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Blood Biomarkers of Expressed and Inducible HIV-1

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Abstract

Objective—To define the relationships between molecular measures of viral persistence in blood (i.e., plasma viremia, cellular HIV-1 DNA, and mRNA) and expressed or inducible virus from resting CD4+T-cells of individuals on suppressive ART.

Design—We compared molecular measurements of HIV-1 in plasma and in uncultured peripheral blood mononuclear cells (PBMC) to the levels of virions produced by either unstimulated or phorbol myristate acetate and ionomycin (PMA/iono)-stimulated PBMC or resting CD4+T-cells from 21 donors on suppressive ART.

Results—We found that unstimulated virion release from cultured resting CD4+T-cells was positively correlated with the levels of plasma viremia in vivo (Spearman rho=0.67, p=0.0017). We also found that levels of both cellular HIV-1 DNA and unspliced HIV-1 mRNA per million uncultured PBMC were positively correlated with the levels of inducible virion release from both PMA/iono-stimulated PBMC (total HIV-1 DNA: rho=0.64, p=0.0017; unspliced HIV-1 RNA: rho=0.77, p<0.001) and PMA/iono-stimulated resting CD4+T-cells (total HIV-1 DNA: rho=0.75, p<0.001; unspliced HIV-1 RNA: rho=0.75, p<0.001).

Conclusions—These results show for the first time that there are strong associations between in vivo measures of HIV-1 persistence and ex vivo measures of spontaneous and inducible virus production from cultured PBMC and resting CD4+T-cells. Findings from this study provide insight into the biology of HIV-1 persistence and suggest methods to guide the evaluation of clinical strategies to reduce the size of the viral reservoir.

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Conflicts of interest: A.T., A.I., J.K., and J.P.M. are employees of Gilead Sciences. D.D.S. is a former employee of Gilead Sciences, and a current employee of Agenovir. S.S.W. is an employee of Quest Clinical Research. J.W.M. is a consultant for Gilead Sciences and owns shares of Co-crystal Pharma, Inc. All other authors declare no relevant conflicts of interest.

Keywords

HIV persistence; HIV reservoir; biomarkers of HIV persistence; persistent HIV viremia; suppressive antiretroviral therapy

Introduction

Antiretroviral therapy (ART) suppresses HIV-1 viral replication, which leads to rapid decreases in plasma viremia and concomitant increases in CD4+T-cell counts [1–4]. Quantifying the persistence of HIV-1 following the initiation of ART has been the subject of much investigation. Most studies have focused on evaluating molecular markers of HIV-1 persistence such as low-level plasma viremia, cellular proviral DNA, or cellular HIV-1 RNA. These studies have demonstrated that levels of viremia fall 4–5 orders of magnitude in the years after beginning ART, but that low levels of HIV-1 RNA (ca. 1 copy/ml) can remain detectable in plasma for 12 or more years after the start of therapy [5–8].

Other studies have focused on evaluating levels of total cellular HIV-1 DNA following the initiation of ART. Previous work has shown an approximately 10- to 15-fold decay of HIV-1 DNA over the first year [9–11]. Gandhi et al. also found that HIV-1 DNA levels continued to decline after 4 years on ART, with a half-life of approximately 13 years [11]. Cellular HIV-1 DNA persists to varying degrees in all subsets of CD4+T-cells, though predominantly in central memory CD4+T-cells [12, 13]. Levels of cellular HIV-1 DNA are lower in those who initiated ART during the primary infection stage, compared with chronic infection [9]; those who initiated ART at the earliest stage of infection (Fiebig I) have lower HIV-1 DNA levels than those who started later (Fiebig II–IV) [14]. Although multiple forms of HIV-1 DNA can be detected during suppressive ART, integrated DNA accumulates prior to the initiation of ART, and tends to dominate the pool of total HIV-1 DNA after long-term suppressive ART [15]. The large majority (>95%) of HIV-1 DNA in individuals on effective ART is defective due to insertions, deletions, or APOBEC-induced hypermutation [16].

Cellular HIV-1 RNA was initially used as a biomarker of progression to AIDS in untreated HIV-1 infection [17–20]. In contrast to the 10- to 15-fold decay in HIV-1 DNA that has been observed during the first 4 years of therapy, cellular HIV-1 RNA has recently been found to decay approximately 525-fold over the first 4 years of ART [11]. Early initiation of ART also leads to significantly lower levels of cellular unspliced HIV-1 RNA compared with patients who begin therapy during chronic infection [21].

In addition to molecular markers of HIV-1 persistence, a reservoir of cells carrying intact (replication-competent) and inducible proviruses persists despite ART at a frequency of approximately 1 cell per million resting CD4+T-cells [22–27], although higher frequencies have recently been reported [28]. The relationships between the HIV-1 latent reservoir and molecular measures of viral persistence have not been thoroughly elucidated. Most studies to date have evaluated the correlations between viral outgrowth assays (QVOA) and molecular measures. Viral outgrowth from resting CD4+T-cells has been reported to correlate with levels of integrated [29, 30] or total HIV-1 DNA in PBMC [30], the ratio of unspliced cellular HIV-1 RNA to DNA in gut-associated CD4+T-cells [29], and the levels of

plasma viremia by the single-copy assay (SCA) [31], but correlations have been inconsistent across studies. A broader assessment of molecular measures that includes both spontaneous and inducible virus production has yet to be performed. Ideally, simple molecular measures would be strongly predictive of the latent HIV-1 reservoir.

Toward this end, we compared molecular measures of cellular HIV-1 nucleic acids to each other and to levels of virion release from cultured PBMC and resting CD4+T-cells isolated from donors on suppressive ART. We hypothesized that the frequency of infected cells, and their transcriptional activity in PBMC, would be correlated with both unstimulated and inducible virion release from resting CD4+T-cells.

Materials and methods

Participant cohort and study samples

Selection criteria for donors in this cross-sectional study were HIV-positive individuals 18 years old on suppressive ART with plasma HIV-1 RNA <50 copies per milliliter for 1 year. Consecutive donors were enrolled by Quest Clinical Research (San Francisco, CA). All donors provided written informed consent for their participation. This study was approved by the Western Institutional Review Board and the Institutional Review Board at the University of Pittsburgh. PBMC and plasma were obtained by leukapheresis and plasmapheresis, respectively.

Isolation of PBMC and resting CD4+T-cells

PBMC were isolated from leukapheresis products by Ficoll-Hypaque density gradient centrifugation. Resting CD4+T-cells (i.e., CD3+CD4+CD25–CD69–HLA–DR–) were isolated by negative selection from PBMC using a custom kit from StemCell Technologies™ containing a cocktail of antibodies targeting CD14, CD16, CD19, CD20, CD25, CD36, CD56, CD69, CD66b, CD123, HLA-DR, and glycoporphin A. This negative selection routinely achieved >95% of cells with a resting CD4+T-cell phenotype by flow cytometric analysis [32]. PBMC were cryopreserved in 90% fetal bovine serum and 10% DMSO and stored in liquid nitrogen for downstream assessment of cellular HIV-1 DNA and RNA. Resting CD4+T-cells were stored at –80°C as cell pellets. Plasma was stored at –80°C for downstream assessment of low-level viremia.

Cell culture conditions

For all ex vivo measures of virus release, freshly isolated PBMC and resting CD4+T-cells were cultured in both unstimulated (i.e., medium only) and stimulated (i.e., stimulation for 7 days with 50 ng/ml phorbol myristate acetate [PMA] and 500 ng/ml ionomycin [iono]) conditions to assess virus release. PBMC were cultured at 15×10^6 cells per well, and the culture supernatant was harvested after 7 days of culture. Resting CD4+T-cells were cultured at 5×10^6 cells per well, and the supernatant was harvested after 6 days of culture. All cells were cultured in deep-well 96-well plates in 1.1 milliliters of RPMI 1640 without phenol red indicator, and contained 10% (volume/volume) fetal bovine serum and 0.6% (v/v) penicillin/streptomycin. To prevent viral propagation, 100 nM efavirenz and 100 nM elvitegravir were

included in the culture medium. Supernatants were stored at -80°C until assessment of virus release by qRT-PCR.

Assessment of virion release in culture supernatants

To assess unstimulated and stimulated virus release, levels of HIV-1 RNA were measured in harvested culture supernatants by qRT-PCR using the Roche COBAS AmpliPrep/TaqMan v2.0 (CAP/CTM) platform [32, 33]. Culture supernatants assessed using CAP/CTM have previously been shown to be free of contaminating HIV-1 DNA [34].

Quantification of low-level viremia

Low-level viremia was quantified using a large-volume adaptation of the SCA targeting the integrase region of the HIV-1 genome as previously described [35]. Briefly, nucleic acid was isolated from large volumes (18–40 ml) of plasma in parallel replicate extractions of ~4 ml. Nucleic acid was isolated by guanidinium hydrochloride/proteinase K incubation followed by guanidinium thiocyanate/glycogen incubation and precipitation of nucleic acid in 100% isopropanol. After washing in 70% ethanol, the nucleic acid was eluted in 5 mM Tris, DTT and RNasin, and cDNA was reverse transcribed, followed by qRT-PCR as previously described [35].

Quantification of cellular HIV-1 RNA and DNA

Total cellular HIV-1 DNA and unspliced cellular HIV-1 RNA were quantified as previously described [36]. Briefly, total nucleic acid was isolated from PBMC or resting CD4+T-cells, and the final extract was split into two aliquots. One aliquot was frozen at -80°C for downstream assessment of total HIV-1 DNA and *CCR5* to control for the total input cell number. The second aliquot was DNase treated to remove all DNA for the quantification of cellular unspliced HIV-1 RNA, and levels of the human gene *IPO8* were quantified to serve as an internal control for the recovery of cellular RNA. The primers and probe used to quantify cellular HIV-1 DNA and RNA were identical to those used for large-volume SCA.

Statistical analysis

All figures (excluding the correlogram) were generated using Prism 6 for Mac OS X. The correlogram was generated in R [37] using the ggplot2 [38] and gplots [39] packages. Spearman's correlation was used to test the significance of all correlations, and the results were corrected for multiple comparisons by setting the false discovery rate at 5%. The limit of detection for large-volume SCA depended on the input volume of plasma assayed: the limit of quantification of the assays for cellular HIV-1 DNA and RNA was one copy of nucleic acid each, and the limit of quantification of the Roche CAP/CTM was 20 copies per milliliter of culture supernatant. Values below the limit of detection of these assays were interpolated as 50% of the limit of detection, and interpolated values were used in all analyses. All statistical analyses were two-sided, and $\alpha < 0.05$ was considered significant.

Results

Study participant characteristics

Table 1 shows characteristics of the donors ($n = 21$). The median age of the participants was 54 years old (interquartile range (IQR): 52–58 years). All participants had CD4+T-cell counts above 500 cells/mm³ (median 725 cells/mm³; IQR: 607–796 cells/mm³); in percentages, a median of 34% of lymphocytes were CD4+T-cells (IQR: 27–40%). Median nadir CD4+T-cell count was 187 (IQR: 92–277) cells/mm³. At the time of the study, all participants were on suppressive ART regimens with HIV-1 RNA in plasma <50 copies per milliliter for 1 (median: 12; IQR: 6–16) years. Median low-level plasma viremia was 1.1 (IQR of 0.05–2.9) HIV-1 RNA copies per milliliter in the 19 participants for which sufficient plasma was available for large-volume iSCA. The median level of total cellular HIV-1 DNA was 283 (IQR: 160–504) copies per 10⁶ PBMC, and the median level of cellular unspliced HIV-1 RNA was 37 (IQR: 13–74) copies per 10⁶ PBMC. The median level of total cellular HIV-1 DNA in resting CD4+T-cell was 1159 (IQR: 547–2378) copies per 10⁶ cells. Results for molecular measures of viral persistence and culture-based measures of virion production are included in supplementary Table 1. Purified resting CD4+T-cells were stored as cell pellets at –80°C, which is not adequate for RNA preservation, thus cellular HIV-1 RNA could not be accurately quantified in resting CD4+T-cells.

Overall assessment of relationships between variables

Figure 1 shows the relationships among 13 different continuous variables using Spearman's correlations for 78 comparisons. We constructed a correlogram to display all the data and relationships simultaneously (data included in the correlogram are also shown in Table 1 and in supplementary Table 1). The bottom half of the correlogram shows scatter plots of the individual variables; the top half is a heat map quantifying the strength of the correlations between variables. Red on the heat map indicates a strong positive correlation, blue represents a strong negative correlation, and white indicates that no correlation was detected. Asterisks highlight the statistically significant relationships between variables at a false discovery rate (FDR) of 5%. In total, 18 of the 78 comparisons were statistically significant. Some variables showed a high degree of interrelatedness, including the frequency of infected cells in PBMC (i.e., total cellular HIV-1 DNA), levels of transcriptional activity in PBMC (i.e., cellular unspliced HIV-1 RNA), spontaneous virus release from resting CD4+T-cells, and inducible virus release from resting CD4+T-cells.

Spontaneous virion release ex vivo correlates with low-level viremia in vivo

We compared low-level viremia to levels of unstimulated virus release from cultured PBMC or resting CD4+T-cells isolated from peripheral blood (Figure 2). The median level of unstimulated virus release from PBMC was 36 (IQR: <20 to 164) HIV-1 RNA copies per milliliter of culture supernatant, whereas the median level of spontaneous virion release from resting CD4+T-cells was 72 (IQR: 27–126) copies per milliliter of culture supernatant. Surprisingly, we found that the in vivo levels of viremia were significantly correlated at the FDR of 5% with spontaneously released virions from resting CD4+T-cells ($\rho=0.67$, $p=0.0017$), and were nearly significantly correlated with spontaneously released virions from cultured PBMC ($\rho=0.50$, $p=0.098$) at this FDR level (without correcting for multiple

comparisons by FDR, this correlation was statistically significant with $\rho=0.50$, $p=0.030$). These results suggest that the spontaneous release of virions from cells obtained from blood may reflect overall virion release and clearance in vivo.

Cellular HIV-1 DNA and unspliced HIV-1 RNA are strongly correlated

We next investigated whether the frequency of infected cells in PBMC was related to their transcriptional activity (Figure 3). Consistent with recently published findings^[36], we found a strong positive correlation ($\rho=0.78$, $p<0.001$) between the levels of total HIV-1 DNA in PBMC and levels of unspliced cellular HIV-1 RNA. These data indicate that a higher frequency of infected cells is associated with higher levels of HIV-1 transcriptional activity.

Unstimulated and stimulated virus release are correlated with cellular HIV-1 DNA and RNA

We next sought to assess whether levels of unstimulated virus release from PBMC and resting CD4+T-cells were related to the frequency of infection and transcriptional activity of infected cells in PBMC (Figure 4). In the 21 study participants evaluated, we found that levels of both total cellular HIV-1 DNA per million PBMC and cellular unspliced HIV-1 RNA per million PBMC were related to unstimulated virus release from PBMC (total HIV-1 DNA: $\rho=0.55$, $p=0.010$; unspliced HIV-1 RNA: $\rho=0.64$, $p=0.0016$). Levels of cellular HIV-1 DNA and RNA in PBMC were also correlated with unstimulated virus release from resting CD4+T-cells (total HIV-1 DNA: $\rho=0.69$, $p<0.001$; unspliced HIV-1 RNA $\rho=0.55$, $p=0.010$). These findings indicate that a higher frequency and a higher level of transcriptional activity of infected cells are associated with higher levels of spontaneous virus release. The frequency of infected resting CD4+T-cells was also significantly associated with spontaneous virion release from PBMC ($\rho=0.73$, $p<0.001$) and from resting CD4+T-cells ($\rho=0.66$, $p=0.0019$) (Figure 1).

We next evaluated whether the frequency of infected cells and transcriptional activity in PBMC were related to induced virus release from PBMC and resting CD4+T-cells stimulated with PMA/iono (Figure 5). The median level of HIV-1 RNA released for stimulated PBMC was 1633 (IQR: 292–6729) copies of HIV-1 RNA per milliliter of culture supernatant, whereas resting CD4+T-cells released a median of 4329 (IQR: 1042–8948) copies of HIV-1 RNA per milliliter of culture supernatant following stimulation. This analysis showed that the frequency of infected cells is related to the level of inducible virus release from both PBMC ($\rho=0.64$, $p=0.0017$) and resting CD4+T-cells ($\rho=0.75$, $p<0.001$). Levels of viral transcription per million PBMC were also positively and significantly correlated with stimulated virus release from PBMC ($\rho=0.77$, $p<0.001$) and resting CD4+T-cells ($\rho=0.75$, $p<0.001$). The frequency of infection of resting CD4+T-cells was also correlated with inducible virion release from PBMC ($\rho=0.53$, $p=0.020$) and resting CD4+T-cells ($\rho=0.71$, $p<0.001$) (Figure 1).

Discussion

To date there have been no published studies that include an integrated assessment of molecular measures of viral persistence in vivo and both spontaneous and stimulated virus release ex vivo. We therefore evaluated in vivo measures of persistence and ex vivo

measures of virus release in donors on suppressive ART. We found that levels of total cellular HIV-1 DNA and cellular unspliced HIV-1 RNA in PBMC were strongly associated with each other, and with unstimulated and stimulated virus release from PBMC and resting CD4+T-cells. We also discovered that unstimulated virus release *ex vivo* from resting CD4+T-cells was correlated with the level of persistent plasma viremia *in vivo*, and that there was a positive association (albeit not statistically significant with a FDR of 5%) between spontaneous release of virions from PBMC and persistent plasma viremia.

Longitudinal single genome sequencing of plasma viral HIV-1 RNA from before the initiation of ART and after years of suppressive ART has revealed no evidence of continued viral evolution [40], demonstrating that ART is largely able to stop viral replication. However, low-level viremia remains detectable on long-term suppressive therapy at a level of approximately 1 copy per milliliter of plasma after up to 7 years of viral suppression [7], regardless of the ART regimen used [41]. The source of this low-level viremia is contentious, and data related to a quantitative relationship between low-level viremia and the size of the viral reservoir are sparse and conflicting [29, 31]. In the current study, we found that levels of unstimulated virus release from resting CD4+T-cells cultured *ex vivo* are significantly correlated with levels of persistent viremia *in vivo* when using large volumes of plasma to accurately quantify viremia. This connection between unstimulated virus release and low-level viremia establishes a link between an *in vivo* measure of active virus release, and an *ex vivo* measure of spontaneous release of virions from resting CD4+T-cells. Although statistical significance was not reached (after correcting for multiple comparisons) between persistent viremia and spontaneous virion release from PBMC, there was a trend towards a positive association. This raises the intriguing possibility that spontaneous virion release from PBMC may be reflective of the balance between virion production and clearance *in vivo*. Future studies will need to evaluate the role effector cells (such as CD8+T-cells and natural killer cells) present in bulk PBMC play in clearance or inhibition of virion producing cells *ex vivo*, and the ways in which this is reflective of *in vivo* clearance of virus producing cells.

We also assessed whether the frequency of infected cells in PBMC was related to the level of viral transcription of unspliced cellular HIV-1 RNA. We found that the frequency of infected cells in PBMC was strongly related to the level of viral transcription in PBMC, which confirms a recently published finding from our group [36]. The frequency of infected, resting CD4+T-cells was also related to the level of total cellular HIV-1 DNA and RNA per million PBMC (Figure 1). These data show that there is proportionality between the frequency of infection and the level of viral transcription. Single-cell analysis will be needed to ascertain whether the source of cellular HIV-1 RNA is many cells transcribing small amounts of HIV-1 RNA, or a few cells transcribing high levels of HIV-1 RNA.

In the current study, we used total virus release from PMA/iono-stimulated PBMC and resting CD4+T-cells as the culture-based measure of latent HIV-1. The gold standard for assessing the size of the latent reservoir is the QVOA, but this assay has substantial barriers to widespread use, including the need for large numbers of resting CD4+T-cells, specialized culture medium, irradiated feeder cells from HIV-negative blood donors, target cells for the propagation of virus, and up to 28 days of co-culture [42]. Recent work has somewhat

simplified the QVOA method, and has suggested that QVOA wells containing virions with properly poly-adenylated virion-associated HIV-1 RNA become positive for p24 after extended culture [43]. Nevertheless, because of the complexity of QVOA, we quantified total, inducible virus release by RT-PCR quantification of HIV-1 RNA in culture supernatants as a simpler approach to measuring latent HIV-1 that is sensitive for virus-producing cells and has a large dynamic range ($3 \log_{10}$). Because the current study did not measure inducible, replication-competent virus production, future work should evaluate the relationship between measures of persistence and virion production described here, and infectious virus outgrowth as measured by the more time- and resource-intensive QVOA.

We found that the frequency and transcriptional activity of infected PBMC are both significantly correlated with inducible virus release from both resting CD4+T-cells and PBMC. These findings are striking, given our recent work showing that only 1.5% of proviruses detectable by qPCR in resting CD4+T-cells could be reactivated to produce virions with one round of stimulation [32]. Although it is well established that many of the proviruses present in resting CD4+T-cells are defective [44], our results suggest that there is a degree of proportionality between the number of infected cells and the amount of virus produced. Similarly, the transcription of HIV-1 RNA in infected cells is correlated with inducible virus release, which demonstrates a proportional relationship between transcription and virus release.

The finding that levels of HIV-1 RNA transcription in PBMC are correlated with virus release following stimulation is consistent with the report that rebound viremia can sometimes be genetically linked to cells transcribing viral RNA prior to the cessation of ART [45]. Work by Rothenberger et al. has also shown that there are many sites of viral expression in lymph nodes across the body during suppressive ART [46], which is in line with the idea that rebound virus may originate from cells that are already transcribing HIV-1 RNA. Alternatively, rebound virus could also come from latently infected cells undergoing stochastic reactivation. Modeling work by Hill et al. [47] has similarly suggested that actively transcribing cells are likely responsible for the rapid viral rebound following the cessation of ART in most people with HIV-1, whereas latently infected cells that have become reactivated are likely responsible for the late viral rebound observed in the Boston patients [48, 49] and the Mississippi baby [50–52].

Our findings are also consistent with recently published *in vivo* assessments of reservoir size in treatment interruption studies, in which Li et al. found that levels of unspliced cellular HIV-1 RNA in PBMC are associated with time to viral rebound [53]. Similarly, Williams et al. found that levels of total HIV-1 DNA in PBMC were associated with time to viral rebound [54].

Combining our results with the two *in vivo* studies described above of the time to viral rebound following ART interruption suggests that simple measures of the frequency of HIV-1 infected cells and their transcriptional activity by q(RT)-PCR in unfractionated and uncultured PBMC can be used to estimate spontaneous and inducible virus production in persons with HIV-1 on suppressive ART. Ultimately, prospective studies of intensively monitored ART cessation and time to viral rebound will be required to validate whether

simple measures of viral persistence in the periphery can accurately predict the size of the viral reservoir, and whether the relationships between molecular viral persistence and culture-based measures of virus production are maintained following interventions designed to reduce the size of the reservoir.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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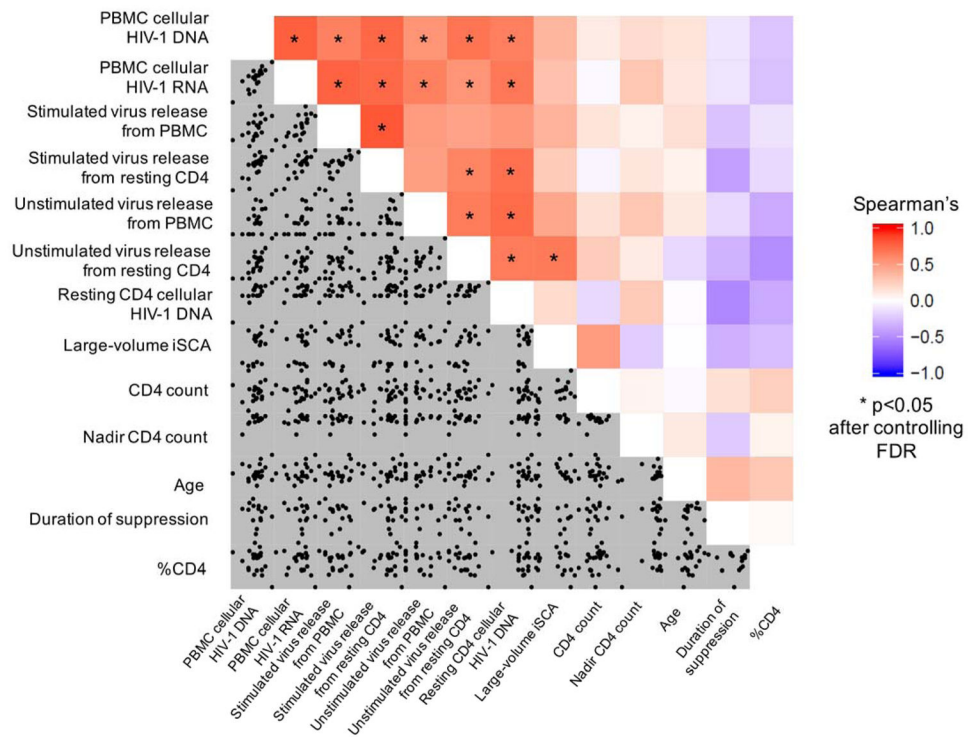


Fig. 1. Correlogram showing the interrelationships between the variables studied

Each of the 13 variables studied is listed on the x- and y-axes. The bottom left-hand portion of the correlogram shows scatter plots between the individual variables, and the top right-hand portion of the plots shows a heat map of correlations between variables. The key on the right-hand side shows the colors corresponding to strong positive correlations (red), strong negative correlations (blue), and neutral correlations (white). Asterisks indicate comparisons that were statistically significant by Spearman's correlation after correcting for multiple comparisons by maintaining a false discovery rate of 5%. In total, 18 of the 78 comparisons were statistically significant. Highly interrelated variables included total cellular HIV-1 DNA, cellular unspliced HIV-1 RNA, and virion release from stimulated resting CD4+T-cells.

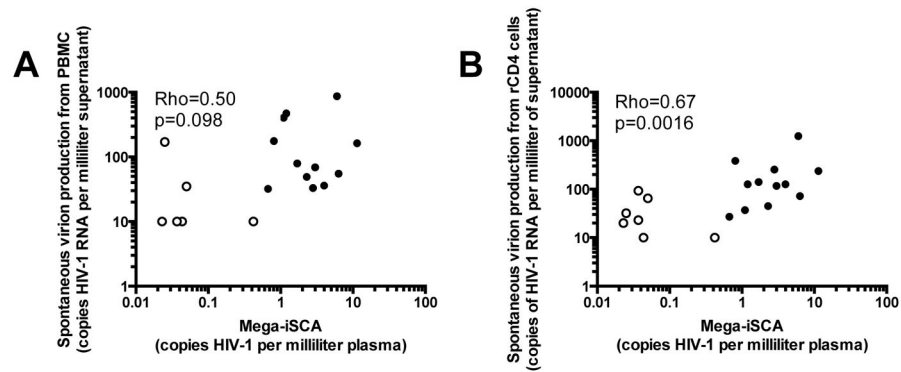


Fig. 2. Unstimulated virus release from cultured PBMC and resting CD4+T-cells correlates with persistent viremia on ART

(a) Unstimulated virus release from 15×10^6 PBMC following 5 days of culture correlated with levels of plasma viremia (HIV-1 RNA) measured by large-volume iSCA ($\rho=0.50$, $p=0.098$). (b) Similarly, unstimulated virus release from 5×10^6 resting CD4+T-cells after 7 days of culture correlated with the levels of plasma viremia by large-volume iSCA ($\rho = 0.67$, $p=0.0016$). In both (a) and (b), open symbols denote samples that had undetectable HIV-1 RNA by large-volume iSCA (interpolated at 50% of the limit of detection based on plasma volume assayed) or CAP/CTM qRT-PCR of culture supernatant (interpolated at 50% of the limit of detection, or 10 copies per milliliter of culture supernatant assayed).

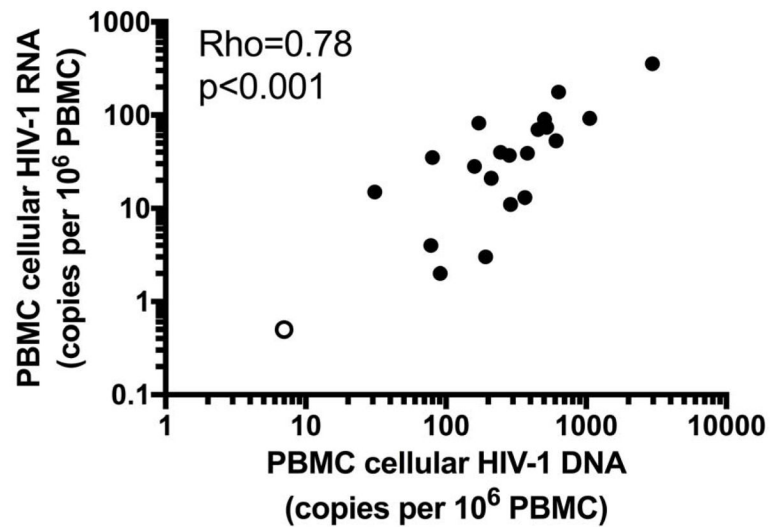


Fig. 3. The frequency of infected cells (cellular HIV-1 DNA) is correlated with HIV-1 transcriptional activity in PBMC (cellular HIV-1 RNA)
Levels of total cellular HIV-1 DNA were significantly correlated with basal levels of unspliced HIV-1 RNA in PBMC that were cryopreserved after processing the leukapheresis product. The open symbol denotes a sample with undetectable HIV-1 RNA, interpolated at 50% of the limit of detection, or 0.5 copies of HIV-1 RNA per million PBMC.

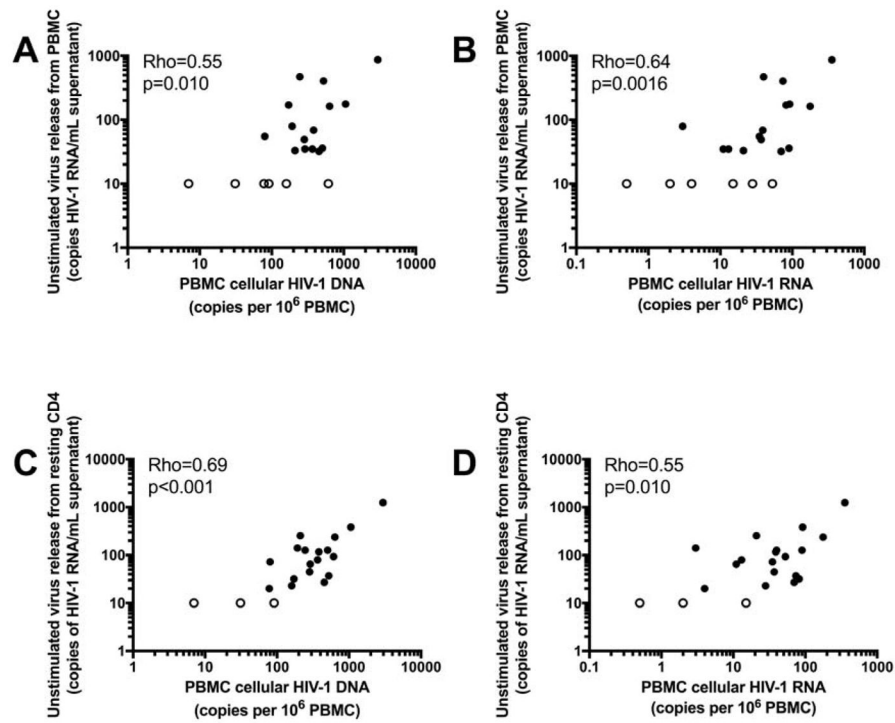


Fig. 4. Spontaneous virion release is correlated with the frequency of infected cells and their transcriptional activity in PBMC

(a) The frequency of infected cells (cellular HIV-1 DNA in PBMC) is correlated with the spontaneous release of virions from unstimulated cultured PBMC ($\rho=0.55$, $p=0.010$). (b) The level of unspliced cellular HIV-1 RNA transcription in PBMC is correlated with spontaneous virion release from PBMC ($\rho=0.64$, $p=0.0016$). (c) The frequency of infected cells in PBMC is correlated with spontaneous virion release from resting CD4+T-cells ($\rho=0.69$, $p<0.001$). (d) The level of unspliced cellular HIV-1 RNA transcription in PBMC is correlated with spontaneous virion release from resting CD4+ T cells ($\rho=0.55$, $p=0.010$). In (a), (b), (c), and (d), open circles represent samples that were undetectable by CAP/CTM qRT-PCR for spontaneous virion release, or one sample that had undetectable levels of cellular unspliced HIV-1 RNA. In all cases of non-detection, values were interpolated as 50% of the limit of detection of the assay (i.e., 10 copies per milliliter of supernatant for CAP/CTM and 0.5 copies of unspliced cellular HIV-1 RNA per million PBMC).

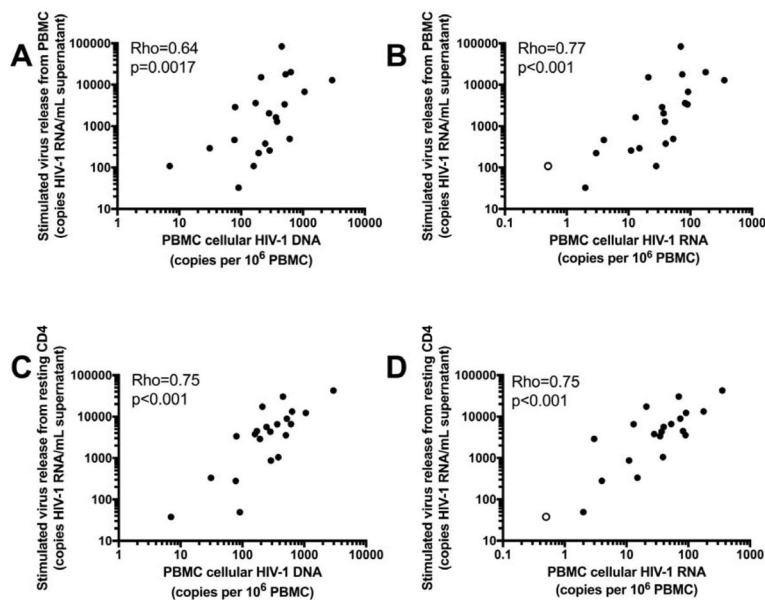


Fig. 5. The frequency of infected cells and their transcriptional activity in PBMC are correlated with the level of inducible virion release from PBMC and resting CD4+T-cells

(a) The frequency of infected cells in PBMC is correlated with the level of inducible virion release from cultured PBMC treated with PMA/iono for 5 days ($\rho=0.64$, $p=0.0017$). (b) The level of cellular unspliced HIV-1 RNA transcription is correlated with the level of inducible virion release from cultured PBMC ($\rho=0.77$, $p<0.001$). (c) The frequency of infected cells in PBMC was correlated with the level of inducible virion release from cultured resting CD4+T-cells treated with PMA/iono for 7 days ($\rho=0.75$, $p<0.001$). (d) The level of cellular unspliced HIV-1 RNA in PBMC is correlated with the level of inducible virion release from resting CD4+T-cells stimulated with PMA/iono ($\rho=0.75$, $p<0.001$). In (b) and (d), one sample (shown as an open circle) had undetectable levels of cellular unspliced HIV-1 RNA. The value of this sample was interpolated as 50% of the limit of detection of the assay, or 0.5 copies per million PBMC.

Table 1

Characteristics of study donors.

Participant ID	Age	Nadir CD4+T-cell count (cells/mm ³)	Duration of virologic suppression (years)	CD4+ T-cell count (cells/mm ³)	Percent CD4+ T-cells in lymphocytes
1	69	210	6.00	742	41.2
2	54	253	19	653	36.3
3	57	300	2	587	36.7
4	55	NA	NA	762	23.8
5	51	0	8	692	17.3
6	54	425	3	607	26.4
7	61	325	16	1022	29.2
8	58	NA	NA	632	23.4
9	50	NA	NA	1345	26.9
10	54	187	12	590	26.8
11	52	67	14	422	28.1
12	53	150	7	958	38.3
13	52	96	1	832	39.6
14	64	NA	NA	725	48.3
15	64	7	15	538	33.6
16	39	325	6	556	39.7
17	56	NA	NA	882	42
18	54	120	15	699	36.8
19	57	190	19	780	39
20	47	NA	NA	796	36.2
21	62	88	24	783	43.5
Median (Interquartile range)	54 (52–58)	187 (92–277)	12 (6–16)	725 (607–796)	34 (27–40)

NA, not available