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Concurrent Measures Of Total And Integrated HIV DNA Monitor Reservoirs And Ongoing Replication In Eradication Trials

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Introduction

Recent clinical reports have rekindled an interest in developing a cure for HIV [1–3]. This renewed interest is fueling several studies aimed at targeting treatment resistant reservoirs in patients with HIV [4–7]. While these studies have already yielded interesting and informative results, more work is needed to determine the efficacy of new therapeutic approaches in clinical trials. In many studies, measuring changes in reservoir size and detecting the onset of viral replication would help evaluate the effects of each therapeutic approach, their efficacy in individual patients, and the mechanisms involved in effecting a cure.

HIV persists in the cells of patients on antiretroviral therapy (ART), creating a reservoir that resurfaces if therapy is discontinued [8–3]. Currently, the infectious units per million (IUPM) assay remains the best accepted method to measure actual HIV reservoirs [14] but PCR measurements of either total or integrated cellular HIV DNA have been proposed as surrogate measures of reservoir size [6]. Although the IUPM assay is considered the gold-standard, this method requires large numbers of viable cells and has a high variance [14] making it difficult to reliably measure small differences in replication competent virus. Measurements of total and integrated HIV DNA require fewer cells and produce results with

smaller errors [15, 16]. However, these methods overestimate reservoir size because they include proviurses that do not contribute to viremia. Furthermore, whether the proportion of replication incompetent DNA is constant over time is unclear. Total HIV DNA further overestimates reservoir size because it includes unintegrated HIV DNA species with variable half lives. Therefore, it remains unclear whether total or integrated DNA would be a better surrogate for measuring the HIV reservoir and how these two measures differ in specific clinical settings.

During viral replication, most of the cell-associated HIV DNA is full-length, linear, and unintegrated [10]. Only a fraction of total HIV DNA is integrated and a smaller fraction still is capable of producing infectious virus [10]. After completion of reverse transcription, unintegrated HIV DNA accumulates in cells if integration does not occur. Since the majority of unintegrated HIV DNA may degrade and contributes little to viral production [17–20], measuring integrated HIV DNA could be a more reliable surrogate of replication-competent virus than measuring total HIV DNA.

It has been previously reported that untreated HIV+ patients (progressors and elite-suppressors) exhibit significantly different levels of total compared to integrated HIV DNA in PBMC [10, 15, 16]. The difference between these measures in patients with ongoing replication [21, 22] denotes that there is detectable unintegrated HIV DNA and that total and integrated HIV DNA are not interchangeable in the presence of ongoing replication. However, since the majority of unintegrated HIV DNA appears to have a short half-life in vitro, it is reasonable to expect that most of the HIV DNA will be integrated over time when replication is completely halted [23]. Therefore, if effective ART controls ongoing replication, the levels of total and integrated HIV DNA should be similar on ART because the majority of unintegrated HIV DNA should disappear over time [23, 24]. Thus, measures of total and integrated HIV DNA have been suggested to be interchangeable surrogates for the reservoir size in PBMC in patients on ART [23, 25, 26]. However, therapies that target reservoirs may induce ongoing replication making it important to determine which measure is a more reliable marker for reservoir size and how often measurements of total and integrated HIV DNA are unequal in patients on ART.

Here, we apply a unique, robust, and sensitive assay for measurements of total and integrated HIV DNA to patient samples from several cohorts in order to compare these surrogates for reservoir size in PBMC in different clinical settings. We evaluated samples from patients before and after ART initiation, samples from patients on effective ART (i.e. patients with < 50 copies/ml of plasma) longitudinally and cross-sectionally, and samples from a recent clinical trial aimed at targeting viral reservoirs. The results of the latter trial are reported in detail elsewhere (currently under review). Briefly, patients on stable ART with undetectable viremia (< 50 copies/ml of plasma) were treated with Peg-IFN-Alpha-2A (IFN- α) plus ART for 5 weeks followed by IFN- α monotherapy during a 12 week ART interruption. A fraction of patients in the study (9/20, 45%) maintained plasma viremia levels below 400 copies/mL while on IFN- α monotherapy for a period of 12 weeks after ART interruption and were termed “responders”. Previous attempts at structured treatment interruptions without concurrent immunotherapy had resulted in control of viremia at this level (<400 copies/ml) in less than 9% of patients, suggesting immunotherapy with IFN- α

may have affected reservoir size [27–29]. Here, we studied the relationship between measures of total and integrated HIV DNA in the responders after IFN- α monotherapy and found a decrease in the levels of integrated but not total HIV DNA. Furthermore, both measures together suggested residual HIV expression and de novo entry and reverse transcription had occurred during IFN- α monotherapy, suggesting an immune mechanism for the reduction in reservoir size. Therefore, measuring multiple viral intermediates may also be useful in other clinical trials aimed at reducing reservoir size.

Methods

Study Subjects/Samples

Frozen PBMC and DNA samples were obtained from subjects recruited by the Clinical Research Center, NIH (Bethesda, MD), the Hospital at the University of Pennsylvania (Philadelphia, PA), or as part of the IFN- α clinical trial (clinicaltrials.org/NCT00594880, Philadelphia, PA). Subjects signed informed consent forms approved by each Institutional Review Board (IRB). The University of Pennsylvania's IRB approved the transfer and use of materials to our laboratory for this research. Patient information was anonymized and all measures for confidentiality were followed. Table 1 summarizes patients' CD4 counts, viral loads, treatment regimens and months on therapy. DNA from participants in the IFN- α clinical trial was isolated using TRI Reagent (Sigma-Aldrich, St. Louis, MO) according to the manufacturer's instructions. For all other samples genomic DNA was extracted using the Blood and Cell Culture Maxi Prep Kit (Qiagen, Valencia, CA).

PCR Assays

Total and integrated HIV DNA assays were performed according to previously published methods [16, 30–33]. HIV specific primers directed at the RU5 region alone, were used to amplify all forms of HIV DNA, annotated here as total HIV DNA. For total HIV DNA measurements, DNA was assayed in duplicates or triplicates at two to four dilutions for RU5 depending on the quantity of cells. We performed qPCR for the cellular DNA sequences encoding albumin to determine the number of cells per ml of extracted DNA. These DNA assays were performed in triplicates at 3-dilutions to rule out the presence of inhibitors. HIV DNA primers designed to detect host-genome repetitive motifs (*Alu*) were paired with HIV-*gag* specific primers to specifically quantify integrated HIV DNA in patient samples as described [30–33]. Our integration standard controls for differences in PCR amplification caused by variations in the distance between the integration sites and host-genome motifs. Thus, we can directly measure total and integrated forms and indirectly detect unintegrated HIV DNA by comparing the levels of total to those of integrated HIV DNA. If a significant difference between total and integrated HIV DNA was detected, we then assayed the samples for 2-LTR circles. Our 2-LTR assay detects 1.4 copies in 150,000 genomes 77% of the time, with a coefficient of variation of <0.30 [16]. 2-LTR assays were negative in all samples measured here. For the 2-LTR assay, DNA samples were measured using 1×10^6 cells in duplicate wells and 3.3×10^5 cells in two additional duplicate wells.

Low-level Plasma Viral Load (Residual viremia)

Individual collaborators measured the levels of plasma RNA in patient samples using previously described methods [34–36].

Statistical Analysis

To determine the ratio at which measures of total and integrated HIV DNA are significantly different (i.e. unintegrated HIV DNA is detectable), we repeatedly measured total and integrated HIV DNA using an “integration only” standard diluted to 100 HIV DNA copies per million genomes. We measured the levels of total and integrated HIV DNA in our standard 7 times (42 *Alu-gag* replicates per integration measurement, and 10 replicates at three dilutions for each measurement of total HIV DNA), and calculated the possible ratios of all measured combinations [15]. In other words, we determined the error in our measurements of total and integrated HIV DNA and the error in the ratio of the two values. Using the most conservative estimates, we calculated that a significant excess would be present when the ratio is greater than 2.1 (i.e. 2.58 standard deviations above the mean of ratios measured in seven independent experiments, $p=0.01$). This ratio conservatively provides the threshold when unintegrated HIV is present as long as the level of integration is greater than 0.0001 HIV copies per PBMC, which was the case in all tested samples.

Spearman coefficients were calculated to evaluate the correlations between levels of total and integrated HIV DNA and low-level viremia (residual HIV). Differences between total and integrated HIV DNA between patients with $VL < 1$ or > 1 copies/ml were determined using a two-tailed Wilcoxon sum rank test. Differences between time points for viremia, total and integrated HIV DNA in patients from the IFN- α study were determined using a two-tailed Wilcoxon signed rank test.

Results

In order to confirm that the majority of unintegrated HIV DNA would disappear upon ART initiation [23], we analyzed longitudinal samples from patients starting ART and calculated the ratio of total to integrated HIV DNA at each time-point (Fig. 1a). A ratio greater than 2.1 denotes a statistically significant difference between total and integrated HIV DNA levels, indicating the presence of detectable unintegrated HIV DNA as explained in the methods and [15]. The calculated ratio of total to integrated HIV DNA in two patients starting ART exhibited a decline after ART initiation and approached 1 within 12 months, consistent with previously published results [23, 25]. We found a significant excess of total HIV DNA at time 0 and 6 months post ART initiation, indicating that there is detectable unintegrated HIV DNA. We also found a significant reduction in integrated HIV DNA 6 months after starting ART in both patients ($p=5.01 \times 10^{-7}$ and 1.18×10^{-6} respectively). We interpret this ~2-fold change to represent the reduction in the frequency of activated CD4+ cells that contain higher levels of integrated HIV that occurs with the initiation of ART.

Next, we measured both total and integrated HIV DNA longitudinally in 4 well suppressed patients on ART (Fig. 1b). As predicted, three of four patients showed consistently similar levels of total and integrated HIV DNA. In contrast, one patient had detectable unintegrated

HIV DNA (ratio of 43) coinciding with an episode of viremia (Fig. 1b, 822 HIV RNA copies per ml of plasma on 1/03). In this patient, viremia was undetectable (<50 copies/ml) when sampled twice before the next apheresis (arrows). At this apheresis there was detectable unintegrated HIV DNA (ratio of 218), in the absence of plasma viremia (Fig. 2b, 8/03). Notably, we did not detect 2-LTR circles during either episode of unintegrated HIV DNA so the persistence of circular DNA does not appear to explain the excess (or apparent long half-life) of unintegrated HIV DNA in this patient. Overall, the data from these patients suggests measures of total and integrated HIV DNA are often similar in patients that are well suppressed on ART, whereas viremic episodes correlate with an increase in the ratio of total to integrated HIV DNA, consistent with the appearance of unintegrated HIV DNA.

We then investigated the association between the residual viremia (<50 copies) and total or integrated HIV DNA as well as the correlation of residual viremia with the presence of unintegrated HIV DNA in samples from a different, cross-sectional cohort of patients on ART who had undetectable viremia (<50 copies/ml) by standard methods (Fig 2). This cohort consisted of 10 patients with residual viremia (<50 copies per ml of plasma) and 10 patients with undetectable viremia (<1 copy per ml of plasma). There was significantly more total ($p=0.02$) and integrated ($p=0.02$) HIV DNA in patients with low level viremia compared to those with undetectable viral load, suggesting that higher integration levels may be associated with higher low-copy plasma viral loads in patients on ART (Fig. 2a,b). Furthermore, when the viral load was >1 but <50, there was a correlation between residual plasma viral load and integrated HIV DNA ($\rho=0.62$, $p=0.03$), but not with total HIV DNA ($\rho=0.06$, $p=.44$), (Fig. 2 c,d). Finally, if we included all of the patients with undetectable viral loads (data not shown), a significant correlation existed between viral load and integrated HIV DNA ($\rho=0.61$, $p=0.002$) and between viral load and total ($\rho=.53$, $p=0.008$), as suggested by others [36, 37]. Thus, integration is more strongly correlated with low level viremia on ART than total HIV DNA measurements.

There was no significant difference in the ratio of the levels of total over integrated HIV DNA in the presence and absence of viremia (Fig. 2e). These results suggest integration measures correlate better with residual viremia and there are different mechanisms associated with viral blips and the presence of unintegrated HIV DNA. For example, a viral blip may reflect stimulation of transcription of the viral reservoir while a new excess of unintegrated HIV DNA likely indicates at least one new round of viral entry and reverse transcription.

Finally, we measured both total and integrated HIV DNA in an IFN- α trial to determine the relevance of quantifying both intermediates in a clinical setting. The results of the clinical trial are detailed elsewhere [38]. Briefly, 23 HIV-infected subjects, virologically suppressed on ART (CD4 count >450 cells/ml) were randomized to receive either 90 or 180 μ g of IFN- α for 5 weeks while receiving ART and then for up to 12 weeks during ART interruption. Study endpoints included rebounding viremia (>400 copies/ml), adverse effects, or completion of the 12 weeks off ART. Total and integrated cellular HIV DNA and plasma viremia (<50 copies/ml) were measured while receiving conventional ART (labeled On ART), after 5 weeks of adding IFN- α to the regular ART regimens (labeled ART and IFN), and after they received IFN- α monotherapy (IFN only) for the next 12 weeks. Since only 9

of 20 primary end-point participants were able to stay on IFN- α monotherapy for 12 weeks while off ART (termed responders), we only measured both HIV intermediates in those patients. Additionally, we had insufficient DNA sample to measure integration in 2 responders, leaving us with 7 patients to report herein. In responders, the levels of integrated HIV DNA decreased during therapy with IFN- α (as described elsewhere), while measurements of total HIV DNA did not decrease over time (Fig. 3). Furthermore, treatment with IFN- α (either alone or with ART) led to an increase in the number of patients with a ratio of total to integrated HIV DNA greater than 2.1 (Fig. 4), suggesting that IFN- α treatment resulted in increased low level replication.

Discussion

The mechanisms underlying the maintenance of HIV reservoirs in patients treated with effective ART are the subject of intense study [6, 39–47]. Here we provide data to suggest that measuring integrated HIV DNA may serve as a more reliable surrogate of reservoir size than measuring total HIV DNA in certain patient populations, especially if ongoing replication occurs. In addition, we suggest that analysis of total and integrated HIV DNA in combination with plasma viremia levels may contribute to our understanding of reservoir maintenance and help determine the mechanisms of action in trials aimed at targeting viral reservoirs.

Current dogma suggests that measuring total HIV DNA should be sufficient to monitor HIV cellular reservoirs given the short half-life of the predominant form of unintegrated HIV DNA. As such, it is reasonable to assume that accurate measurements of total and integrated HIV DNA would eventually become equal in most patients starting antiretroviral therapy. While our data is consistent with this hypothesis (Figure 1A), it appears as though it may take more than six months for the majority of unintegrated HIV DNA to disappear in patients starting antiretrovirals for reasons that remain to be determined [23, 48]. There are at least two potential explanations for the slow decay of unintegrated HIV DNA: (1) the *in vivo* half-life of unintegrated HIV DNA maybe much longer than expected from *in vitro* experiments [23] and (2) ongoing replication may persist during the initial period of therapy due to insufficient drug levels in sanctuary sites [48–50] or intermittent adherence to therapy. In either case, cells with unintegrated DNA may migrate to the peripheral blood from other anatomical sites [48–50]. Thus, an excess of unintegrated HIV DNA in the absence of plasma viremia may be seen if cells from these other sites traffic into circulation carrying unintegrated HIV DNA. Notably, the half-life of circular forms also continues to be debated, but is clearly longer than for unintegrated HIV DNA [51–57]. Understanding why unintegrated HIV DNA decays slower *in vivo* than *in vitro* after initiating ART may lead to a better understanding of reservoir persistence.

We were next interested in how often total and integrated HIV DNA were different over time in patients on long term ART. Our data suggest that in most well controlled patients, total and integrated HIV DNA were similar at many time points (Figure 1b). Interestingly, we were able to capture a difference in total and integrated HIV DNA at the same time an episode of detectable plasma viremia appeared. This indicates concurrent measurements of total and integrated HIV DNA may help identify periods of viral replication in patients on

ART. Consistent with our previous results (Figure 1a), unintegrated HIV DNA was still detected approximately four months after plasma viremia returned to undetectable levels. This presence of unintegrated HIV DNA may support the notion that unintegrated HIV could have a prolonged half-life *in vivo* perhaps related to the presence of circular forms. Alternatively, it may reflect a second burst of viral replication that was not detected by monitoring plasma viremia every 3 months, which is also reasonable since there should be more rapid clearance of viral RNA than viral DNA [58]. This second burst could be due to intermittent adherence.

As recent studies noted a correlation between levels of total HIV DNA and low-level viremia measured by ultrasensitive measures [36, 37], we wanted to determine whether the same was true for integrated HIV DNA. Therefore, we examined a cross-sectional sampling of patients on ART (Figure 2). Interestingly, integrated but not total HIV DNA measures significantly correlated with the level of plasma viremia in this patient cohort (Figure 2c,d). This discrepancy with the literature is likely due to the number of patients studied [36, 37]. To the best of our knowledge, our report is the first to show a better correlation between the level of integrated HIV DNA and low-level plasma viremia.

Measuring total and integrated HIV DNA concurrently may also provide important mechanistic information about HIV persistence. For example, total and integrated HIV DNA together, along with viral load, may help distinguish expression of latent proviruses from ongoing replication [12]. For instance, complete control of viral replication may result in the extinction of unintegrated HIV DNA over a period of time (Fig. 1a), making the ratio of total and integrated HIV DNA closer to one during a period of complete latency (Fig. 1b). At this point, the production of viral particles upon cellular stimulation could result in detectable RNA levels in plasma, without changing the ratio of total to integrated HIV DNA in cells. However, the presence of ongoing replication results in synthesis of intracellular unintegrated DNA leading to an increase in the ratio of total to integrated HIV DNA (Fig. 3). Identifying this process early in patients undergoing therapeutic changes could be valuable for understanding HIV persistence. For example, histone deacetylase inhibitors added to ART may result in viral expression without ongoing replication or they might stimulate new rounds of replication. These two possibilities could be distinguished using these methods but may not be distinguishable by viremia measures alone.

While our data indicate integrated HIV DNA correlates with low level viremia, another important question is the relationship between integration and replication competent proviruses (IUPM). Current estimates suggest that roughly one percent of integrated HIV DNA is replication competent at any time [10], but it remains unclear if the fraction of replication competent virus is constant. In other words, it is unclear if integrated HIV DNA is a strong surrogate for replication competent virus. There are three reasons why integrated HIV DNA may not give rise to replication competent virus: (1) the viral promoter may be so mutated that no transcription occurs (2) epigenetic silencing may prevent HIV expression or (3) the provirus may be mutated so that it does not produce infectious virus but may still express viral proteins. Integrated HIV DNA in setting 1 is unlikely to be relevant to treatment of HIV infection. On the other hand, integrated HIV DNA in settings 2 and 3 may be relevant. Epigenetic changes may occur over time and thus release replication competent

virus; in addition, replication incompetent mutated HIV proviruses might express HIV proteins that may have toxicity for cells or may make the cells susceptible to immune pressure. Given that the proportion of integrated HIV DNA that exists in the three possibilities mentioned is unclear, the relevance of changes in integrated HIV DNA remains unclear. Unfortunately, we did not have enough cells to examine the relationship between integration and IUPM here. Nonetheless, our data suggest integrated HIV DNA can provide useful insights into the latent reservoir.

Finally, we examined the usefulness of measuring both total and integrated HIV DNA in a clinically relevant setting. To do this, we measured total and integrated HIV DNA in patients participating in a clinical trial where ART was interrupted in the presence of IFN- α immunotherapy. As expected, these patients initially exhibited similar levels of total and integrated HIV DNA while on ART. However, when IFN- α was administered for 5 weeks with ART and for 12 weeks after ART interruption there was a decrease in the level of integrated HIV DNA in responders. These patients exhibited a decrease in integrated HIV DNA per CD4+ T cell (also per PBMC and per μ l of blood), without a similar decrease in total HIV DNA (Figure 3). Rather than contradicting our integration data, total HIV DNA instead provides important insight into the decline in integrated HIV DNA and suggests an immune mechanism.

As integration alone decreased, our data suggest a mechanism by which integrated HIV DNA would be preferentially removed. Immune clearance of cells expressing HIV proteins meets this criteria. Thus, IFN- α might enhance immune mediated killing of cells that contain integrated HIV DNA. First, studies have demonstrated that integrated HIV DNA is more effectively expressed than unintegrated HIV [59, 60]. Furthermore, we have recently shown that latently infected cells can express HIV proteins without leading to spreading infection [61]. Thus, it is reasonable that integrated HIV DNA would be preferentially cleared by an immune response. Finally, it is possible that IFN- α either enhances target susceptibility to CTL (e.g. IFN- α could increase HIV presentation) or enhances effector function. In fact, IFN- α has previously been shown to augment both CTL and NK effector function, [62–64].

One major concern with this hypothesis is how we managed to detect a decrease in integrated HIV DNA if most proviruses are defective. As previously described, it is possible that defective proviruses not capable of producing infectious virus might still be capable of expressing some viral proteins and thus be susceptible to an immune response. It is also possible that epigenetic silencing of proviruses may be reversed by IFN- α (allowing viral protein expression) but are not captured by the IUPM assay (Siliciano, R. 2012. “HIV Eradication: Understanding the magnitude of the problem.” Towards an HIV Cure International AIDS Society’s Pre-Conference Symposium, Washington D.C. Ronald Reagan Building).

Why then did we not detect a decrease in total HIV DNA if in fact cells with integrated HIV DNA were cleared? There are two possibilities. First, it is possible that the decrease in integration levels occurred alongside a period of de novo infection (HIV entry and reverse transcription) in new target cells. Given that the patients were no longer taking ART, this hypothesis seems reasonable. Notably, viral RNA increased slightly during the treatment

interruption and the ratio of total over integrated also increased (Figure 4), both of which are also consistent with the idea that a low level of de novo infection occurred after ART was stopped. A second possibility is that total in fact did decrease due to the drop in integrated HIV DNA, but the presence of unintegrated DNA before IFN- α made it difficult to detect with a total HIV DNA assay. However, our integration assay has a sufficiently small variance (error) that it can detect the difference as it specifically measures the HIV DNA intermediate that was cleared—integrated HIV DNA. It is also possible that both of these scenarios occurred in different patients. In any case, measuring integrated HIV DNA provided important information that total HIV DNA did not.

While it may be tempting to attribute a decrease in integrated HIV DNA to an IFN- α mediated restriction to HIV infection as it is known to inhibit infection [65–68], it is difficult for this restriction to result in a reduction in integrated HIV DNA. Instead, a restriction based mechanism would likely result in similar levels of integration that did not increase after ART was stopped. For this reason, we favor an immune based mechanism for clearance of integrated HIV DNA. However, it remains possible that IFN- α 's ability to restrict HIV infection may play a role. Specifically, we envision that during the structured treatment interruption, IFN- α may restrict (or reduce) HIV replication and thereby may enhance the chance that a boosted immune response could control HIV before immune exhaustion prevents control of viremia.

A final possibility is that integrated HIV DNA did not decrease and lower levels in PBMC were due to the expansion of CD4+T and non CD4+T cells or to changes in trafficking. Given that total HIV DNA did not decrease along with integrated makes both of these scenarios less likely. These explanations would require significant differences in expansion or trafficking of cells with integrated compared to unintegrated HIV DNA. Furthermore, we found a significant reduction in integrated HIV DNA when we normalized to CD4+ cells, PBMC and to uL of blood, thus making selective expansion less likely. Finally, neither expansion nor trafficking differences should result in enhanced viral control as seen in these IFN- α responders.

Whether IFN- α enhances the effectiveness of the immune response, enhances the expression of HIV, or works by other means awaits future studies. In any case, concurrent measurements of total and integrated HIV DNA in these and other eradication trials may help to identify therapies that are effective against reservoirs and may provide clues to their mechanism of action.

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Figure 1. The Ratio of Total to Integrated HIV DNA in patients on ART

a: The Ratio of Total to Integrated HIV DNA Approaches 1 Within One Year of ART

Initiation. The top bar graphs depict the total and integrated HIV DNA in PBMC samples from 2 HIV+ patients during the first year of ART. Samples were collected at time 0 (Off-ART), and following 6 months, and 12 months of ART. Black bars indicate the copies of total HIV DNA per µl and gray bars indicate the copies of integrated HIV DNA per µl. The numbers in the graph indicate the ratio of total to integrated HIV DNA at each time point. A ratio greater than 2.1 indicates the presence of unintegrated HIV DNA; marked with an

asterisk (*). The bottom line graphs depict the CD4+ T cell count per μl at each time-point tested.

b: Measurements of Total and Integrated HIV DNA in Longitudinal samples from 4 patients on ART. PBMC from patients who maintained good control of plasma viremia were obtained for concurrent measurements of total and integrated HIV DNA. Patients had maintained viremia at <50 copies per mL of plasma when monitored every three months for more than one year prior to inclusion in the study. The CD4+ T cell count is plotted against the right y-axis for each patient. Asterisks (*) denote samples in which the ratio of total to integrated HIV DNA is greater than 2.1, indicating the presence of unintegrated HIV DNA. Viral load remained below 50 copies/mL blood in all patients three of four patients. In one patient who had one episode of viremia (823 copies/mL) on 1/03 (V) two samples exhibited a ratio of total to integrated HIV DNA >2.1 (*). Arrows denote two of the time points (4/7/03 and 6/30/03) at which viremia was undetectable after the one viremic episode.

Figure 2. Patients with higher levels of both total and integrated HIV DNA have statistically higher low level plasma viremia in a cross-sectional cohort of patients on ART
Total and integrated HIV DNA was measured in CD4+ T cells from patients on ART. Residual plasma viremia was measured by ultra-sensitive assays to levels between 1 and 50 copies/mL. Higher plasma viremia levels also correlated with higher levels of integrated HIV DNA, but not total HIV DNA. The ratio of total to integrated HIV DNA was not statistically different in the presence and absence of viremia.

Figure 3. Total and Integrated HIV DNA in patients from IFN-α trial

PBMC from patients that maintained low levels (<400 copies/ml) of plasma viremia during 12 weeks of monotherapy with IFN-α were analyzed for levels of total and integrated HIV DNA at baseline on ART (On ART), on ART plus IFN-α (ART and IFN), and after monotherapy with IFN-α (IFN only). The levels of total HIV DNA did not change over time, but there was a significant decrease in the level of integrated HIV DNA in patients who responded to IFN-α.

Figure 4. Changes in residual viremia mimic changes in the ratio of total to integrated HIV DNA in patients responding to IFN- α

The number of patients with and without a ratio of total to integrated HIV DNA greater than 2.1 is shown for each time-point (a). The increase in the number of patients with detectable unintegrated HIV DNA corresponded to changes in plasma viremia as measured by ultra-sensitive methods at similar time-points (b).

Table 1

Patient Data

Figure	Patient No	Age	Gender	Year of Dx	ART	CD4 count (cells per ul)	Months with VL <50 on ART
(at time-points measured)							
1a	1	41	M	2003	atazanavir, abacavir, lamivudine	558–869	na
	2	52	M	1995	fosamprenavir, ritonavir, lamivudine, zidovudine	516–833	na
1b	1	dna	dna	1989	lamivudine, stavudine, indinavir	1234–2112	36
	2	dna	dna	1995	lamivudine, stavudine, atazanavir	1409	156
	3	dna	dna	1993	lamivudine, stavudine, atazanavir	386–864	48
	4	dna	dna	1985	dna	312–780	dna
2	1	54	M	1999	tenofovir, kivexa, sustiva	490	46
	2	53	M	1985	kivexa, nevirapine, kaletra	650	112
	3	50	M	1982	truvada, Prezista, Intelence	920	71
	4	33	M	2002	truvada, nevirapine	180	63
	5	50	M	2004	kivexa, sustiva	540	76
	6	42	M	2001	truvada, isentress, celsentri	810	40
	7	51	M	1986	truvada, kaletra, nevirapine dna	660	66
	8	8	M		dna	630	dna
	9	58	M	1987	atrilpla	330	123
	10	57	M	1989	atrilpla, integrase	820	142
	11	59	M	1993	isentress, kaletra, sustiva	350	138
	12	39	M	1993	Prezista, ritonavir, isentress, Intelence	410	33
	13	40	M	1999	truvada, nevirapine	730	132
	14	47	M	1989	kivexa, Prezista, ritonavir, Intelence	580	160
	15	51	M	1992		380	34
	16	57	M	1987	tenofovir, lamivudine, nevirapine	910	107
	17	30	M	2002	kivexa, kaletra, integrase	800	92
	18	41	M	1997	truvada, nevirapine	400	47
	19	48	M	1997	truvada, isentress	510	15
	20	41	M	1998	atrilpla	540	dna
3/4	1	43	F	2003	emtricitabine, tenofovir, efavirenz	545–925	na

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Figure	Patient No	Age	Gender	Year of Dx	ART	CD4 count (cells per ul) (at time-points measured)	Months with VL <50 on ART
	2	47	M	2002	tenofovir, emtricitabine, ritonavir, atazanavir	383–542	na
	3	50	M	1994	lopinavir, ritonavir, efavirenz, abacavir, lamivudine	440–1191	na
	4	28	M	2002	lopinavir, ritonavir, tenofovir, emtricitabine	509–937	na
	5	39	F	1994	fosamprenavir, abacavir, lamivudine	584–908	na
	6	42	M	1998	emtricitabine, tenofovir, efavirenz	710–1358	na
	7	45	M	2001	fosamprenavir, ritonavir, abacavir, lamivudine, tenofovir	649–1263	na

na=not applicable
dna=data not available