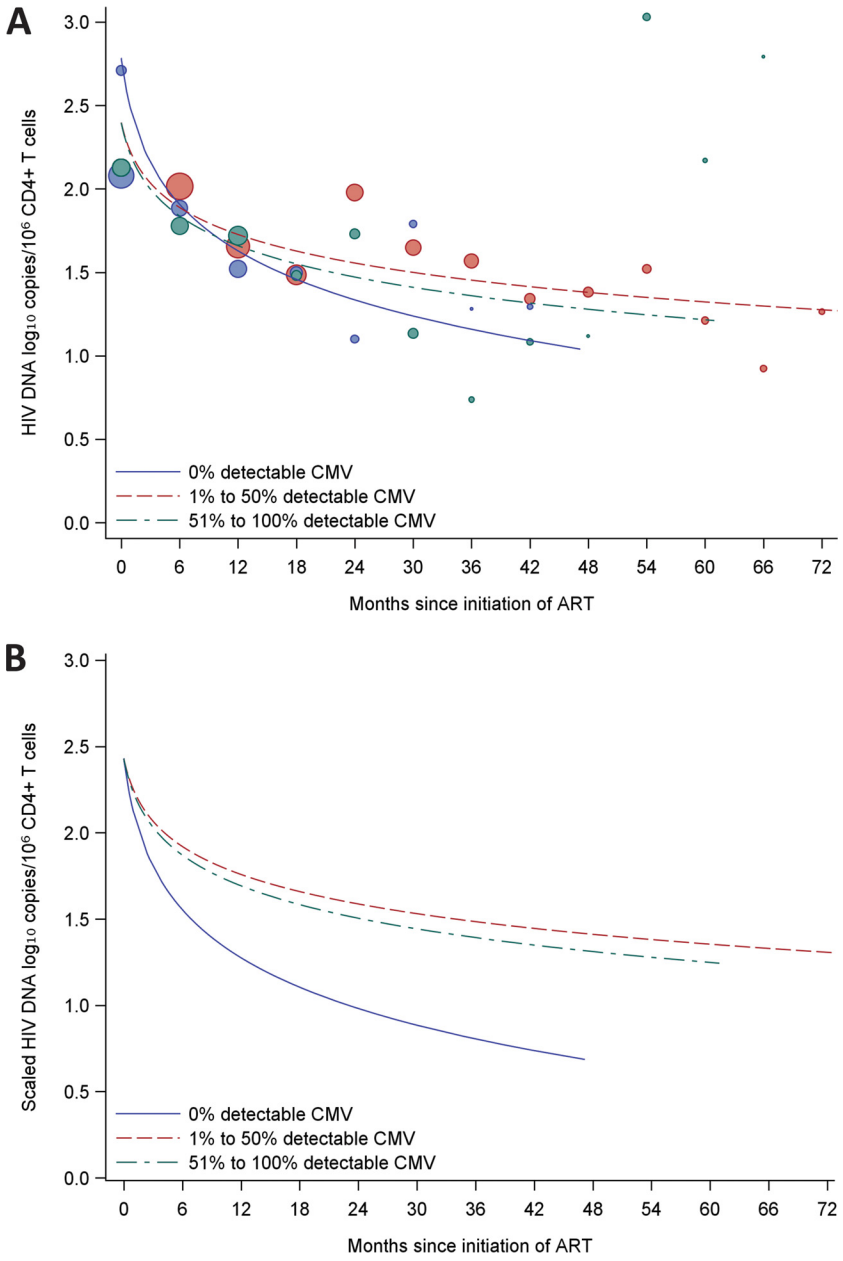

FIG 2 (A) Mean HIV DNA decay for the group with no detectable CMV, the group with 1 to 49% detectable CMV, and the group with 50 to 100% detectable CMV. The size of the bubbles corresponds to the number of time points used to calculate each mean. (B) To compare the differences in the decay of HIV DNA for each CMV group, the predicted values were scaled so that each HIV DNA curve starts at the predicted mean value at baseline.

asymptomatic CMV and EBV replication in blood cells is associated with HIV DNA levels over time, we investigated a large, uniquely well-characterized cohort of HIV-infected MSM who started ART during the early phases of HIV infection and sustained stable HIV RNA suppression. Overall, our study found that asymptomatic replication of CMV in PBMC was the strongest variable associated with lower rates of decay of the HIV DNA reservoir after the early initiation of ART. Further, detectable CMV in PBMC accounted for a 0.12-$\log_{10}$-slower HIV DNA decay for each doubling in months of follow-up between participants. The only other factor associated with slower HIV DNA decay rate was later initiation of ART. In fact, HIV DNA levels declined 0.08 $\log_{10}$ copy/10^6 $CD4^+$ T cells faster for each doubling of months on ART in participants who started ART within 3 months from EDI than for those with a later ART start. Interestingly, higher baseline HIV DNA levels before ART and detectable EBV DNA were both associated with increased HIV DNA levels during suppressive ART but not with HIV DNA decay over time. Further, higher levels of EBV DNA were associated with greater HIV DNA levels only when the CD4/CD8 ratio was lower than 1, suggesting that a conserved immune system might be protective against the EBV effect on HIV DNA levels. A similar interaction was not present between CMV and CD4/CD8 ratio.

Although the current study design does not allow causality to

be inferred, it does support the theory that persistent CMV replication at low levels could contribute to the stability of the HIV DNA reservoir during suppressive ART even when ART is initiated during the earliest phases of HIV infection. The observed association between higher HIV DNA levels and presence of detectable EBV DNA could suggest that coinfection with HHV in general might contribute to HIV persistence. Through millions of years of coexistence, HHV have developed numerous strategies to modulate the human immune response, including impeding antigen-reactive immune cells from eradicating virus-infected cells (24). It is reasonable to hypothesize that other coinfecting pathogens, like HIV, could exploit this particular immune environment to promote HIV pathogen persistence and replication, for example, through upregulation of immune-inhibitory molecules like interleukin 10 (IL-10) and programmed cell death protein 1 (PD-1), which are also involved in HIV persistence (25, 26).

Persistence of the HIV DNA reservoir during ART has been attributed to two possible factors, which could contribute separately or in combination. First, residual or intermittent HIV RNA replication replenishes the pool of infected cells over time (2, 3). Second, HIV-infected cells undergo homeostatic or antigen-driven proliferation (4). To investigate possible mechanisms underlying the connection between presence of CMV DNA and slower HIV DNA decay, we investigated if the presence of CMV DNA was associated with higher levels of cellular HIV RNA or more detectable 2-LTR circles during ART, suggesting increased residual HIV RNA replication. We found no association between the presence of cellular CMV DNA and any HIV RNA transcription or with detectable 2-LTR circles. Highlighting differences between blood and other mucosal sites (i.e., genital), this study differed from our previous cross-sectional study of chronically HIV-infected individuals (10), in which the presence of genital CMV DNA replication was associated with higher levels of HIV msRNA but not HIV usRNA. While this difference might be a consequence of different cohorts and sampling strategies, the consistent lack of association between CMV DNA and HIV usRNA in both studies suggests that a different mechanism likely connects asymptomatic CMV replication and HIV persistence. An alternative explanation connecting CMV and HIV persistence might be an increase in antigen-driven or bystander clonal expansion of HIV-infected lymphocytes. Also, the relationship between CMV replication and slower HIV decay might be that CMV enhances the infection of longer-lived cell types (like central memory cells), which decay more slowly than effector T cells (27). Both these hypotheses need to be tested in future studies. On the other hand, high levels of EBV DNA were associated with increased HIV usRNA, suggesting that EBV might be associated with increased HIV transcription during suppressive ART.

This study had a number of limitations. Most importantly, because this was an observational study, we cannot establish a definitive causal relationship between CMV and EBV reactivation and HIV DNA levels, and the extent of immune activation during treated HIV infection could be a determinant of both HHV shedding and slower HIV DNA decay. Similarly, slower HIV DNA decay might be the cause of increased CMV shedding. However, participants with and without CMV replication presented similar baseline characteristics, including peak HIV RNA, pre-ART HIV DNA levels, and rates of EBV DNA replication in PBMC, arguing against generalized inflammation within the detectable-CMV group.

It is not clear if the observed increase in HIV DNA levels in $CD4^+$ T cells represents replication-competent latent provirus or which cellular subset carries the integrated HIV DNA in relation to detectable CMV DNA. While previous work suggested that CMV-specific cells are not preferentially infected with HIV DNA, compared to HIV-infected cells (28, 29), their relative contribution to the maintenance of the HIV reservoir during long-term suppressive ART has not been evaluated. It is conceivable that continuous stimulation of a CMV-specific immune response by persistent subclinical replication (while HIV RNA is suppressed with ART) might lead to a preferential expansion of these populations. Alternatively, bystander inflammation (often seen during CMV infection) (24) could sustain a proliferation of HIV-infected $CD4^+$ T cells or enhanced infection of longer-lived cell types (27). It is also not clear if the presence of detectable CMV DNA in PBMC is a surrogate of low-level CMV replication or just latent CMV DNA present in circulating monocytes. Another limitation is that our study only evaluated levels of cellular HIV RNA transcription in PBMC, and we cannot exclude that a difference in cellular transcription might exist in another compartment (e.g., lymph tissue).

Despite these limitations, this study provides important insights regarding connections between asymptomatic HHV replication and the HIV DNA levels and complements our previous findings by providing longitudinal data on a well-characterized cohort of individuals during early HIV infection. Specifically, it demonstrated that even among individuals who started ART early during HIV infection, asymptomatic CMV reactivation was associated with slower HIV DNA decay. Additionally, it demonstrated that this effect was not unique to CMV but might be also associated with detection of EBV, although the mechanisms are likely different. Since almost all HIV-infected individuals and especially HIV-infected MSM are infected with multiple HHV (12), future studies are needed to determine if persistent CMV and EBV replication could be targeted as a strategy to reduce the size of the latent HIV reservoir. Further, since our study only evaluated CMV and EBV, future studies should evaluate if other persistent coinfecting viruses might also promote HIV DNA persistence.

ACKNOWLEDGMENTS

We are grateful to all the participants in the San Diego Primary Infection cohort (SD PIC), the CFAR Genomic, Translational Virology and Flow Cytometry Cores. We also acknowledge Susanne May for great input and revision of the statistical analysis. The HIV RNA quantification standard was obtained through the NIH AIDS Research and Reference Reagent Program, DAIDS, NIAID: HIV VQA RNA Quantification Standard from the DAIDS Virology Quality Assurance Program. The primer and probe for quantification of herpesviruses as well as the plasmids and quantification standards were kindly provided by Fred Lakeman and Rich Whitley.

S. Gianella participated in the study design, performed the laboratory experiments, participated in the data analyses for this study and wrote the primary version of the manuscript; C. M. Anderson performed the primary data analysis and wrote the primary version of the manuscript; S. R. Var performed laboratory experiments and participated in data analysis; S. M. Lada, M. V. Vargas, M. F. Oliveira, and M. Massanella performed laboratory experiments and participated in data analysis; D. D. Richman, D. M. Smith, and S. J. Little enrolled participants, collected and archived the samples, participated in the study design and data analyses, and revised the manuscript; and J. Pérez-Santiago and M. C. Strain participated in the study design and data analyses and revised the manuscript. All authors read and approved the final manuscript.

S. Gianella, C. M. Anderson, S. R. Var, S. M. Lada, M. V. Vargas, M. F. Oliveira, J. Pérez-Santiago, M. C. Strain, and M. Massanella do not have any commercial or other associations that might pose a conflict of interest. D. D. Richman has served as a consultant for Chimerix, BMS, Gilead, Gen-Probe, and Monogram. D. M. Smith has received grant support from ViiV Pharmaceuticals and consultant fees from Gen-Probe and Testing Talent Services. S. J. Little has received grant support from Gilead.

FUNDING INFORMATION

Creative and Novel Ideas in HIV Research (CNIHR) provided funding to Sara Gianella under grant number P30-AI027763. HHS | National Institutes of Health (NIH) provided funding to Douglas D. Richman under grant numbers AI036214, AI007384, and CARE U19 AI096113. HHS | National Institutes of Health (NIH) provided funding to Sara Gianella under grant numbers 5UM1AI068636-09 and CTRI UL1TR000100. HHS | NIH | National Institute of Allergy and Infectious Diseases (NIAID) provided funding to Davey M. Smith under grant number AI100665. HHS | NIH | National Institute on Drug Abuse (NIDA) provided funding to Davey M. Smith under grant number DA034978. Foundation for the National Institutes of Health (FNIH) provided funding to Susan J. Little under grant number R24AI106039. HHS | NIH | National Institute of Mental Health (NIMH) provided funding to Davey M. Smith under grant number MH097520.

This work was supported primarily by a grant from the National Institutes of Health through the University of California, San Francisco-Gladstone Institute of Virology & Immunology Center for AIDS Research (P30-AI027763; CNIHR grant to Sara Gianella) and by the Department of Veterans Affairs, the James B. Pendleton Charitable Trust, and additional grants from the National Institutes of Health: AI100665, MH097520, DA034978, AI036214, AI007384, UL1TR000100, MH101012, R24AI106039, CARE U19 AI096113, and 5UM1AI068636-09. This work was also supported by amfAR grant 108537 with support from the Foundation for AIDs Research (FAIR). Sara Gianella received support from Interdisciplinary Research Fellowship in NeuroAIDS grant R25-MH081482, awarded to Mariana Cherner. Marta Massanella and Michelli F. Oliveira were supported by Beatriu de Pinos (AGAUR) and CNPq-Brazil postdoctoral fellowships, respectively. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

REFERENCES

1. **Richman DD, Margolis DM, Delaney M, Greene WC, Hazuda D, Pomerantz RJ.** 2009. The challenge of finding a cure for HIV infection. Science **323:**1304–1307. http://dx.doi.org/10.1126/science.1165706.
2. **Buzón MJ, Massanella M, Llibre JM, Esteve A, Dahl V, Puertas MC, Gatell JM, Domingo P, Paredes R, Sharkey M, Palmer S, Stevenson M, Clotet B, Blanco J, Martinez-Picado J.** 2010. HIV-1 replication and immune dynamics are affected by raltegravir intensification of HAART-suppressed subjects. Nat Med **16:**460–465. http://dx.doi.org/10.1038/nm.2111.
3. **Chun TW, Nickle DC, Justement JS, Large D, Semerjian A, Curlin ME, O'Shea MA, Hallahan CW, Daucher M, Ward DJ, Moir S, Mullins JI, Kovacs C, Fauci AS.** 2005. HIV-infected individuals receiving effective antiviral therapy for extended periods of time continually replenish their viral reservoir. J Clin Invest **115:**3250–3255. http://dx.doi.org/10.1172/JCI26197.
4. **Chomont N, El-Far M, Ancuta P, Trautmann L, Procopio FA, Yassine-Diab B, Boucher G, Boulassel MR, Ghattas G, Brenchley JM, Schacker TW, Hill BJ, Douek DC, Routy JP, Haddad EK, Sekaly RP.** 2009. HIV reservoir size and persistence are driven by T cell survival and homeostatic proliferation. Nat Med **15:**893–900. http://dx.doi.org/10.1038/nm.1972.
5. **Hunt PW.** 2012. HIV and inflammation: mechanisms and consequences. Curr HIV/AIDS Rep **9:**139–147. http://dx.doi.org/10.1007/s11904-012-0118-8.
6. **Hatano H.** 2013. Immune activation and HIV persistence: considerations for novel therapeutic interventions. Curr Opin HIV AIDS **8:**211–216. http://dx.doi.org/10.1097/COH.0b013e32835f9788.
7. **Massanella M, Fromentin R, Chomont N.** 16 November 2015. Residual inflammation and viral reservoirs: alliance against an HIV cure. Curr Opin HIV AIDS http://dx.doi.org/10.1097/COH.0000000000000230.
8. **Dan JM, Massanella M, Smith DM, Spina CA, Schrier R, Daar ES, Dube MP, Morris SR, Gianella S.** 25 January 2016. Effect of cytomegalovirus and HIV transcription on CD57 and PD-1 T cell expression during suppressive ART. Acquir Immune Defic Syndr http://dx.doi.org/10.1097/QAI.0000000000000936.
9. **Gianella S, Massanella M, Wertheim JO, Smith DM.** 2015. The sordid affair between human herpesvirus and human immunodeficiency virus. J Infect Dis **212:**845–852.
10. **Gianella S, Massanella M, Richman DD, Little SJ, Spina CA, Vargas MV, Lada SM, Daar ES, Dube MP, Haubrich RH, Morris SR, Smith DM, California Collaborative Treatment Group 592 Team.** 2014. Cytomegalovirus replication in semen is associated with higher levels of proviral HIV DNA and CD4+ T cell activation during antiretroviral treatment. J Virol **88:**7818–7827. http://dx.doi.org/10.1128/JVI.00831-14.
11. **Gianella S, Smith DM, Vargas MV, Little SJ, Richman DD, Daar ES, Dube MP, Zhang F, Ginocchio CC, Haubrich RH, Morris SR, Team C.** 2013. Shedding of HIV and human herpesviruses in the semen of effectively treated HIV-1-Infected men who have sex with men. Clin Infect Dis **57:**441–447. http://dx.doi.org/10.1093/cid/cit252.
12. **Gianella S, Morris SR, Anderson C, Spina CA, Vargas MV, Young JA, Richman DD, Little SJ, Smith DM.** 2013. Herpesviruses and HIV-1 drug resistance mutations influence the virologic and immunologic milieu of the male genital tract. AIDS **27:**39–47. http://dx.doi.org/10.1097/QAD.0b013e3283573305.
13. **Gianella S, Anderson CM, Vargas MV, Richman DD, Little SJ, Morris SR, Smith DM.** 2013. CMV DNA in semen and blood is associated with higher levels of proviral HIV DNA. J Infect Dis **207:**898–902.
14. **Gianella S, Strain MC, Rought SE, Vargas MV, Little SJ, Richman DD, Spina CA, Smith DM.** 2012. Associations between virologic and immunologic dynamics in blood and in the male genital tract. J Virol **86:**1307–1315. http://dx.doi.org/10.1128/JVI.06077-11.
15. **Little SJ, Frost SD, Wong JK, Smith DM, Pond SL, Ignacio CC, Parkin NT, Petropoulos CJ, Richman DD.** 2008. Persistence of transmitted drug resistance among subjects with primary human immunodeficiency virus infection. J Virol **82:**5510–5518. http://dx.doi.org/10.1128/JVI.02579-07.
16. **Le T, Wright EJ, Smith DM, He W, Catano G, Okulicz JF, Young JA, Clark RA, Richman DD, Little SJ, Ahuja SK.** 2013. Enhanced CD4+ T-cell recovery with earlier HIV-1 antiretroviral therapy. N Engl J Med **368:**218–230. http://dx.doi.org/10.1056/NEJMoa1110187.
17. **Strain MC, Lada SM, Luong T, Rought SE, Gianella S, Terry VH, Spina CA, Woelk CH, Richman DD.** 2013. Highly precise measurement of HIV DNA by droplet digital PCR. PLoS One **8:**e55943. http://dx.doi.org/10.1371/journal.pone.0055943.
18. **Massanella M, Gianella S, Lada SM, Richman DD, Strain MC.** 2015. Quantification of total and 2-LTR (long terminal repeat) HIV DNA, HIV RNA and herpesvirus DNA in PBMCs. Bio Protoc http://www.bio-protocol.org/e1492.
19. Reference deleted.
20. **Schmid A, Gianella S, von Wyl V, Metzner KJ, Scherrer AU, Niederöst B, Althaus CF, Rieder P, Grube C, Joos B, Weber R, Fischer M, Günthard HF.** 2010. Profound depletion of HIV-1 transcription in patients initiating antiretroviral therapy during acute infection. PLoS One **5:**e13310. http://dx.doi.org/10.1371/journal.pone.0013310.
21. **Shan L, Rabi SA, Laird GM, Eisele EE, Zhang H, Margolick JB, Siliciano RF.** 2013. A novel PCR assay for quantification of HIV-1 RNA. J Virol **87:**6521–6525. http://dx.doi.org/10.1128/JVI.00006-13.
22. **Parrinello CM, Sinclair E, Landay AL, Lurain N, Sharrett AR, Gange SJ, Xue X, Hunt PW, Deeks SG, Hodis HN, Kaplan RC.** 2012. Cytomegalovirus immunoglobulin G antibody is associated with subclinical carotid artery disease among HIV-infected women. J Infect Dis **205:**1788–1796. http://dx.doi.org/10.1093/infdis/jis276.
23. **Gianella S, Morris SR, Tatro E, Vargas MV, Haubrich RH, Daar ES, Dube MP, Richman DD, Little SJ, Smith DM.** 2014. Virologic correlates of anti-CMV IgG levels in HIV-1 infected men. J Infect Dis **209:**452–456. http://dx.doi.org/10.1093/infdis/jit434.
24. **Freeman ML, Lederman MM, Gianella S.** 26 January 2016. Partners in crime: the role of CMV in immune dysregulation and clinical outcome during HIV infection. Curr HIV/AIDS Rep http://dx.doi.org/10.1007/s11904-016-0297-9.

25. **Hatano H, Jain V, Hunt PW, Lee TH, Sinclair E, Do TD, Hoh R, Martin JN, McCune JM, Hecht F, Busch MP, Deeks SG.** 2013. Cell-based measures of viral persistence are associated with immune activation and programmed cell death protein 1 (PD-1)-expressing CD4+ T cells. J Infect Dis **208:**50–56.
26. **Said EA, Dupuy FP, Trautmann L, Zhang Y, Shi Y, El-Far M, Hill BJ, Noto A, Ancuta P, Peretz Y, Fonseca SG, Van Grevenynghe J, Boulassel MR, Bruneau J, Shoukry NH, Routy JP, Douek DC, Haddad EK, Sekaly RP.** 2010. Programmed death-1-induced interleukin-10 production by monocytes impairs CD4+ T cell activation during HIV infection. Nat Med **16:**452–459. http://dx.doi.org/10.1038/nm.2106.
27. **Appay V, van Lier RA, Sallusto F, Roederer M.** 2008. Phenotype and function of human T lymphocyte subsets: consensus and issues. Cytometry A **73:**975–983.
28. **Casazza JP, Brenchley JM, Hill BJ, Ayana R, Ambrozak D, Roederer M, Douek DC, Betts MR, Koup RA.** 2009. Autocrine production of beta-chemokines protects CMV-specific CD4 T cells from HIV infection. PLoS Pathog **5:**e1000646. http://dx.doi.org/10.1371/journal.ppat.1000646.
29. **Douek DC, Brenchley JM, Betts MR, Ambrozak DR, Hill BJ, Okamoto Y, Casazza JP, Kuruppu J, Kunstman K, Wolinsky S, Grossman Z, Dybul M, Oxenius A, Price DA, Connors M, Koup RA.** 2002. HIV preferentially infects HIV-specific CD4+ T cells. Nature **417:**95–98. http://dx.doi.org/10.1038/417095a.