

Persistence of intact HIV-1 proviruses in the brain during antiretroviral therapy

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Abstract

HIV-1 reservoir cells that circulate in peripheral blood during suppressive antiretroviral therapy (ART) have been well characterized, but little is known about the dissemination of HIV-1-infected cells across multiple anatomical tissues, especially the central nervous system (CNS). Here, we performed single-genome, near full-length HIV-1 next-generation sequencing to evaluate the proviral landscape in distinct anatomical compartments, including multiple CNS tissues, from 3 ART-treated participants at autopsy. While lymph nodes and, to a lesser extent, gastrointestinal and genitourinary tissues represented tissue hotspots for the persistence of intact proviruses, we also observed intact proviruses in CNS tissue sections, particularly in the basal ganglia. Multi-compartment dissemination of clonal intact and defective proviral sequences occurred across multiple anatomical tissues, including the CNS, and evidence for the clonal proliferation of HIV-1-infected cells was found in the basal ganglia, in the frontal lobe, in the thalamus and in periventricular white matter. Deep analysis of HIV-1 reservoirs in distinct tissues will be informative for advancing HIV-1 cure strategies.

eLife assessment

This **important** study uses near full-length HIV-1 sequencing to examine proviral persistence in various tissues derived from three individuals who received antiretroviral therapy until time of death. Intact as well as defective HIV-1 proviruses are found at various anatomical sites including the central nervous system; the results are **convincing** and relevant for our understanding of latent viral reservoirs, especially in the brain.

Introduction

Combination antiretroviral therapy has extended the life expectancy of people living with HIV (PLWH) to near-normal levels, but it is clear that antiretroviral therapy (ART), in its present form, does not lead to a sustained, drug-free remission of HIV-1 infection; instead, a long-lived reservoir

of virally-infected cells persists despite ART (1, 2) and can effectively fuel rebound viremia in case of treatment interruptions. Hence, antiretroviral therapy needs to be taken life-long; finding a way to eliminate HIV-1 reservoir cells remains critical to finding a cure for HIV-1 infection.

HIV-1 reservoir cells that circulate in the peripheral blood have been well-described in recent years (3). These circulating reservoir cells mostly consist of memory CD4 T cells that persist life-long and harbor chromosomally-integrated viral DNA, also referred to as a “provirus”. The pool size of HIV-1-infected cells during suppressive antiretroviral therapy is frequently maintained or replenished by clonal proliferation of viral reservoir cells, during which an identical viral DNA copy is passed on to daughter cells (4–7). Important advances in recent technology development, including single-genome near full-length proviral sequencing, have demonstrated that the vast majority of HIV-1 DNA species encountered in ART-treated persons are defective (8–10), mostly due to errors occurring during reverse transcription of viral RNA into DNA. Such errors can lead to large deletions, premature stop codons, or defects in the viral packaging signal region; moreover, specific host proteins such as APOBEC-3G/3F can induce lethal hypermutations into the proviral sequence. The ability of viral reservoir cells to persist indefinitely is frequently attributed to very limited or absent proviral gene transcription; this viral latency protects infected cells from host immune responses and reduces possible cytopathic effects associated with viral gene expression. However, recent studies have emphasized that proviral gene expression can be ongoing in some HIV-1-infected cells during antiretroviral therapy (11), typically when proviruses are integrated in immediate proximity to activating chromatin marks (12).

Much less is currently known about HIV-1 reservoir cells in other body compartments that are more difficult to access for analytic purposes. Lymph nodes and lymphoid tissues in the gastrointestinal tract harbor the vast majority of all lymphocytes and are likely to represent a prime location for the persistence of virally-infected cells (13–16); however, studies that interrogate viral reservoir cells in these tissues remain limited. Even less is known about HIV-1 persistence in other organs, and, in particular, in the CNS, although recent studies have started to explore viral reservoir cells in such specific body compartments (17). In the present study, we used single-genome proviral sequencing to conduct a detailed analysis of HIV-1 proviral sequences in autopsy samples from up to n=18 different organ systems from three study participants.

Methods

Study Participants

HIV-1-infected study participants were recruited at the Massachusetts General Hospital (MGH) and the Brigham and Women’s Hospital in Boston, MA. Fresh tissues were sampled during routine autopsy according to protocols approved by the Institutional Review Board and cryopreserved for future study according to standard protocols. The clinical characteristics of study participants are summarized in **Figure S1**.

HIV-1 DNA quantification by IPDA

Tissue samples were dissected and subjected to genomic DNA extraction using the DNeasy Blood and Tissue Kit (QIAGEN DNeasy, #69504). HIV-1 DNA was analyzed by the Intact Proviral DNA Assay (IPDA), using primers and probes described previously (18). PCR was performed using the following program: 95 °C for 10 min, 45 cycles of 94 °C for 30 s and 59 °C for 1 min, 98 °C for 10 min. The droplets were subsequently read by the QX200 droplet reader (Bio-Rad), and data were analyzed using QuantaSoft software (Bio-Rad).

Near full-length HIV proviral sequencing

Genomic DNA diluted to single HIV-1 genome levels based on Poisson distribution statistics and IPDA results was subjected to near full-genome HIV-1 amplification using a one-amplicon approach (9 [DOI](#), 19 [DOI](#)). PCR products were visualized by agarose gel electrophoresis. Amplification products were individually subjected to Illumina MiSeq sequencing at the MGH DNA Core facility. The resulting short reads were de novo assembled using Ultracycler v1.0 and aligned to HXB2 to identify large deleterious deletions (<8000 bp of the amplicon aligned to HXB2), out-of-frame indels, premature/lethal stop codons, internal inversions, or 5'-LTR defect (≥ 15 bp insertions and/or deletions relative to HXB2), using an automated in-house pipeline written in Python scripting language (<https://github.com/BWH-Lichterfeld-Lab/Intactness-Pipeline> [DOI](#)). The presence/absence of APOBEC-3G/3F-associated hypermutations were determined using the Los Alamos HIV Sequence Database Hypermut 2.0 program. The sequences of individual genes were extracted by GeneCutter, and the start codons of Gag, Pol, and Env were examined and considered. Viral sequences that lacked all defects listed above were classified as “genome-intact”. Multiple sequence alignments were performed using MUSCLE (20 [DOI](#)). Phylogenetic analyses were conducted using MEGA X, applying maximum likelihood approaches. Viral sequences were considered clonal if they had completely identical consensus sequences; single-nucleotide variations in primer binding sites were excluded for clonality analysis. When viral DNA sequences were undetectable, data were reported as LOD (limit of detection), calculated as 0.5 copies per maximum number of cells tested without target identification. The sensitivity of proviral species to broadly-neutralizing antibodies (bnAb) was estimated by calculating the number of amino acid signature sites associated with sensitivity to four bnAb classes within the env amino acid sequence from each intact provirus, as previously described (21 [DOI](#)).

Data Analysis and Statistics

Data are summarized as bar graphs. Phylogenetic relationships were evaluated using maximum-likelihood phylogenetic trees. Images were prepared using Adobe Illustrator. HIV-1 tropism was computationally inferred using Geno2pheno (<https://coreceptor.geno2pheno.org/> [DOI](#)). HIV-1 tropism was classified as “CCR5” if the false-positive rate (FPR) predicted by Geno2pheno was >2%, however, 92% of our proviral sequences meeting this definition had a FPR score >10%; proviruses were considered “CXCR4-tropic” if FPR was <2%. Due to confidentiality concerns, proviral sequencing data cannot be publicly released, but will be made available upon reasonable request and after signing an institutional data-sharing agreement.

Results

Frequency of intact and defective proviruses in tissue compartments

To investigate the proviral landscape across multiple anatomical tissues, including the central nervous system (CNS), we focused on 3 participants from whom post-mortem tissue samples were available for HIV-1 research. Tissue samples from the CNS and other organs were collected by a rapid (<24 hour) autopsy after death. The clinical and demographic characteristics of these study participants are shown in **Figure S1A**. All study participants adhered to antiretroviral treatment until death; plasma viral loads were undetectable by commercial assays in study participants 1 and 2, in whom 14 and 15 different tissue sections were sampled, respectively (**Figure S1B**). Organ-specific tissues analyzed in these two study participants included lymph node, spleen, colon, liver, pancreas, kidney, thyroid gland, and adrenal gland; in the female study participant 1, ovarian and uterus tissues were analyzed, while in the male study participant 2, prostate and testicular tissues were studied. In both of these study participants, four different CNS tissue sections (basal ganglia, thalamus, frontal lobe, and occipital lobe) were collected for investigation.

In study participant 3, plasma viral load was 136 copies/ml at the time of death; 5 different tissue sections from the CNS (basal ganglia, thalamus, occipital lobe, frontal lobe, periventricular white matter) were analyzed in this person.

Near full-length single-template next-generation HIV-1 proviral sequencing was performed to profile the proviral reservoir landscape at single-molecule resolution in tissue samples. The number of cells analyzed from each organ in each participant is listed in **Figure S1B**. In total, 846.53, 425.54, and 199.66 million cells were assayed in study participants 1, 2, and 3, respectively, resulting in 1471.73 million cells analyzed in all study participants combined. A total of 1497 individual proviral sequences were amplified, of which $n=497$ were selected for next-generation sequencing based on their amplicon sizes on gel electrophoresis; the remaining sequences were classified as proviruses with large deletions. All amplicons ($n=74$) from CNS tissues were sequenced, regardless of their length. Using a previously-described computational pipeline to identify lethal defects in proviral sequences, we identified 48 proviruses (3.21% of all proviruses) that met our criteria for genome-intactness (**Figure 1A-C**); this number is consistent with the small number of genome-intact proviruses detected in previous studies. Many sequences, both genome-intact and defective, were identified multiple times, consistent with clonal proliferation of infected cells (**Figure 1B**), as reported in prior work (7, 9, 10, 22).

To evaluate HIV-1 persistence in selected tissue compartments, the frequencies of total, intact, and defective HIV-1 proviruses in each tissue were analyzed (**Figure 2A-C**). Intact proviral sequences were only detected in 8 tissue sites, including basal ganglia, periventricular white matter, lymph node, spleen, colon, kidney, prostate, and thyroid (**Figure 2B**). The numbers of intact HIV-1 sequences in these 8 tissues varied from 0.01 to 0.6 copies per million cells. Consistent with previous studies, the frequency of intact proviral sequences was highest in the lymph node in participant 1 (0.52 intact proviruses/million cells) and participant 2 (0.58 intact proviruses/million cells), followed by kidney, spleen, colon, and basal ganglia in participant 1 and by prostate, spleen, thyroid in participant 2. Intact proviruses were detected in the basal ganglia in study participant 1 (frequency of 0.015/million cells) and in study participant 3 (0.030 intact proviruses/million cells). Moreover, one intact provirus was also detected in periventricular white matter in participant 3 (0.027 intact proviruses/million cells), in whom analysis was limited to brain tissues. No intact proviruses were detected in the CNS tissues of study participant 2, despite analyzing 66.29 million cells. Together, these results indicate that intact HIV-1 proviruses are preferentially detected in lymphoid and gastrointestinal (GI) tissues. The frequency of intact proviruses in the CNS is comparatively low; however, this study is the first one to document the presence of genome-intact proviral sequences in CNS tissues using near full-length proviral sequencing.

Defective proviral sequences were detected in all analyzed tissue samples except for the thalamus from participant 1 (**Figure 2C**). In participant 1, the frequency of defective proviral sequences was highest in lymph nodes (5.7 defective proviruses/million cells), followed by colon, spleen, and kidney. In participant 2, the frequency of defective proviral sequences was highest in lymph nodes (13.0 defective proviruses/million cells), followed by prostate, colon, and spleen. Notably, the prostate had a very high frequency of virally-infected cells. The ratio of intact to defective proviral species was relatively high among sequences isolated from the basal ganglia of participants 1 and 3 (**Figure S2**). Taken together, these results demonstrate the highest frequencies of defective proviruses in lymph nodes, in the colon and in the prostate. CNS tissues contained relatively low frequencies of proviral sequences, compared to other tissue sites; however, defective proviruses were isolated in all but one of the analyzed 13 different CNS samples.

Phylogenetic associations and clonality

A series of prior studies demonstrated large sequence-identical clones of intact and defective proviruses in the peripheral blood of ART-treated study participants. In our subsequent analysis, we studied the dissemination of clonal proviral sequences across different tissues. In participant 1, a total of 24 intact proviral sequences were detected. Two large clones of intact proviruses were

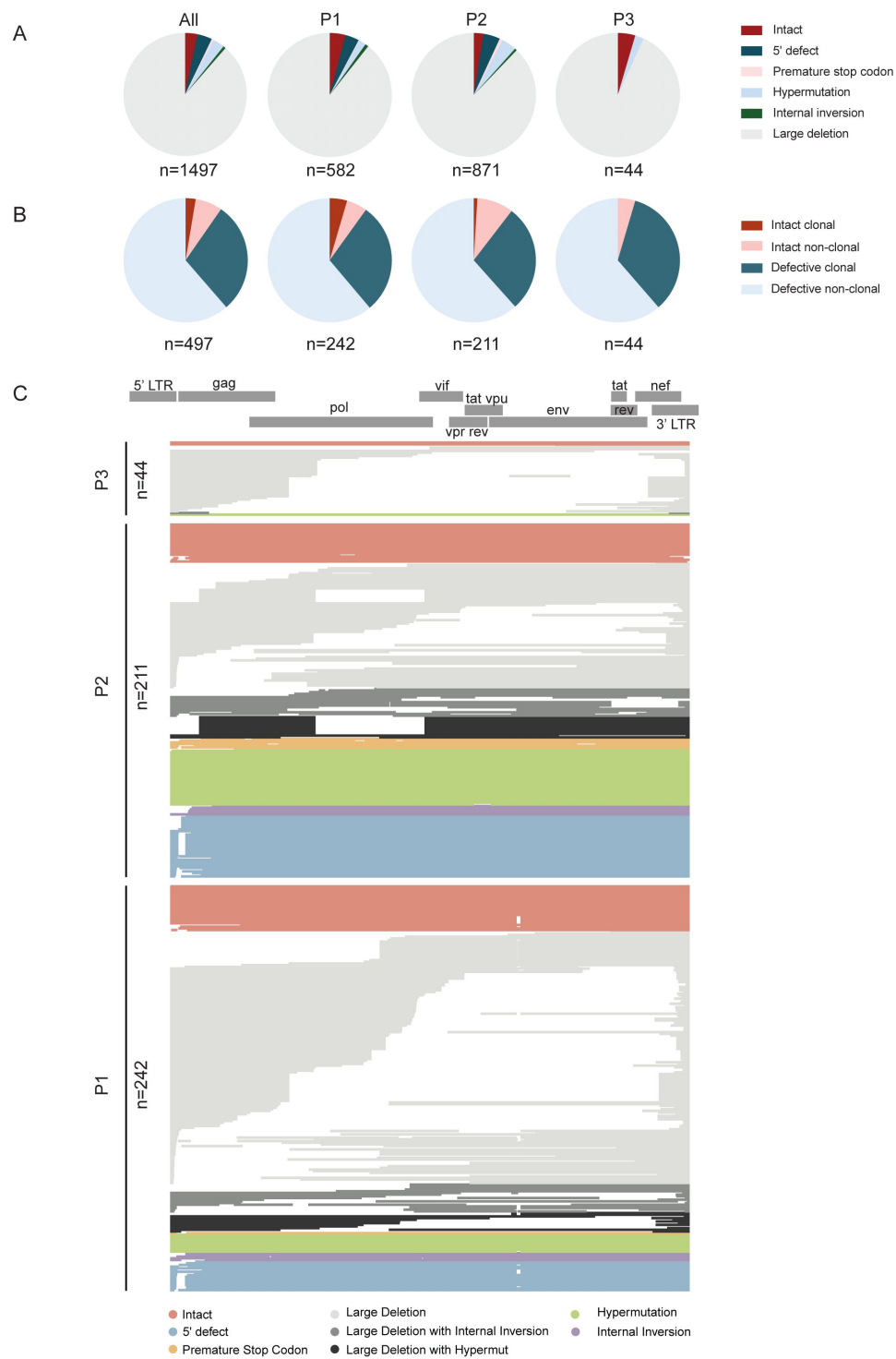


Figure 1.

Proviral sequence classification in all analyzed HIV-1-infected cells from 3 study participants.

(A) Pie charts reflecting proportions of proviruses classified as intact or defective. All proviruses identified by single-genome, near-full-length, next-generation sequencing and by counting amplification products in agarose gel electrophoresis were included. The total number of individual sequences included is listed below each pie chart. (B) Pie charts indicating proportions of intact and defective proviruses detected once (classified as non-clonal) and detected multiple times (classified as clonal). The total number of proviral sequences identified by single-genome, near-full-length, next-generation sequencing is listed below each pie chart. (C) Virograms summarizing individual HIV-1 proviral sequences aligned to the HXB2 reference genome from each participant; color coding reflects the classification of proviral sequences.

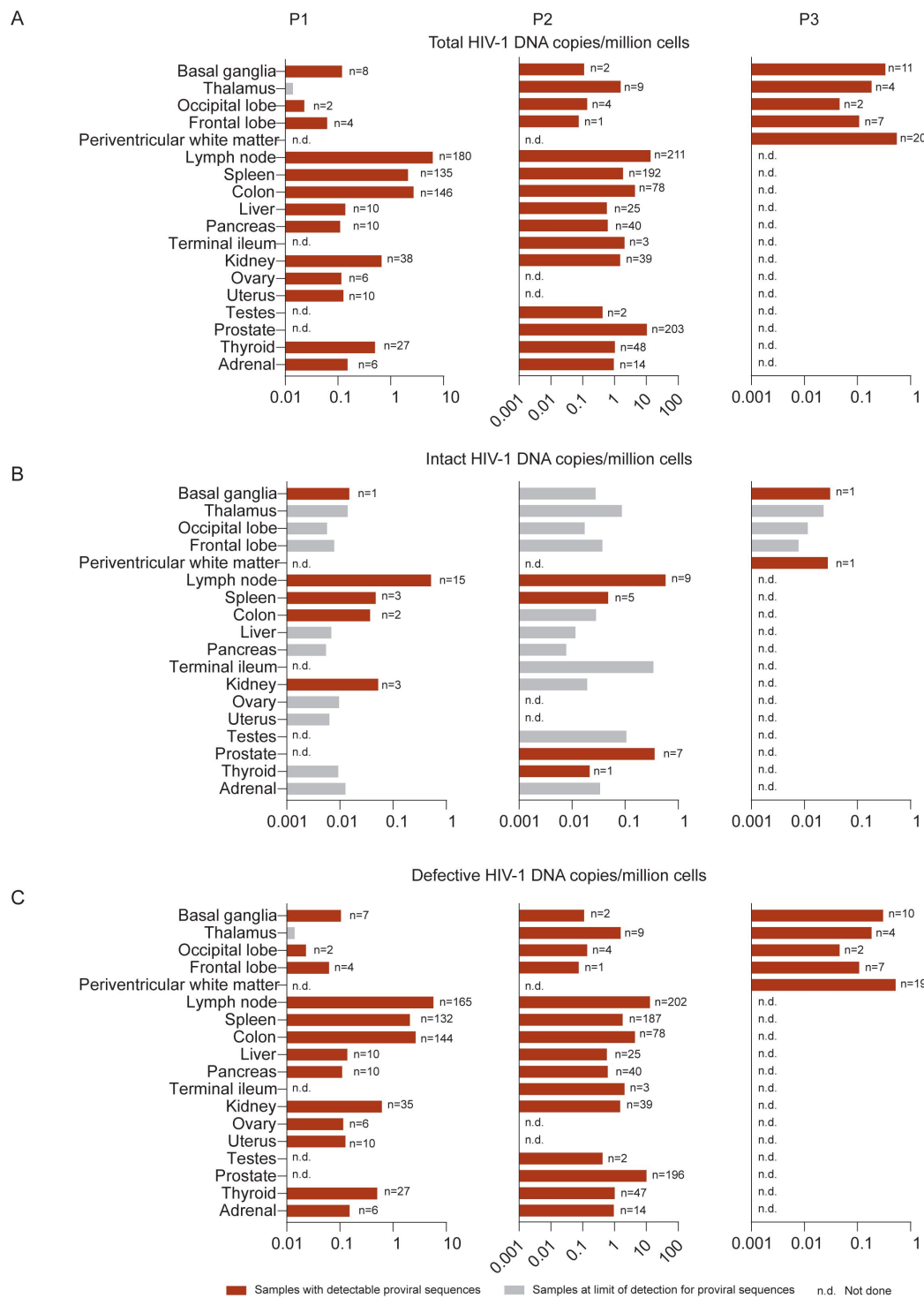


Figure 2.

Distribution of total, intact, and defective HIV-1 proviruses in individual tissue compartments.

Bar diagrams reflect relative frequencies of total (A), intact (B), and defective (C) proviruses in all analyzed tissues in study participants 1-3. The total number of individual proviral sequences determined by single-genome, near-full-length, next-generation sequencing and by counting amplification products in agarose gel electrophoresis from each tissue site of each participant is listed aside each bar. The red bars reflect samples with detectable proviral sequences; grey bars reflect samples at limit of detection for proviral sequences, calculated as 0.5 (single genome near-full-length PCR) copies per maximum number of cells tested without target identification (see Materials and Methods for details). N.d. (not done) indicates that the samples were not available from the indicated tissue sites.

observed, one of which included sequences detected in kidney, lymph node, and spleen samples (**Figure 3A-B**). The other clone involved intact proviral sequences from basal ganglia and lymph node tissues. Among 218 defective proviruses sequenced in participant 1, 14 clones were observed across multiple tissues (**Figure 3C**). In participant 2, among 22 intact proviral sequences, only one clone with two member sequences was identified; both of these clonal sequences were located in the prostate (**Figure 3A-B**). Fourteen clones of defective proviruses were observed across multiple tissues in participant 2 (**Figure 3C**).

Clonal proviral sequences in CNS tissues were detected in all three study participants. In study participant 1, members of a clone of defective proviruses were observed in the basal ganglia and the frontal lobe (**Figure 4A-B**). In participant 2, two clones of defective proviruses were noted in the thalamus, one clone was detected in cells from the basal ganglia, and members of a fourth clone were detected in the thalamus and the occipital lobe (**Figure 4C-D**), demonstrating rather extensive evidence for clonal proliferation of virally-infected cells in the brain. Two of the 44 proviral sequences detected in the central nervous system of participant 3 met the criteria for genome-intactness; one of those was isolated from the basal ganglia and one from periventricular white matter. Defective proviruses, most of them harboring large deletions, were detected in all 5 CNS tissues from participant 3 (**Figure 4E-F**). One large clone involving 9 defective proviral sequences in participant 3 was broadly distributed across different brain tissues, encompassing sequences in the occipital lobe, basal ganglia, thalamus, and periventricular white matter. The other clone of defective proviruses was only detected in the periventricular white matter. Again, these data suggest that clonal proliferation is a rather common feature of HIV-1 reservoir cells in the CNS.

Viral tropism and immune selection footprints

Viral tropism was evaluated based on the env V3 region of the proviruses using the Geno2pheno algorithm. Notably, all proviral sequences containing the env V3 region of participant 1 (n=130, 53.7%) and 3 (n=6, 13.6%) were predicted to be CCR5-tropic (**Figure 5A**). In participant 2, 62.1% of proviral sequences (n=131) were predicted to be likely CXCR4-tropic, while 18.5% (n=39) were classified as CCR5-tropic; the remaining 19.4% (n=41) were classified as undetermined due to the lack of env V3 regions in this study participant (**Figure 5A**). Notably, approximately half of the 131 proviruses with predicted CXCR4 tropism from study participant 2 (n=66, 50.4%) were isolated from the prostate, followed by the spleen (n=38, 29.0%), lymph node (n=20, 15.3%) and thyroid gland (n=3, 2.3%) (**Figure 5B-C**). Among all proviruses (n=16) from CNS tissues of participant 2, only one provirus with a large deletion, isolated from the occipital lobe, had predicted CXCR4-tropism; the tropism of other proviruses from the CNS was unknown due to the lack of env V3 regions. As an additional analysis step, we evaluated footprints of immune selection pressure and mutations resulting in resistance to antiretroviral agents in intact proviral sequences from our study subjects. We noted that the frequency of viral amino acid residues associated with resistance to broadly-neutralizing antibodies did not notably differ among sequences isolated from different tissue compartments (**Figure S3**). We did not observe sequence variations consistent with escape from antiretroviral agents in any of the intact proviral sequences analyzed here.

Discussion

The lifelong persistence of viral reservoir cells makes HIV-1 infection an incurable disease that necessitates indefinite antiretroviral suppression therapy. However, the location of HIV-1 viral reservoir cells across different tissues has been difficult to assess in the past, due to the limited availability of tissue samples. Recent studies, pioneered by investigators of the “Last Gift Cohort”, have catalyzed investigations to characterize HIV-1 sequences in diverse organ systems, specifically in the CNS (23, 24). In our study, we used single-genome near full-length proviral

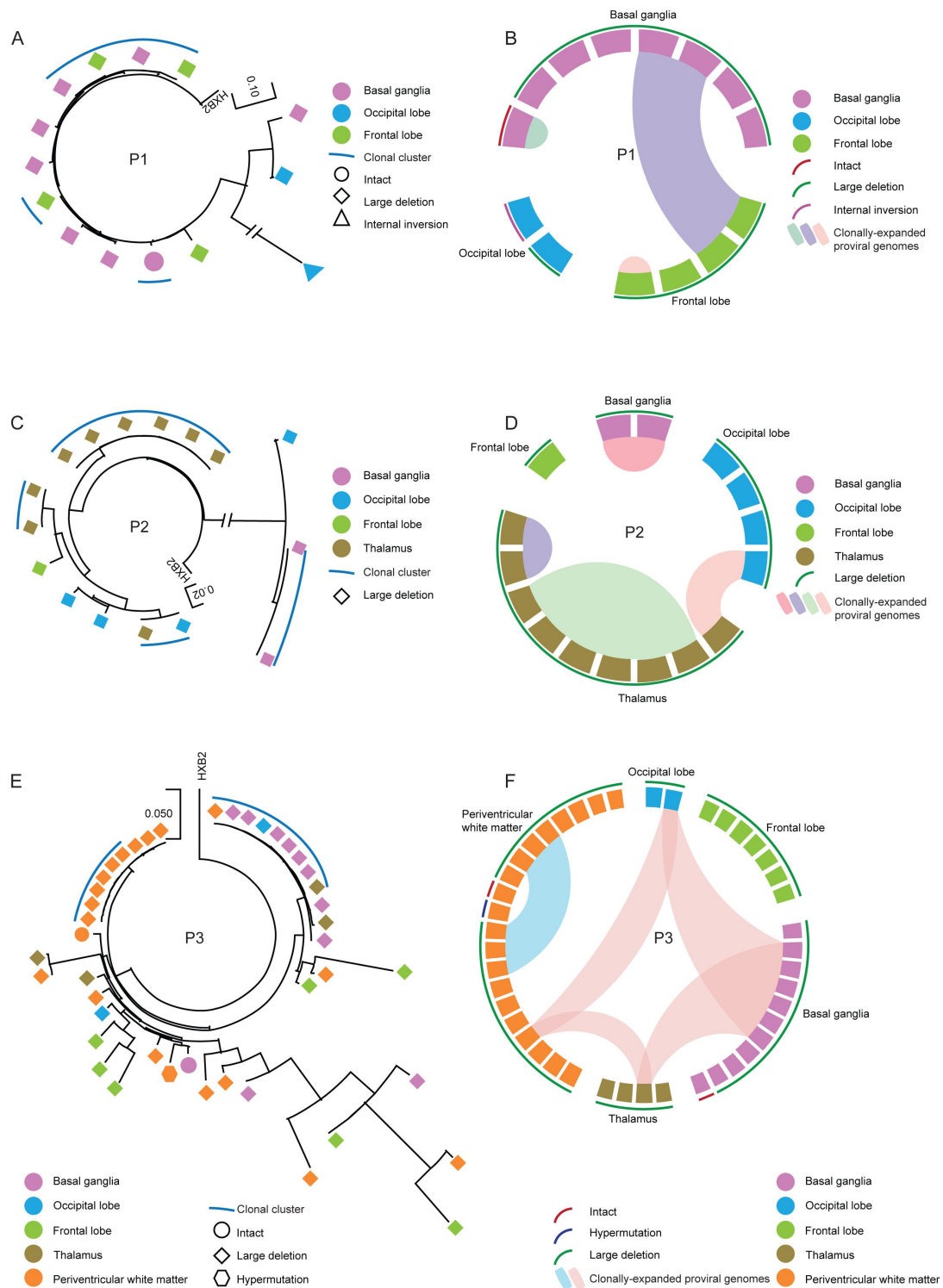


Figure 4.

Dissemination of HIV-1-infected cells across CNS tissues.

(A, C, and E) Circular maximum likelihood phylogenetic trees of all proviral sequences derived from CNS tissues of the 3 study participants (A, participant 1; C, participant 2; E, participant 3). Color coding reflects tissue origins. Clonal sequences, defined by complete sequence identity, are indicated by blue arches. (B, D, and F) Circos plot reflecting the clonality of all proviral sequences isolated from CNS tissues of 3 participants (B, participant1; D, participant 2; F, participant 3). Each symbol reflects one provirus. Clonal sequences, defined by complete sequence identity, are highlighted. Color-coded arches around the plots indicate types of proviral sequences.

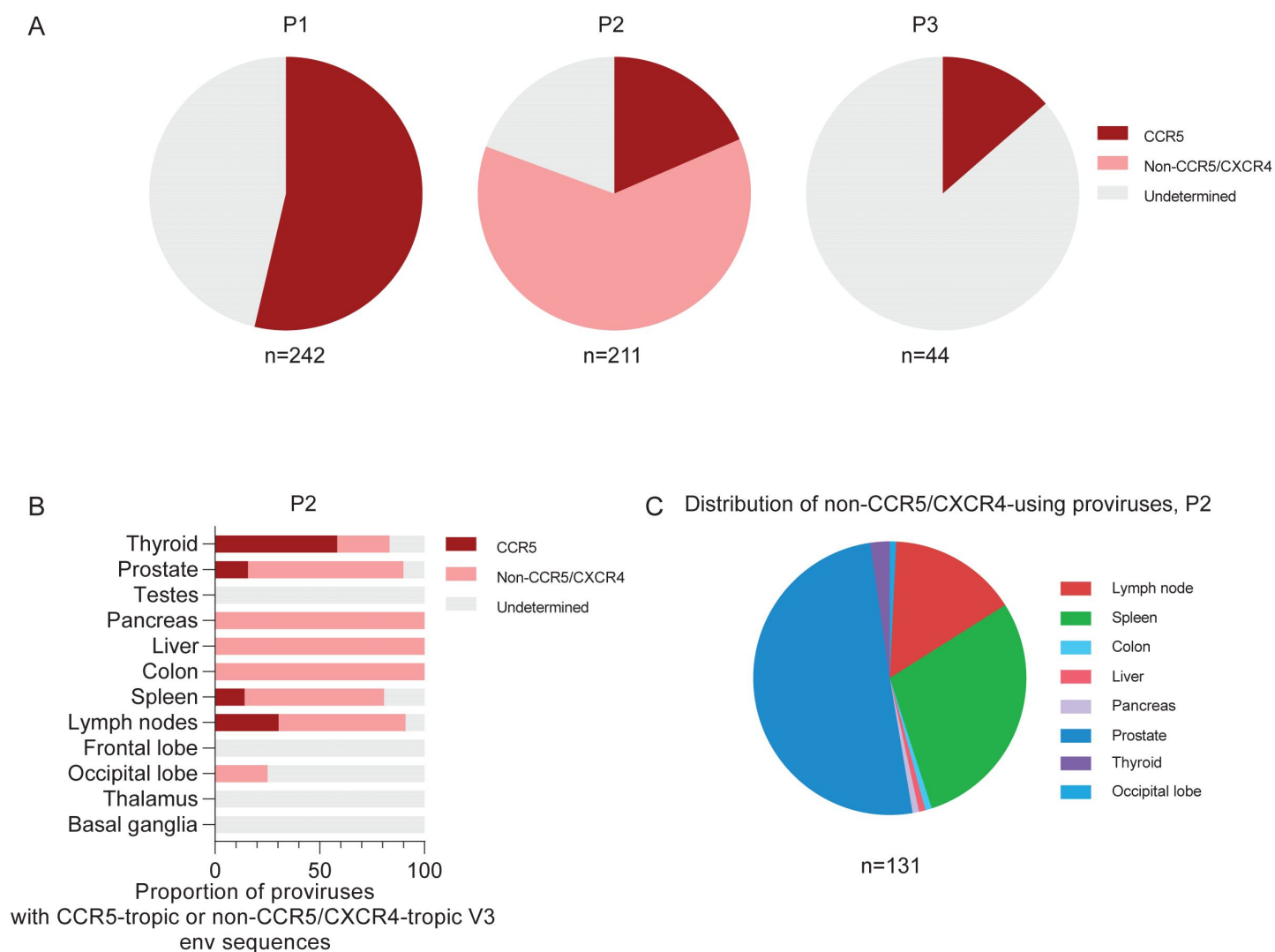


Figure 5.

HIV-1 tropism analysis of proviral sequences.

(A) Pie charts indicating the proportions of all proviruses from each participant with CCR5-tropic or non- CCR5/CXCR4-tropic V3 envelope sequences are shown. The total number of proviral sequences included in this analysis is listed below each pie chart. (B) The proportions of proviruses with CCR5-tropic or non-CCR5/CXCR4-tropic V3 env sequences in each tissue from participant 2 are shown. (C) Pie charts indicating the proportions of all non- CCR5-tropic proviruses from participant 2 are shown. Color coding reflects tissue origins. The total number of analyzed proviral sequences is listed below the pie chart. HIV-1 tropism was computationally inferred using Geno2pheno (<https://coreceptor.geno2pheno.org/>). HIV-1 tropism was classified as “CCR5” if the false-positive rate (FPR) predicted by Geno2pheno was >2% and “CXCR4” if FPR was <2%.

sequencing to evaluate the distribution of HIV-1 reservoir cells in multiple anatomical compartments from autopsy samples of three individuals living with HIV-1 and receiving antiretroviral therapy until the time of their decease. Consistent with previous studies (25, 26), intact proviruses were readily detected in lymph nodes; moreover, we detected relatively high frequencies of intact proviruses in the colon, likely reflecting viral infection of CD4 T cells residing in gut-associated lymphoid tissues (GALT). Of note, our study is among the first investigations to identify near full-length proviral sequences from the central nervous system in two study participants, supporting the hypothesis that CNS cells can serve as reservoirs for long-term HIV-1 persistence despite antiretroviral therapy. Importantly, we noted that large clones of virally infected cells were broadly disseminated across multiple tissues, and, in selected cases, involved cells from the CNS; this suggests that HIV-1 reservoir cells seeded to the brain via hematogenous spread can proliferate in the local tissue microenvironment of the central nervous system. Together, our work suggests that HIV-1 reservoir cells harboring intact proviruses are broadly distributed across multiple anatomical locations, involving lymphoid tissues, gastrointestinal tissues, genitourinary tissues, and, importantly, central nervous system tissues.

HIV-1 can invade the central nervous system (CNS) within days after infection, as demonstrated in animal (27) and in human studies (28). Most likely, infection of CNS cells occurs as a result of transmigration of infected CD4 T cells and, possibly, macrophages across the blood-brain-barrier (BBB) (29, 30), a process that may be facilitated by the increased permeability of the BBB during the initial, highly-replicative stage of HIV-1 infection. Infected cells that successfully enter the CNS may frequently be short-lived, however some infected CD4 T cells in the brain may persist long-term. Moreover, invading infected cells can transmit the virus to resident CNS cells via effective cell-to-cell transmission (30). At least three different CNS cell types seem to be susceptible to HIV-1 infection: perivascular macrophages, microglial cells, and astrocytes, although the role of the latter as HIV-1 target cells is more controversial (31–33). Yet, due to the difficulties in accessing brain tissues for analytic purposes, the role of the central nervous system in HIV-1 persistence during antiretroviral therapy remained largely unknown for a long time. Using the intact proviral DNA assay (IPDA), a ddPCR-based technique allowing to identify proviruses with a high probability of being genome-intact, previous investigators identified intact proviruses in 6 out of 9 ART-treated persons (17), although the precise proviral DNA sequence and their possible clonality was not assessed with this technology. In our study, a total of 13 CNS tissue samples from three study participants were analyzed, including specimens from the basal ganglia, thalamus, occipital lobe, frontal lobe, and periventricular white matter. In two study persons (participants 1 and 3), intact proviral sequences were detected in basal ganglia, suggesting that HIV-1 may preferentially persist in this anatomical compartment in the CNS; an additional intact provirus was detected in periventricular white matter. Notably, the intact provirus from basal ganglia in one of our study persons (participant 1) was clonal with 4 intact proviruses from the lymph node, indicating, to our knowledge for the first time, that CNS tissue can be involved in the multi-compartment dissemination of large clones of HIV-1 proviruses in ART-treated persons. Moreover, in multiple instances, we observed clones of HIV-1-infected cells that were distributed across different autologous CNS tissues, specifically in study participant 3, suggesting local spread of virally infected cells through clonal proliferation within the immune microenvironment of the CNS.

Our study did not allow to determine which cell types were infected by HIV-1 and responsible for clonal expansion of viral reservoir cells in the CNS; however, it is possible that infected microglia are involved. Microglial cells originate from erythromyeloid progenitors in the yolk sac and colonize the developing CNS during embryogenesis (34); these cells act as the main innate immune cell population of the CNS. Due to their long half-life (typically several years), their ability to divide and self-renew, and their high cell-intrinsic susceptibility to HIV-1 (35), these cells may represent a primary cellular site for long-term HIV-1 persistence in the CNS. In particular, self-renewal through homeostatic proliferation in microglia (36) may support HIV-1 persistence through clonal expansion. A recent study indeed identified HIV-1 DNA and RNA in microglia cells

from autopsies from ART-treated PLWH who did not have specific (HIV or non-HIV associated) CNS pathology (37). Evidence for HIV-1 persistence in CD68+ myeloid cells, most likely microglia cells, was also described by previous investigators (17). That said, the presence of clonal proviral sequences shared between the CNS and lymphoid tissues suggests that migrating CD4 T cells infected with R5-tropic viruses may infect the brain as “Trojan horses”, and then potentially clonally expand in situ in the CNS; this hypothesis is consistent with recent findings from Kincer et al (38). In the future, it may be possible to capture the phenotypic characteristics of HIV-1 reservoir cells from the CNS directly with single-cell assays that permit combined assessments of the phenotype and the proviral sequence; an example for such an assay system was recently described (39).

In our study, CXCR4-tropic proviruses were exclusively detected in participant 2. Compared to the other two participants who began ART shortly after HIV-1 diagnosis, participant 2 was diagnosed with HIV-1 25 years prior to starting antiretroviral therapy and died 10 months after ART commencement; therefore, viral CXCR4 tropism most likely resulted from “coreceptor switch” frequently occurring during advanced stages of immune deficiency (40). Whether the preferential persistence of CXCR4-tropic viruses in study participant 2 was associated with our inability to detect intact proviruses in the CNS in this person is unclear; however, prior studies suggested that CCR5 can act as the principal co-receptor for HIV-1 isolates in the brain (41, 42). Moreover, rebound viremia in cerebrospinal fluid after ART interruption is mostly fueled by CCR5-tropic virus (38), further supporting the assumption that R5-tropic viruses are better adjusted to persist in the brain. Another notable finding in our study was the high number of HIV-1 proviruses isolated from the prostate, which had the second highest proviral frequency among all analyzed tissues in study participant 2, second only to lymph node samples. Other studies also reported that the prostate can represent a tissue reservoir for HIV-1 (24). We were unable to identify the precise cell type harboring HIV-1 in the prostate, but it is possible that myeloid cells may harbor HIV-1 in this location. A prior study indeed demonstrated that intact, replication-competent HIV-1 can persist in myeloid cells from the urethra, located in immediate anatomical proximity to the prostate (43).

Our study has several limitations. Importantly, this work includes only 3 participants, and brain tissues were the only samples available from participant 3. Moreover, due to limited tissue sizes, very few cells were assayed from the terminal ileum and testes; prior studies suggested high HIV-1 DNA levels in these 2 tissues during suppressive antiretroviral therapy (44, 45). Another limitation was that peripheral blood samples were not available from the 3 participants, which made it impossible to study phylogenetic associations between tissue reservoirs of HIV-1 relative to viral species circulating in peripheral blood. Moreover, we cannot fully exclude contamination of tissue samples with cells from peripheral blood. However, after autopsy, the tissue samples were washed thoroughly with PBS to eliminate blood as much as possible. Notably, we failed to detect intact proviral sequences from over 100 million liver cells of 2 participants, despite the fact that the liver contains about 13% of all human blood supply at any given time point, arguing against contamination of tissues with blood cells.

In sum, this study provides a deep analysis of tissue reservoirs for HIV-1 that includes a detailed assessment of HIV-1 sequences in CNS tissues. Our work supports the persistence of genome-intact HIV-1 in many tissues, including CNS tissues, emphasizing the difficulties in finding strategies to effectively eliminate HIV-1 from the human body in clinical settings.

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Author contributions

Sample collection and preservation: SIC, IHS, SM HIV-1 sequencing assays: WS, YR Data analysis, interpretation, presentation: WS, CG, XL, XGY, ML Study concept and supervision: IHS, SM, XGY and ML

Conflicts of Interest

The authors declare that conflicts of interest do not exist.

Supplemental Material

Supplemental Figure 1: (A) Clinical and demographical data of study participants. (B) Cell numbers analyzed from each tissue of each study participant.

Supplemental Figure 2: (A) Bar diagrams reflect the ratios of intact to defective HIV-1 proviruses in all analyzed tissues in study participants 1-3. The red bars reflect samples with detectable proviral sequences; grey bars reflect samples at limit of detection for proviral sequences, calculated as 0.5 proviral copies per maximum number of cells tested without target identification (see Materials and Methods for details). N.d. (not done) indicates that the samples were not available from the indicated tissue sites.

Supplemental Figure 3: (A-F) Numbers of broadly neutralizing antibody (bnAb) sensitivity (A, C, and E) and resistance (B, D, and F) signature sites in intact proviral sequences from indicated tissues from each participant are shown. Each dot represents one intact provirus. Amino acid residues associated with susceptibility or resistance to bnAbs were inferred based on the study by Bricault (21 [↗](#)).

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Reviewer #1 (Public Review):

Despite durable viral suppression by antiretroviral therapy (ART), HIV-1 persists in cellular reservoirs in vivo. The viral reservoir in circulating memory T cells has been well characterized, in part due to the ability to safely obtain blood via peripheral phlebotomy from people living with HIV-1 infection (PWH). Tissue reservoirs in PWH are more difficult to sample and are less well understood. In this small (n=3) autopsy study, Sun and colleagues use an advanced genetic sequencing technique to characterize HIV-1 that persists in human tissues despite antiretroviral therapy. The authors describe isolation and genetic characterization of HIV-1 reservoirs from a variety of tissues including the central nervous system (CNS) obtained from three recently deceased individuals at autopsy. They identified clonally expanded proviruses in the CNS in all three individuals.

Strengths of the work include the study of human tissues that are under-studied and difficult to access, and the sophisticated near-full length sequencing technique that allows for inferences about genetic intactness and clonality of proviruses. The small sample size (n=3) is a drawback. Furthermore, two individuals were on ART for just one year at the time of autopsy and had T cells compatible with AIDS, and one of these individuals had a low-level detectable viral load (Figure S1). This makes generalizability of these results to PWH who have been on ART for years or decades and have achieved durable viral suppression and immune reconstitution difficult.

While anatomic tissue compartment and CNS region accompany these PCR results, it is unclear which cell types these viruses persist in. As the authors point out, it is possible that these reservoir cells might have been infiltrating T cells from blood present at the time of autopsy tissue sampling. Cell type identification would greatly enhance the impact of this work. Overall, this small, thoughtful study contributes to our understanding of the tissue distribution of persistent HIV-1, and informs the ongoing search for viral eradication.

Reviewer #2 (Public Review):

The authors were trying to survey reservoir viral sequences in different anatomical sites in the body, with the brain being of special interest. This is a study that is technically demanding and here is well done, providing insights that prompt new and more sophisticated questions.

The authors use end-point dilution PCR to identify individual proviruses that can then be sequenced with high accuracy. These are high quality data sets but given the technical requirements of this approach the number of sequenced proviruses is limiting given the scope of questions this study addresses. Nonetheless, there is a lot of data here to draw many useful conclusions.

It will be important to realize how clones of infected T cells can move around the body, including into the CNS compartment. It will also be important to remember that there are limits in sampling depth of proviruses in any one tissue meaning the failure to detect something has a limit in sensitivity of detection when trying to interpret a negative result.

As noted in the next section, it is important to emphasize that there is another entry phenotype beyond X4 that will ultimately be important in interpreting these results. Macrophage-tropic viruses are often found in the CNS compartment and it will be important to understand whether these CNS-derived sequences are macrophage-tropic viruses there

infecting macrophages and microglia or if they are all T-tropic viruses brought in by wandering infected T cells (or both).

Author Response

The following is the authors' response to the original reviews.

We are very grateful to the reviewers for their insightful and detailed analysis of our work, in particular to reviewer 2. We also would like to thank the Elife editorial team for organizing this form of public review and debate, which we believe will be of interest to the science community.

Reviewer #1 (Public Review):

Despite durable viral suppression by antiretroviral therapy (ART), HIV-1 persists in cellular reservoirs in vivo. The viral reservoir in circulating memory T cells has been well characterized, in part due to the ability to safely obtain blood via peripheral phlebotomy from people living with HIV-1 infection (PWH). Tissue reservoirs in PWH are more difficult to sample and are less well understood. Sun and colleagues describe isolation and genetic characterization of HIV-1 reservoirs from a variety of tissues including the central nervous system (CNS) obtained from three recently deceased individuals at autopsy. They identified clonally expanded proviruses in the CNS in all three individuals.

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While anatomic tissue compartment and CNS region accompany these PCR results, it is unclear which cell types these viruses persist in. As the authors point out, it is possible that these reservoir cells might have been infiltrating T cells from blood present at the time of autopsy tissue sampling. Cell type identification would greatly enhance the impact of this work. Several other groups have undergone similar studies (with similar results) using autopsy samples (links below). These studies included more individuals, but did not make use of the near-full length sequencing described here. In particular, the Last Gift cohort, based at UCSD and led by Sara Gianella and Davey Smith, has established protocols for tissue sampling during autopsy performed soon after death.
<https://pubmed.ncbi.nlm.nih.gov/35867351/> <https://pubmed.ncbi.nlm.nih.gov/37184401/>

We agree with reviewer 1 that studies to identify specific cell types that harbor intact HIV-1 in individual tissue compartments would be very informative; our group has recently initiated such studies.

Overall, this small, thoughtful study contributes to our understanding of the tissue distribution of persistent HIV-1, and informs the ongoing search for viral eradication.

We thank reviewer 1 for these encouraging remarks.

Reviewer #2 (Public Review):

The manuscript by Sun et al. applies the powerful technology of profiling viral DNA sequences in numerous anatomical sites in autopsy samples from participants who maintained their antiviral therapy up to the time of death. The sequencing is of high quality in using end-point dilution PCR to generate individual viral genomes. There is a thoughtful discussion, although there are points that we disagree with. This is an important data set that increases the scope of how the field thinks about the latent reservoir with a new look at the potential of a reservoir within the CNS.

We greatly appreciate the comments by reviewer 2 and would like to thank them for their detailed and very knowledgeable analysis of this paper.

1. The participants are very different in their exposure to HIV replication and disease progression. Participant 1 appears to have been on ART for most of the time after diagnosis of infection (16 years) and died with a high CD4 T cell count. The other two participants had only one year on ART and died with relatively low CD4 T cell counts (under 200). This could lead to differences in the nature of the reservoir. In this regard, the amount of DNA per million cells appears to be about 10-fold lower across the compartments sampled for participant 1. Also, one might expect fewer intact proviruses surviving after 16 years on ART compared to only 1 year on ART. The depth of sampling may be too limited and the number of participants too few to assess if these differences are features of these participants because of their different exposures to HIV replication. On the positive side, finding similarities across these big differences in participant profiles does reinforce the generalizability of the observations.

Many thanks for pointing this out. We also noticed that the total number of HIV-1 proviruses is smaller in our study participant 1 (who had been on ART for 16 years), compared to study persons 2 and 3 with more limited treatment durations (1-2 years), however, due to the small number of study persons, we think we cannot use these results for inferring how treatment duration influences viral reservoir size in tissues.

1. The following analysis will be limited by sampling depth but where possible it would be interesting to compare the ratio of intact to defective DNA. A sanctuary might allow greater persistence of cells with intact viral DNA even without viral replication (i.e. reduced immune surveillance). Detecting one or two intact proviruses in a tissue sample does not lend itself to a level of precision to address this question, but statistical tests could be applied to infer when there is sampling of 5 or more intact proviruses to determine if their frequency as a ratio of total DNA in different anatomical sites is similar or different. This would allow adjustment for the different amount of viral DNA in different compartments while addressing the question of the frequency of intact versus defective proviruses. One complication in this analysis is if there was clonal expansion of a cell with an intact genome which would represent a fortuitous overrepresentation intact genomes in that compartment.

We have performed the analysis suggested by reviewer 2 and included a diagram reflecting the ratio of intact/defective proviruses as a new supplemental figure (Figure S2). Unfortunately, we do not feel comfortable to draw any real conclusions from this additional analysis; the sample sizes are simply too limited.

1. The key point of this work is that the participants were on therapy up to the time of death ("enforcing" viral latency). The predominance of defective genomes is consistent with this assumption. Is there data from untreated infections to compare to as a signature of whether the viral DNA population was under selective pressure from therapy or not?

Presumably untreated infections contain more intact DNA relative to total DNA. This would represent independent evidence that therapy was in place.

We agree that an analysis of autopsy samples from untreated persons living with HIV-1 would be of great interest, and are actively collaborating with neuropathologists from multiple sites to obtain such samples. Yet, we are not convinced that selection pressure on reservoir cells during ART can be appropriately identified through quantitative virological assays. Rather, we feel that the selection of proviruses can be best assessed when qualitative parameters, including proviral integration sites and their position relative to host epigenetic chromatin features, are evaluated.

1. There are several points in Figure 5 to raise about V3 loop sequences. The analysis includes a large number of "undetermined" sequences that did not have a V3 loop sequence to evaluate. We would argue it is a fair assumption that the deleted proviruses have the same distribution of X4 and R5 sequences as the ones that have a V3 sequence to evaluate. In this view it would be possible to exclude the sequences for which there is no data and just look at the ratio of X4 and R5 in the different compartments, specifically does this ratio change in a statistically significant way in different compartments? The authors use "CCR5 and non-CCR5" as the two entry phenotypes. The evidence is pretty strong that the "other" coreceptor the virus routinely uses is CXCR4, and G2P is providing the FPR for X4 viruses. Perhaps the authors are trying to create some space for other coreceptors on microglia, but we are pretty sure what they are measuring is X4 viruses, especially in this late disease state of participant 2. Finally, we have previously observed that the G2P FPR score of <2 is a strong indicator of being X4, FPR scores between 2 and 10 have a 50% chance of being X4, and FPR scores above 10 are reliably R5 (PMID27226378). In addition, we observed that X4 viruses form distinct phylogenetic lineages. The authors might consider these features of X4 viruses in the evaluation of their sequences. Specifically, it would be helpful to incorporate the FPR scores of the reported X4 viruses.

Many thanks for these thoughts. We have now included FPR scores for all sequences and considered sequences with FPR score <2 as X4-tropic. Among 497 proviral sequences derived from all three participants, only 14 proviral sequences had FPR scores between 2 and 10 and their tropism was classified as CCR5 in the new Figure 5. We agree that viral tropism analysis of proviral sequences from the CNS would be of particular interest for study subject 2; however, most brain-derived sequences from that person had large deletions in the env region, precluding an analysis of viral tropism.

1. We have puzzled over the many reports of different cell types in the CNS being infected. When we examined these cell types (both as primary cells and as iPSC-derived cells), all cells could be infected with a version of HIV that had the promiscuous VSV-G protein on the virus surface as a pseudotype. However, only macrophages and microglia could be infected using the HIV Env protein, and then only if it was the M-tropic version and not the T-tropic version (PMID35975998). RNAseq analysis was consistent with this biological readout in that only macrophages and microglia expressed CD4, neurons and astrocytes do not. From the virology point of view, astrocytes are no more infectable than neurons.

We appreciate these comments. As described in our discussion, we agree that the role of astrocytes as target cells for HIV-1 infection is highly controversial; we look forward to future opportunities to evaluate HIV sequences in sorted astrocytes from autopsy tissues.

1. The brain gets exposed to virus from the earliest stages of infection but this is not synonymous with viral replication. Most of the time there is virus in the CSF but it is present at 1-10% of the level of viral load in the blood and phylogenetically it looks like

the virus in the blood, most consistent with trafficking T cells, some of which are infected (PMID25811757). The fact that the virus in the blood is almost always T cell-tropic in needing a high density of CD4 for entry makes it unlikely that monocytes are infected (with their low density of CD4) and thus are not the source of virus found in the CNS. It seems much more likely that infected T cells are the "Trojan Horse" carrying virus into the CNS.

We appreciate the reviewer's referral to Greek mythology and agree that the hypothesis of infected T cells acting as "Trojan horses" is more intuitive and better supported by available data. We have adjusted our discussion accordingly.

1. While all participants were taking antiretroviral therapy at the time of their death, they were not all suppressed when the tissues were collected. The authors are careful not to mention "suppressive ART" in the text, which is appreciated. However, the title should be changed to also reflect this fact.

Thanks for pointing this out. From our perspective, ART is never fully suppressive, as low-level viremia (below the detection threshold of commercial PCR assays) is detectable in almost all ART-treated persons. As such, it is not clear to us that "suppressive" necessarily implies suppression below the detection limits of commercial PCRs. We argue that ART can also be suppressive when plasma viral loads are in the range of 100 copies/ml, as they are in our study subject 3. Nevertheless, we have changed the title to avoid confusion.

Reviewer #1 (Recommendations For The Authors):

I encourage the authors to compare their autopsy and tissue sampling procedures to those used by The Last Gift researchers and consider including references to this ongoing study. If the authors plan to continue in this line of research, the field would greatly benefit from a collaboration that would bring together their excellent and advanced PCR technique with the larger sample size offered by The Last Gift. Lastly, is there some way to simultaneously determine cell type when NFL sequencing is performed?

We look forward to collaborating with investigators from the Last Gift Cohort in the future and have integrated additional references in the manuscript to acknowledge their work. At the current stage of technology development, we think that sorting of infected cells based on canonical markers of defined cell populations is the preferred approach for identifying phenotypic properties of infected cells; however, expansion of the PheP-Seq assay (Sun et al., Nature 2023), may facilitate this process in the future.

Reviewer #2 (Recommendations For The Authors):

1. The authors have chosen to lump all R5 viruses together in terms of their entry phenotype, giving all viruses an equal chance of infecting all potentially susceptible cell types. This ignores the fact that normal HIV is selected to infect cells, requiring a high density of CD4 as is found on T cells. We use the term R5 T cell-tropic to describe "normal" HIV. The ability to efficiently enter cells that have a low density of CD4, such as macrophages and microglia, involves the evolution of a distinct phenotype, termed macrophage tropism (PMID24307580, and work of others). This happens most often in the CNS where T cells are infrequent thus potentiating evolution to infect an alternative cell type. This change in entry phenotype is dramatic and, like X4 viruses, results in phylogenetically distinct lineages (PMID22007152). There are no sequence signatures for M-tropic viruses as there are for X4 viruses, but the fact that there are sequences shared between the CNS and lymphoid tissue makes it much more likely that there are T cells migrating around the body, including into the CNS, that are carrying R5 T cell-tropic

virus with them, with the cells potentially clonally expanding in situ in the CNS. The persistence of a potential CNS T cell reservoir was the point we were trying to make in our recent paper (ref. 38), not only that these CSF rebound viruses were R5 viruses but they were selected for replication in T cells as seen by their dependence of a high density of CD4 for entry. This is the conclusion one would reach if clonally expanded viral sequences were shared between two lymphoid compartments. It is not necessary to ascribe properties of infection and clonal amplification to microglia cells when a more parsimonious explanation is that there are low levels of T cells in the CNS, especially in the absence of entry phenotype data showing these sequences encode an M-tropic entry phenotype. As is the authors are just adding to the unproven belief that virus in the CNS must be in myeloid cells, which in this case in particular we suspect is the wrong interpretation.

We are impressed by reviewer 2's recent work, suggesting the viral reservoir in the CNS may primarily consist of clonally-expanded R5 T-cell tropic viruses. We have adjusted our discussion to emphasize this possibility, and to highlight that viral entry phenotyping data will be informative for better understanding viral persistence in the brain.

1. The authors noted that the frequency of intact proviruses is highest in the lymph nodes of 2/2 participants for which they had lymph node samples, relative to the other tissues examined. They thus conclude, "Together, these results indicate that intact HIV-1 proviruses are preferentially detected in lymphoid and gastrointestinal (GI) tissues." However, an examination of Figure 2 reveals that the total HIV copy number is highest in the lymph nodes of these two people. Thus, it doesn't seem like HIV is preferentially intact in the lymph nodes as much as they sampled more provirus from that tissue and therefore were able to detect more intact proviruses.

We have adjusted our manuscript to indicate that the highest numbers of intact HIV-1 proviruses were present in lymph nodes, both in terms of absolute numbers and after normalization to the total numbers of cells analyzed.

1. In Figure 1A, the legend should be changed so that "PMSC" is spelled out as "premature stop codon" for ease of reading. This is done for Figure 1B.

We have corrected this issue as suggested by the reviewer.

1. The pie charts in Figure 5 could be better labeled for ease of interpreting. In Figure 5C, instead of just labeling it as "P2" it could be "Distribution of CXCR4-using proviruses, P2", as an example. As it stands, it is hard to know what the figure is describing without reading the text.

We have changed this accordingly.

1. While all participants were taking antiretroviral therapy at the time of their death, they were not all suppressed when the tissues were collected. The authors are careful not to mention "suppressive ART" in the text, which is appreciated. However, the title should be changed to also reflect this fact.

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Editorial comments:

In addition to the reviewers suggestion, we feel that adding more information on how you define intact proviral sequence, e.g. are only disrupted essential genes or also in accessory genes considered? Previous studies have shown that brain-derived HIV-1 strains are usually CCR5-tropic, show high affinity for the CD4 receptor and frequently contain defective vpu genes. Some information and discussion if the brainderived sequences confirm these previous finding seems of significant interest.

As described in our previous work (e. g. Lee et al, JCI 2017; Jiang et al, Nature 2020), accessory genes are not considered in our definition of “genome intactness”; this is consistent with approaches other investigators have chosen (e. g. Hiener et al, Cell Reports 2017). Within the genome intact sequences we identified in the CNS in our study persons, we found no evidence for deletions of vpu sequences; this has been emphasized in the revised manuscript.