Persistent HIV-1 replication is associated with lower antiretroviral drug concentrations in lymphatic tissues

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Antiretroviral therapy can reduce HIV-1 to undetectable levels in peripheral blood, but the effectiveness of treatment in suppressing replication in lymphoid tissue reservoirs has not been determined. Here we show in lymph node samples obtained before and during 6 mo of treatment that the tissue concentrations of five of the most frequently used antiretroviral drugs are much lower than in peripheral blood. These lower concentrations correlated with continued virus replication measured by the slower decay or increases in the follicular dendritic cell network pool of virions and with detection of viral RNA in productively infected cells. The evidence of persistent replication associated with apparently suboptimal drug concentrations argues for development and evaluation of novel therapeutic strategies that will fully suppress viral replication in lymphatic tissues. These strategies could avert the long-term clinical consequences of chronic immune activation driven directly or indirectly by low-level viral replication to thereby improve immune reconstitution.

drug levels | pharmacokinetics | FDC network

Combination antiviral therapy (ART) to suppress HIV-1 replication and reduce plasma viremia to below the limits of detection in peripheral blood (PB) has reduced mortality and dramatically improved quality of life for patients. However, immune reconstitution, measured by changes in the size of populations of CD4 T cells, is often incomplete, even after years of therapy (1–3). During apparently effective therapy, CD4 T-cell populations in PB mononuclear cells (PBMCs), lymph node (LN), and gut-associated lymphoid tissue (GALT) remain abnormally low and innate and adaptive immunity is not fully restored (4). Levels of T-cell activation and innate system activation are often higher than that observed in well-matched uninfected adults (5, 6). These persistent abnormalities may contribute to abnormal vaccine responses (7, 8), a higher than normal incidence of non-AIDS-related cancers (9, 10) and increased risk for clinical conditions associated with chronic inflammation (e.g., cardiac disease, clotting disorders, pulmonary hypertension, emphysema, and stroke) (11–18). Thus, improvements over current approaches to treatment of HIV infection that more fully restore normal immune function might significantly improve health and life expectancy.

To that end, we explore here the hypothesis that antiretroviral drug (ARV) concentrations might be insufficient to fully suppress replication in the lymphoid tissue compartments, which are the principal sites where virus is produced, stored as complexes on the follicular dendritic cell network (FDCn) (19–21), and persists in latently infected cells during ART (19, 20, 22). This hypothesis builds first on the link between the size of the reservoir and the degree of inflammation, arguing that persistent virus production during ART could sustain immune activation (IA) and downstream pathological consequences (23, 24), and second on drug distribution studies in animal models of AIDS in which drug concentrations in tissues have been shown to differ from PB levels (25, 26). Supporting this argument is the observation that some (but not all) intensification schemes with the integrase inhibitor raltegravir demonstrated a transient increase in 2LTR circles and decreases in IA, suggesting ongoing replication in a tissue site that is not reflected by measures in PB (27, 28).

We prospectively treated 12 subjects with ARVs and performed multiple samplings of LN, ileum and rectum, and PB after initiating ART to determine intracellular (IC) concentrations of the ARVs in these tissues and to assess the impact of treatment on virus production, measured by reduced numbers of productively infected HIV-1–RNA$^+$ cells and HIV-1 RNA in virions associated with the FDCn. Ten of the subjects were naïve to ART, and two subjects had been previously treated but had been off therapy for >1 y. In all subjects, commercial genotyping assays confirmed that the virus isolated from their plasma was sensitive to the planned ART. Subjects received tenofovir disoproxil fumarate (TDF)/emtricitabine (FTC) in combination with efavirenz (EFV; $n = 6$), atazanavir (ATV)/ritonavir (RTV) ($n = 4$), and darunavir (DRV)/RTV ($n = 2$). Subjects were followed for 6 mo with LN, ileum, and rectal biopsies obtained just before initiation of ART (month 0; M0), and again at months 1, 3, and 6 (M1, M3, and M6). PB was obtained at monthly intervals and a 24-h pharmacokinetic study in PB was done at M3.

Significance

We show that HIV continues to replicate in the lymphatic tissues of some individuals taking antiretroviral regimens considered fully suppressive, based on undetectable viral loads in peripheral blood, and that one mechanism for persistent replication in lymphatic tissues is the lower concentrations of the antiretroviral drugs in those tissues compared with peripheral blood. These findings are significant because they provide a rationale and framework for testing the efficacy of new agents and combinations of drugs that will fully suppress replication in lymphatic tissues. More suppressive regimens could improve immune reconstitution, as well as provide the effective regimens needed for functional cure and eradication of infection.

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Results

Decreased Drug Concentrations in Lymphatic Tissue. Table 1 provides demographic details of the cohort. We performed 714 determinations of ARV drug concentrations in plasma and 592 analyte determinations for IC drug concentrations in PBMCs and in mononuclear cells (MNCs) from the LN, ileum, and rectum. We used validated ultra-HPLC with mass spectrometry detection (29–31). We chose to measure IC ARV concentrations from MNCs of lymphatic tissue (LT) instead of tissue homogenates because: (*i*) tissue homogenates are a complex mixture of extracellular and IC fluids and particles; (*ii*) they are easily contaminated by blood present in tissue vessels; and (*iii*) there is inhomogeneous distribution of drug in distinct cellular and anatomic compartments of the tissue (32). The latter is particularly important because the relevant information is whether the drug is actually available for antiretroviral activity inside an infected cell.

The expected concentrations in plasma of the ARVs were achieved in all subjects. At M3, the mean concentrations (±SD) measured 24 h after observed dose concentrations were as follows: tenofovir (TFV), 47.4 ± 12.4 ng/mL; FTC, 63.1 ± 14.6 ng/mL; ATV, 377 ± 337 ng/mL; DRV, 1,310 ± 87 ng/mL; RTV, 56.5 ± 32.4 ng/mL; and EFV, 1,750 ± 994 ng/mL. Fig. 1 shows the IC for the five drugs studied in each compartment over time. All five drugs were uniformly detectable at therapeutic concentrations in PBMC samples (n = 241) and were consistent over the 6-mo duration of the study. In LT, IC ARV concentrations were quantifiable in 61% (71/116) of LN samples, 87% (101/116) of ileal samples, and 98% (117/119) of samples from the rectum and differed markedly from PB. Compared with concentrations in PBMCs, the IC concentration of all five ARVs was lower in the LT compartment, particularly in the LN. For example, compared with average PBMC concentration, the average LN concentrations were 80% lower for TFV–diphosphate (DP), −66% for FTC–triphosphate (TP), −100% for ATV, −99% for DRV, and −94% for EFV (all $P < 0.0001$). In all compartments and for all drugs, the variability in concentrations within a subject over time (percent coefficient of variation; CV) was less than variability (CV) across all patients. In PBMCs, within-subject variability ranged from 38% to 88%, whereas interpatient variability was 83–133%. The ranges for within-subject and interpatient variability, respectively, in the other compartments were as follows: LN, 75–93% and 193–287%; ileum, 78–130% and 140–387%; and rectum, 63–140% and 109–296%. There was evidence for drug-specific compartmentalization, consistent with other studies (33, 34). For example, FTC-TP concentrations were higher than TFV-DP concentrations in PBMCs and in the LN, but were lower than TFV-DP in ileal and rectal MNCs; IC of ATV was higher in rectal than in ileal MNCs. IC concentrations of the ARVs in PBMCs did not predict concentrations in LN, ileal, and rectal MNCs.

It is unlikely that these compartmental differences in drug concentrations are attributable to methodological differences, timing issues, or adherence to ARVs by the participants. From the point that cells were obtained, PBMCs and MNCs from LN, ileum, and rectum were rapidly processed in exactly the same way, from lysis of the cellular matrix through the analysis of the supernatant for the IC analytes of interest. Moreover, the fact that drug concentrations were quantifiable and, for some drugs such as TFV-DP, higher in rectum or ileum than in PBMCs argues against processing as the cause of the low concentrations in LN, because differences in processing between PB and tissue compartments would likely affect all analytes, not just select ones by compartment. The compartmental differences in IC concentration are also unlikely to be the result of poor patient adherence to their ARV regimens. All participants throughout the 6-mo study had PBMC concentrations consistent with adherence and with the long IC half-life of TFV-DP (~100 h) but not with taking this medication just before clinic visits (35). Thus, these IC PBMC concentrations indicate a behavior pattern of consistent adherence and have been used as an objective measure of adherence in other studies (36).

Table 1. Demographic characteristics of the cohort

Characteristic	Value
Male, %	100
Age at HIV diagnosis, y (range)	27 (3–44)
CD4 at entry, cells per μL (range)	467 (327–620)
Plasma VL, copies per mL (range)	34, 783 (2,530–157,000)
Ethnicity	8 white, 3 AA, 1 H

AA, African American; H, Hispanic.

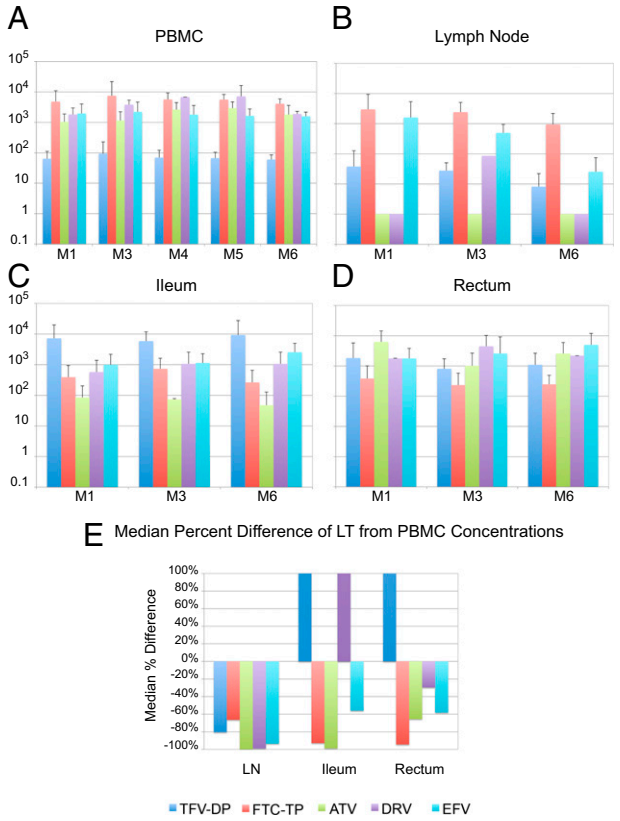

Fig. 1. IC ARV concentrations by compartment and month of therapy. Mean (and SD error) IC concentrations (log scale) for TFV-DP, FTC-TP, ATV, DRV, and EFV are shown for PBMCs (*A*), LN MNCs (*B*), ileal MNCs (*C*), and rectal MNCs (*D*). For *B*, where values were below the limit of quantitation (BLQ), a value of 1 has been assigned for illustration purposes; for example, all LN samples for ATV had IC concentrations that were BLQ. (*E*) Overall median percent difference between the concentration in PBMCs and those in the LN, ileum, and rectum, respectively, for each of the five drugs from all samples obtained during the 6 mo of therapy in the individual subjects. The scale is truncated at +100%. Actual values >100% were as follows: TFV-DP, 2,229%, and DRV, 1,318% in the ileum; and TFV-DP, 599%, and DRV, 149% in the rectum. In the LN, concentrations were uniformly lower than PBMCs for all drugs: TFV-DP concentrations, −80%; FTC-TP, −66%; ATV, −100%; DRV, −99%; and EFV, −94% (all $P < 0.0001$). TFV-DP, TFV-diphosphate; FTC-TP, FTC-triphosphate.

Continued HIV-1 Production in LT During Treatment. All 12 subjects had the expected reduction in plasma viral load (VL) (Fig. 2), but the lower IC concentrations of ARVs, especially in LN, were consistent with the hypothesis that they might be insufficient to fully suppress replication. We sought evidence for this hypothesis by assessing persistent virion production by in situ hybridization (ISH) to determine the frequency and location of MNCs containing HIV-1 RNA in LT and by quantifying the amount of virus bound to the FDCn using, quantitative image analysis (QIA) (37,

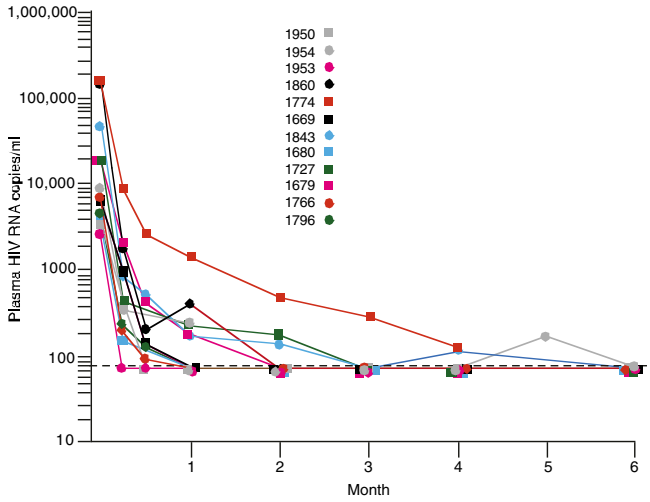

Fig. 2. Plasma HIV RNA is plotted against time. All patients except one (1,774) had a prompt reduction in plasma VL to <48 copies per milliliter. Note that the lower limit of detection was 75 copies per milliliter for patients 1,669; 1,679; and 1,680 through M5, M2, and week 2, respectively when the Siemens Medical Solutions Diagnostics Versant HIV-1 RNA 3.0 Assay (bDNA) was in use in the clinical laboratory where samples were analyzed. All other samples were analyzed by using the Roche COBAS AmpliPrep/COBAS TaqMan HIV-1 test, and the lower limit of detection was 48 copies per milliliter (dotted line).

38). These in situ measurements directly quantify cells producing virus in the tissues, because virions have been detected and quantified in the viral RNA$^+$ (vRNA$^+$) CD4$^+$ T cells (39) that are the principal host cells for virus production in LTs in HIV-1 and simian immunodeficiency virus infections (19, 20, 22, 38, 40, 41). Thus, enumerating vRNA$^+$ cells is also a measure of productively infected cells. The FDCn pool of virus also measures virus production because the FDCn pool is directly correlated with the size of the population of productively infected cells (38), and decays in proportion to the reduction in the number of vRNA$^+$ productively infected cells during ART (40). The FDCn pool is of particular importance for assessing the relationships between drug concentrations and efficacy in inhibiting virus production in LTs for two reasons: (*i*) the size of the pool and thus dynamic range over which changes in production can be measured—total body estimates of FDC-bound virus by QIA for a 70-kg individual before treatment—is 50 billion virions, which is 10^2 to $>10^4$ times the VL in PB or the number of infected cells in an LT snapshot (19, 38); and (*ii*) the FDCn pool is a cumulative record of previous virus production (19) that reflects previous production from infected cells that have died or have been cleared by immune mechanisms and hence would not be scored in an LT snapshot of vRNA$^+$ cells.

The measurements of vRNA$^+$ cells and the FDC pool revealed striking visual and quantitative evidence of continued virus production in LNs in four of the subjects, despite plasma HIV-1 RNA levels in PB below the limit of detection of 48 copies per mL. The FDC pool and numbers of HIV-1 RNA$^+$ cells showed significant correlation in all three compartments (Table 2) and decayed exponentially during the first month in all of the subjects with a mean half-life of 2 wk in LN and 3 wk in both GALT compartments (Table 3). This finding is in excellent agreement with previous measurements of the second phase decay of the FDC pool after initiation of ART and reflects the balance between continued production by CD4$^+$ T cells (22, 40) before initiating ART, deposition of virus on the FDCn, and the complex kinetics of virus release from the FDCn (41). However, in four of nine subjects from whom we had sufficient samples for analysis through M6, the rate of decay of virus from the FDC pool and reduction in HIV RNA$^+$ cells then either slowed or increased in one subject (Fig. 3). The most dramatic example of the virus production and reaccumulation of vRNA in virions associated with the FDCn is illustrated for subject 1,669 (Fig. 4), but the slowed rate of the reduction in the size of the FDC pool for the other three subjects also reflects substantial virus production and deposition to balance release of virions from the FDCn pool, given the logarithmic scale of the decay.

These results were consistent with the hypothesis that drug concentrations in the LTs were suboptimal to fully suppress viral replication. To further test this hypothesis, we assessed the association between drug concentrations in lymphoid tissues and the decay rates and size of the FDC pool. We pooled data from all compartments and modeled the size of virion RNA-associated with the FDCn as a function of IC drug concentrations. Significant negative correlations were found for TFV-DP (P = 0.0242) and FTC-TP (P = 0.0204) (Fig. 5). Thus, IC drug concentrations predicted the size of the FDC pool of virions. When considering the LN alone, there was also an association between TFV-DP (P = 0.0027) and EFV (P = 0.0023) IC concentrations and the decay rate (half-life) of the LN FDCn pool of virions. Similarly, the estimated half-life of 1.9 wk for total vRNA (vRNA in cells and in virions associated with the FDCn pool) was significantly negatively associated (P = 0.0346) with TFV-DP concentration when accounting for the effect of time.

Discussion

In this study of 12 well-characterized subjects following initiation ART, we found that drug penetration for many commonly used ARVs was lower in lymphoid tissue cells than that observed in blood cells. We also show that in four subjects the rate of decay of virus from the FDCn slowed or increased in one subject (1,669) between M1 and M6, and that this change in the rate of reduction in the size of the FDCn pool, which reflects continued virus production during ART, correlated with the lower ARV drug levels in LTs over 6 mo of therapy. Thus, measures of virus replication in blood do not necessarily reflect the impact of ARVs on virus production at its principal source in lymphatic compartments. We show here several examples in which virus was undetectable in PB, but the rate of decay of virus from the FDCn pool slowed, or, in one patient, the size of the FDC pool even increased, indicating ongoing virus production. Collectively, our findings support the hypothesis that ARV concentrations in LT can be insufficient to fully suppress HIV-1 replication and that measuring VL in PB will not necessarily reflect virus production at its source in tissues.

Why would IC drug concentrations differ between blood and lymphatic compartments, under the generally accepted assumption that there is continual trafficking between blood and lymphatic compartments? In fact, blood and lymphatic compartments cannot be assumed to be in constant equilibrium. There are several studies documenting impaired T-cell trafficking in HIV infection, especially in GALT (42–44), and recent

Table 2. Correlation between vRNA in FDC and vRNA$^+$ cells

Compartment	*r*	*P*
LN	0.34	0.0001
Ileum	0.36	0.0091
Rectum	0.42	0.0012

Table 3. Rate of virus decay by compartment

Compartment	Half-life, wk	Lower limit	Upper limit
LN	2.12	1.73	2.74
Ileum	3.39	2.51	5.20
Rectum	3.28	2.46	4.94

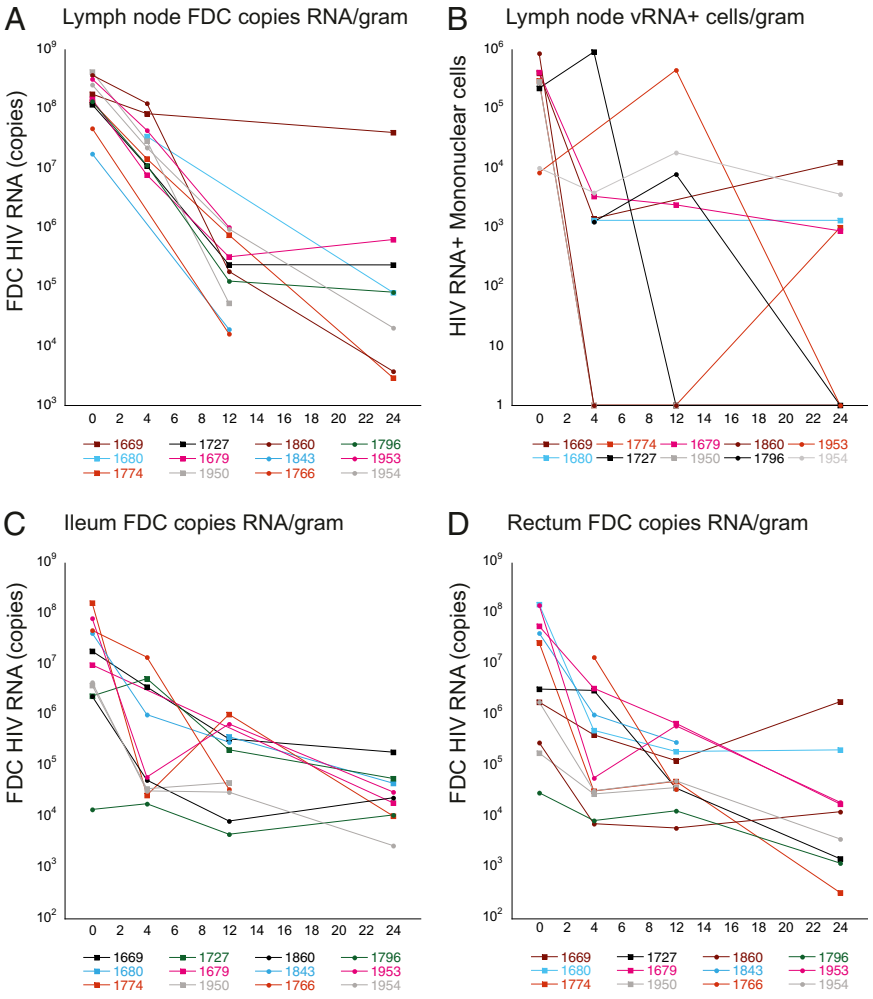

Fig. 3. Decay of the FDCn pool and individual vRNA$^+$ cells in LN under combination antiretroviral therapy. (*A* and *B*) As described in the text, for the 12 patients as a group, the FDCn pool of virions decayed exponentially with the expected half-life of ~2 wk (*A*; Table 2), and there was the expected correlation between vRNA in the FDCn pool and vRNA$^+$ cells (*B*; Table 3). Note that for four of the patients, the FDCn pool decayed as expected between baseline and M3 and then plateaued or increased (identified in *A* with a box drawn around the M6 data points). (*C* and *D*) The decay rates of vRNA in the FDCn of ileum (*C*) and rectum (*D*) are shown. They have slightly longer half-lives but still show good correlation with the number of vRNA$^+$ MNCs in the adjacent parafollicular T-cell zone.

direct estimates in ART-treated HIV-1–infected patients indicate that CD4$^+$ T cells in PB represent only 5.5% of the pool of CD4$^+$ T cells that potentially could traffic to PB (45). It is also generally assumed that lymphatic fluid composition is similar to plasma, and therefore another reason to think that drug concentrations in blood and lymphoid compartments should be similar. However, again there are data that challenge this assumption. The protease inhibitor indinavir is cleared more quickly from lymph compared with plasma (33) and provides one example of differences in drug distribution between the lymphatic system and PB.

We think that the different physicochemical properties affecting penetration into the intestinal lymphatic system of the ARVs (nucleoside reverse transcriptase inhibitors, nonnucleoside reverse transcriptase inhibitors, and protease inhibitors) used in this study, provide a scientific basis for some of the differences in concentrations between PBMCs and LT. The relevant physicochemical properties include molecular weight, particle size, lipophilicity [log octanol/water partition coefficients (log *P* values)], and long-chain triglyceride solubility. Factors described in the literature particularly associated with greater absorption via the intestinal lymphatic system are higher molecular weights, particle size, and log $P > 5$ (46–48). All of the ARVs administered in this study are considered low-molecular-weight drugs, with molecular weights ranging from 247.25 to 720.96. No ARV had an experimental log $P > 5$. Collectively, these physiochemical properties predict poorer penetration into the lymphatic system. Cerebrospinal fluid (CSF) penetration is also known to be dependent upon physicochemical characteristics such as lipophilicity, molecular weight, and protein binding. We explored the relationship between the observed LN penetration of these ARVs and CSF concentrations reported in the literature. A strong correlation ($r = 0.92$) was observed, indicating that low LN penetration correlated with low CSF penetration.

A series of studies with the protease inhibitor indinavir are illustrative of the mechanisms of LN penetration. Indinavir (low molecular weight; log $P < 5$) is more rapidly cleared from lymph than plasma after oral administration to rats (49), and LN MNC concentrations of indinavir determined in three HIV-infected patients were found to be low: ~25% those in blood and very similar to the CSF penetration (50). Administration to macaques of a lipid-associated formulation of indinavir, with higher molecular weight, greater lipophilicity, and a larger particle size that favors distribution into lymph flow and accumulation in LNs, greatly enhanced delivery to the LNs, increasing concentrations to >250% those of blood and increasing in vitro anti-HIV potency by twofold (50). These data clearly illustrate that physicochemical properties of the ARVs are important determinants of LT penetration and provide leads to identifying drugs and regimens with improved penetration and anti-HIV activity. Patient-specific factors, such as host genetic variability in drug metabolizing enzymes or transporter function and LN fibrosis, seem likely to be determinants of drug penetration as well. Our sparse knowledge of these drug- and patient-specific factors precludes predictions about LT penetration of other ARVs. Thus, although the integrase inhibitor raltegravir has been shown to achieve ileal and rectal concentrations in tissue homogenates from healthy volunteers that are higher than those in plasma, and might

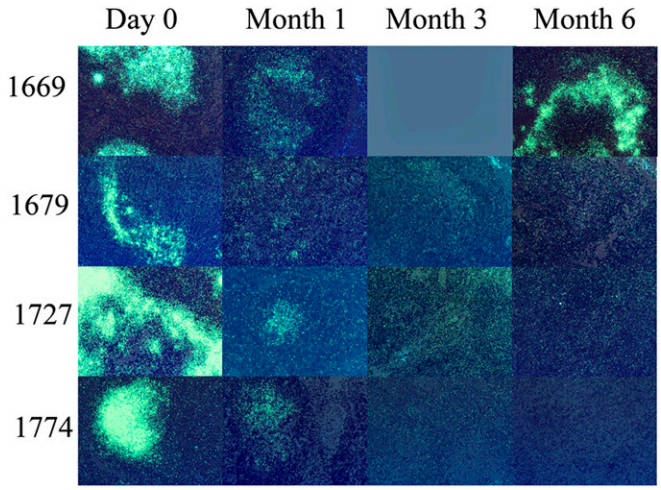

Fig. 4. The different rates of decay of HIV RNA from the FDCn of LN are illustrated in four subjects at baseline, M1, M3, and M6 (M3 LN is missing for subject 1,669 because a LN was not found during the procedure). Subject 1,669 demonstrates no significant decrease over a 6-mo interval. In contrast, subjects 1,679 and 1,727 demonstrated an initial decline and then the copies of HIV RNA on the FDCn declined more slowly at almost a flat rate. In contrast, in subject 1,774, the decay of the FDCn pool was continuous. These three patterns (no decay, initial decay and then leveling off, and continuous decay) were seen in these 12 subjects. Of note, the first two patterns are indicative of ongoing replication as the decreased rate of decay of the FDCn pool or increases in the pool indicate replenishment from virus production.

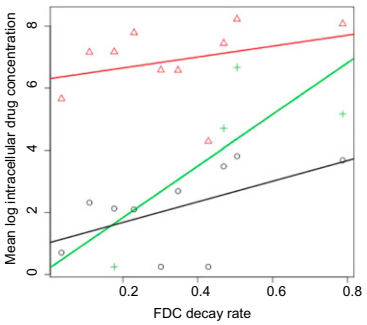

Fig. 5. Representation of the association between the decay rate of virions from the FDC pool and the mean quantity of drug for TFV-DP (black), FTC-TP (red), and EFV (green), showing faster decay of virions with higher concentrations of drug. Mixed-effects models detected significant negative associations between the magnitude of the FDC pool and drug concentrations for TFV-DP ($P = 0.0242$) and FTC-TP ($P = 0.0204$), averaging over all compartments and between the decay rate of the virions from the FDC pool and the quantity of TFV-DP ($P = 0.0027$) and EFV ($P = -0.0023$) in LN.

suggest similarly good penetration into LNs, the low CSF penetration of raltegravir might suggest otherwise (51, 52). Therefore, studies of other ARVs, such as those described here, are necessary to determine relevant IC concentrations in LTs.

We recognize that 6 mo is a relatively short time frame to assess continuing replication in the LTs not apparent in PB, but we note the current Department of Health and Human Services (DHHS) guidelines stated goal for antiretroviral therapy of a plasma VL < 50 copies per milliliter in 12–24 wk was achieved. In fact 5 of 12 (42%) subjects had undetectable plasma VL at the week-4 visit and 8 of 12 (67%) at the week-8 visit. It is also important to note that all DHHS-approved regimens are associated with good virologic responses in most patients, and thus it would not be prudent at this time to alter clinical management based on our results. Rather, it is our longer-term view that insufficient drug concentration in LT allows ongoing, low-level replication sufficient to maintain a state of immune activation, which could impair immune reconstitution, and thus further studies are warranted to: (*i*) develop and assess new drugs and new combinations of drugs that will fully suppress virus replication in LTs; and (*ii*) determine if they do indeed decrease immune activation and improve immune reconstitution. Here the 6-mo treatment window is advantageous to study the effects of these new treatment regimens because of the increased reliability of the evaluations afforded by statistically significant numbers of HIV RNA$^+$ cells and HIV RNA in virions associated with the FDCn and the documented relationship between decay rates and LN compartmental drug concentrations. Waiting for later time points to assess efficacy would be problematic because there might be considerably less evidence of replication, but persistent replication nonetheless at low levels sufficient to sustain IA.

In summary, our findings—that measuring drug concentrations in plasma or in PBMCs does not predict those in lymphoid compartments where most viral replication actually occurs and that viral replication persists in LT of some patients—provide a compelling case and rationale to develop new ART strategies that will fully suppress virus production at its source. In this way, the long-term consequences of persistent virus production for reservoir replenishment and tissue pathologies that restrict immune reconstitution can be averted, and the foundations can be laid for a potential functional cure for HIV-1 infection.

Materials and Methods

The full description of the methods for cohort selection and protocol procedures can be found in *SI Material and Methods*. Clinical laboratory studies (e.g., CD4 and plasma HIV RNA) were carried out in laboratories certified to complete these studies, and details can be found in *SI Material and Methods*, as can the details of tissue processing. Methods for ISH and QIA have been published and are also described in *SI Material and Methods* (37, 38, 40, 53, 54). The analytical pharmacology methods have been published using validated methods (55) and are reviewed in *SI Material and Methods*. All statistical analyses were completed using mixed-effects models in conjunction with permutation tests that are described in *SI Material and Methods*.

T.W.S., A.T.H., C.V.F., M.S., and D.C.D. developed the overall conceptual design of the protocols and performed analyses; T.W.S. was responsible for management of the project and coordination of information and data between investigators; M.R. was responsible for regulatory oversight, recruiting patients, and coordinating all procedures and follow-up; A.T. managed day-to-day activities of the protocol and assisted with all patient procedures; J.A. and T.E.S. processed and shipped patient samples (blood, lymph node, ileum, and rectum); G.J.B. and J.G.C. performed all surgical procedures; A.K. performed all colonoscopy procedures; the C.V.F. laboratory developed methods for intracellular and tissue assessment of drug concentration, and all samples were analyzed by these methods in the C.V.F. laboratory; K.S., S.W.W, and K.P. performed detection of HIV replication by in situ hybridization in the A.T.H. laboratory; A.S.P. provided advice and assistance on the analysis of the decay of the follicular dendritic cell pool; and C.R. completed the data management and statistical analyses.

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