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MAJOR ARTICLE

Sequence Analysis of Inducible, Replication-Competent Virus Reveals No Evidence of HIV-1 Evolution During Suppressive Antiviral Therapy, Indicating a Lack of Ongoing Viral Replication

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Background. Persistence of HIV-1 in reservoirs necessitates life-long antiretroviral therapy (ART). There are conflicting data using genetic analysis on whether persistence includes an actively replicating reservoir with strong evidence arguing against replication.

Methods. We investigated the possibility of ongoing viral evolution during suppressive therapy by comparing near full-length viral genomic sequences using phylogenetic analysis of viral RNA in plasma before therapy initiation early after infection and from virus induced to grow from the latent reservoir after a period of suppressive ART. We also focused our analysis on evidence of selective pressure by drugs in the treatment regimen and at sites of selective pressure by the adaptive immune response.

Results. Viral genomes induced to grow from the latent reservoir from 10 participants with up to 9 years on suppressive ART were highly similar to the nearly homogeneous sequences in plasma taken early after infection at ART initiation. This finding was consistent across the entire genome and when the analysis focused on sites targeted by the drug regimen and by host selective pressure of antibody and cytotoxic T cells. The lack of viral evolution away from pretherapy sequences in spite of demonstrated selective pressure is most consistent with a lack of viral replication during reservoir persistence.

Conclusions. These results do not support ongoing viral replication as a mechanism of HIV-1 persistence during suppressive ART.

Keywords. HIV-1 persistence; latent reservoirs; phylogenetic analysis; suppressive therapy; viral evolution.

Highly active antiretroviral therapy (ART) has led to a significant reduction in morbidity and mortality associated with HIV-1 infection by inhibiting viral replication and reducing the level of plasma HIV-1 RNA to below the detection limit of sensitive clinical assays (<50 copies/mL) [1]. However, eradication of HIV-1 from people with HIV-1 (PWH) by current treatment regimens has been unsuccessful by 3 measures. First, residual low-level viremia (LLV) persists in people while on therapy [2–7], and this low-level viremia cannot be suppressed with therapy intensification [8–10]. By definition, this low-level viremia is in the form of virus particles, and these populations can consist of both replication-competent and defective viruses [11–14]. Second, infectious HIV-1 can be induced to replicate from latently infected memory CD4+ T cells [15–18], providing a mechanism for the long-term persistence of infectious viral genomes. However, the number of infected cells that can be induced to produce virus in culture significantly underestimates the number of cells with intact proviral genomes [19–26]. Third, HIV-1 quickly returns to pretherapy levels of viremia in the vast majority of people on therapy if therapy is discontinued [27–35].

There are 2 distinct mechanisms that could account for the persistence of infectious HIV-1 in the face of antiviral therapy that is otherwise suppressive of viremia in the blood. One mechanism involves low-level persistent viral replication, perhaps in sanctuary sites with low drug penetrance [36]. Several studies have described the evolution of viral sequences in people taking combination antiviral therapy and suggested that this provides evidence for a replicating viral reservoir [37, 38]. This interpretation has been challenged [39, 40]. Other

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studies have failed to detect viral evolution during suppressive antiviral therapy and noted the lack of evolution of drug resistance as further evidence that the virus is not replicating in the face of selective pressure [41–49].

Most of the viral DNA that persists in long-lived cells is defective [23], a feature that confounds the study of viral evolution on therapy. However, the use of replication-competent virus induced from latently infected cells similarly showed a lack of viral evolution during suppressive therapy [44]. A feature of HIV-1 transmission is that most of the time a single viral variant establishes the new infection [50, 51], which makes the initial viral population highly homogeneous. Several studies have used this simplified starting point to examine viral evolution after therapy was initiated early after infection and found none [41, 49]. We have combined these strategies of using the simplified viral populations that exist when therapy is initiated early after infection with a focus on replication-competent virus that is maintained in the latent reservoir to address the question of whether there is detectable ongoing viral replication during suppressive therapy. We measured evolutionary divergence by surveying across the entire viral genome using sequences derived from plasma at the time of therapy initiation and from virus induced in culture to replicate from the latent reservoir after an average of 4.5 years of therapy. Our study involved 10 participants who initiated therapy early after infection and who had been infected with a single variant. We did not detect any evidence of viral evolution in these participants while on suppressive therapy.

METHODS

Participants

Participants in the UNC Chapel Hill acute HIV-1 cohort were identified as having been recently infected with HIV-1 [52]. We selected a group of participants who initiated suppressive antiretroviral therapy within 2 months of the estimated date of infection; as a result of the early initiation of therapy, they had relatively small latent reservoirs as measured by only low levels of inducible virus from peripheral cells when examined after a period on therapy (≤0.2 infectious units per million cells [IUPM]) using the virus outgrowth assay (VOA) [53]. This group was screened for the complexity of the viral population at the time of diagnosis to identify participants who had been infected with a single virus based on the low sequence complexity of the viral population at the time of therapy initiation [54]. This resulted in a cohort of 10 participants for whom viremic plasma samples were available at the time of therapy initiation, and outgrowth virus at end-point dilution from the VOA was available after an average of 4.5 years of suppressive therapy (Supplementary Table 1). We included specimens from 1 untreated viremic participant with plasma collected early after infection, then 2.2 years later as a control participant.

5′- and 3′-Half Genome Amplification

RNA was isolated from blood plasma and culture supernatants derived from VOA using a QIAamp viral RNA kit (Qiagen). Isolated viral RNA was reverse-transcribed into cDNA using Superscript III Reverse Transcriptase (Invitrogen) and an oligo-$(dT)_{20}$ primer (Invitrogen) to copy the 3′-half of the genome, or using a gene-specific primer to copy the 5′-half of the genome. To amplify 5′- and 3′-half genomes, cDNA isolated from pretherapy plasma and culture supernatant from VOA wells was subjected to end-point dilution polymerase chain reaction (PCR) amplification or bulk PCR, respectively. For cDNA isolated from plasma samples, end-point dilution PCR was performed according to an established protocol [55]. Briefly, cDNA was diluted such that only a fraction of the PCR wells were positive for a DNA product, consistent with the presence of a single template, and nested PCR was performed using Platinum Taq High Fidelity Polymerase (Invitrogen). The primers and PCR conditions to generate 5′- and 3′-half genomes are described in Supplementary Table 2. Final PCR products were separated in a 1.2% agarose gel, and gel-purified PCR products were directly subjected to Sanger sequencing using multiple primers to generate overlapping sequences. Bulk cDNA isolated using viral RNA from VOA wells (at end-point dilution indicative of a single virus growing out) was directly amplified using nested PCR, and final PCR products were separated on a 1.2% agarose gel. Gel-purified PCR products generated from viral RNA sequences were sequenced directly using Sanger sequencing or were analyzed using the PacBio sequencing platform following the protocol previously described (see Supplementary Table 2 for primers and PCR conditions) [56].

Phylogenetic and Statistical Analyses

Viral genomes sequenced by Sanger sequencing were processed as follows. Contigs were assembled using Gap5. All chromatograms were manually inspected for the presence of double peaks, and sequences with double peaks were excluded from further analysis. VOA viruses sequenced by PacBio sequencing were processed as in the previous publication [56]. Viral sequences from both assays were pooled for the downstream phylogenetic analysis. Sequences were examined for the presence of hypermutations using the Los Alamos Hypermut tool (https://www.hiv.lanl.gov/content/sequence/HYPERMUT/hypermut.html), and hypermutated sequences were discarded and not used in further analysis. Sequences containing premature stop codons and indels that changed open reading frames (ORFs) were removed from downstream analyses as well. A consensus sequence was generated using the multiple amplicons sequenced from each pretherapy plasma sample to approximate the sequence of the transmitted/founder virus in each person. The 5′- or 3′-half genome sequences derived from the pretherapy plasma samples, the VOA wells, and the pretherapy

consensus sequence were aligned using MAFFT, version 7, and maximum-likelihood (ML) phylogenetic trees were constructed in MEGA 7.0 [13]. To calculate genetic divergence of the viral populations in each person, pairwise distance between individual sequences and the pretherapy plasma consensus sequence was computed in MEGA 7.0, and the average genetic distance from the pretherapy plasma consensus sequence was calculated for each group of sequences (pretherapy plasma sequences or VOA well sequences). A 2-sided Mann-Whitney test was performed to compare average distances between pretherapy plasma and VOA using GraphPad Prism, version 6 (GraphPad Software, Inc., San Diego, CA, USA).

In a separate analysis, the V1-V2 variable region of the viral *env* gene was excised from each 3′-half genome sequence and analyzed by phylogenetic analysis to enhance the sensitivity of detection of sequence differences. All sequence data have been deposited at GenBank (accession numbers PP405628-PP405941).

Drug Resistance Mutations

All 5′-half genome sequences were analyzed for drug resistance mutations using the HIVdb program, Stanford University HIV Drug Resistance Database (https://hivdb.stanford.edu/).

CD8+ T-cell Mapping

All 18-mer peptides (n = 384), overlapping by 10 amino acids were synthesized (Sigma-Genosys) to match the entire HIV-1 clade B consensus sequence (https://www.hiv.lanl.gov/content/sequence/HIV/CONSENSUS/Consensus.html). Interferon-γ ELISpot assays were performed on peripheral blood mononuclear cells from 4 participants following the protocol previously described [57]. Spot-forming unit (SFU) values >4 times the background were used as a threshold to define positive T-cell responses [58].

RESULTS

Phylogenetic Analysis of 5′- and 3′-Half Genomes After Extended Therapy

Participants who had a nearly homogeneous virus population at the time of initiation of therapy were selected to maximize the sensitivity to detect viral evolution over time while on therapy. Pretherapy plasma samples were subjected to deep sequencing analysis to determine the number of viral lineages in the blood sample (data not shown), and participants who had been infected with more than a single variant were excluded from the study. The participants spent an average (range) of 4.5 (2.1–8.9) years on therapy before undergoing leukopheresis to isolate cells for use in the quantitative viral outgrowth assay (VOA) (Supplementary Table 1); this assay involves diluting cells until only a fraction of the wells are positive for virus outgrowth (end-point dilution), consistent with the culture supernatant of the virus-positive wells containing a single outgrowth virus [53, 59]. To compare full-length viral genome sequences from pretherapy plasma early after infection and from the latent reservoir after years of therapy (from the culture supernatants of the VOA wells), 5′- and 3′-half genomes were generated from the pretherapy samples by end-point dilution of the cDNA copies of viral RNA in nested PCR and from the VOA wells by bulk PCR of the cDNA copies of viral RNA. A total of 137 half genome sequences from the 10 pretherapy plasma samples and 125 half genome sequences from VOA wells were used to examine genetic changes in the viral population during suppressive therapy. For comparison, 47 half genome sequences (generated at end-point dilution) obtained from longitudinal plasma samples (early after infection and 2.2 years later) collected from 1 untreated person (CT-1) with HIV-1 were included in the analysis as a positive control for the detection of viral sequence evolution.

To assess genetic relationships between sequences obtained before and after extended therapy, 2 ML phylogenetic trees representing 5′-half genomes and 3′-half genomes were generated using sequences from both the pretherapy plasma samples (open circles/red) and VOA wells (filled circles/blue) for each participant. A consensus sequence (arrow/green) was generated using all sequences derived from the pretherapy plasma sample to approximate the transmitted/founder virus sequence, and this was included in each tree (Figure 1). Sequences obtained from VOA wells representing virus outgrowth from the reservoir were identical or highly genetically similar to the baseline pretherapy plasma sequences for each participant, indicating no discernible evolutionary divergence from the pretherapy sequence as surveyed across the entire genome after an average of 4.5 years of therapy (Figure 1*A*; Supplementary Figure 1). Furthermore, sequences from VOA wells were intermingled with those from pretherapy plasma without showing obvious clustering, another indication of a lack of viral evolution. In contrast, sequences from the plasma sample collected at a later time point (Figure 1*B* filled circles/purple) from 1 viremic participant were genetically distinct from the baseline sequences at diagnosis and heterogeneous compared with the baseline sequences, which are indicative of viral evolution in the absence of therapy (Figure 1*B*; Supplementary Figure 1). Note that each scale bar represents 1 nucleotide (nt) change per 5000 nts unless otherwise specified.

To further investigate viral evolution during suppressive therapy, genetic divergence between the 2 time points, pretherapy plasma and post-therapy VOA wells, was measured by computing the genetic distance between individual sequences and the pretherapy plasma consensus sequence (approximating the transmitted/founder virus). The average genetic distance (AGD) from each plasma consensus sequence representing sequences from pretherapy plasma and VOA wells is shown in Figure 2. A significant increase in AGD between plasma and

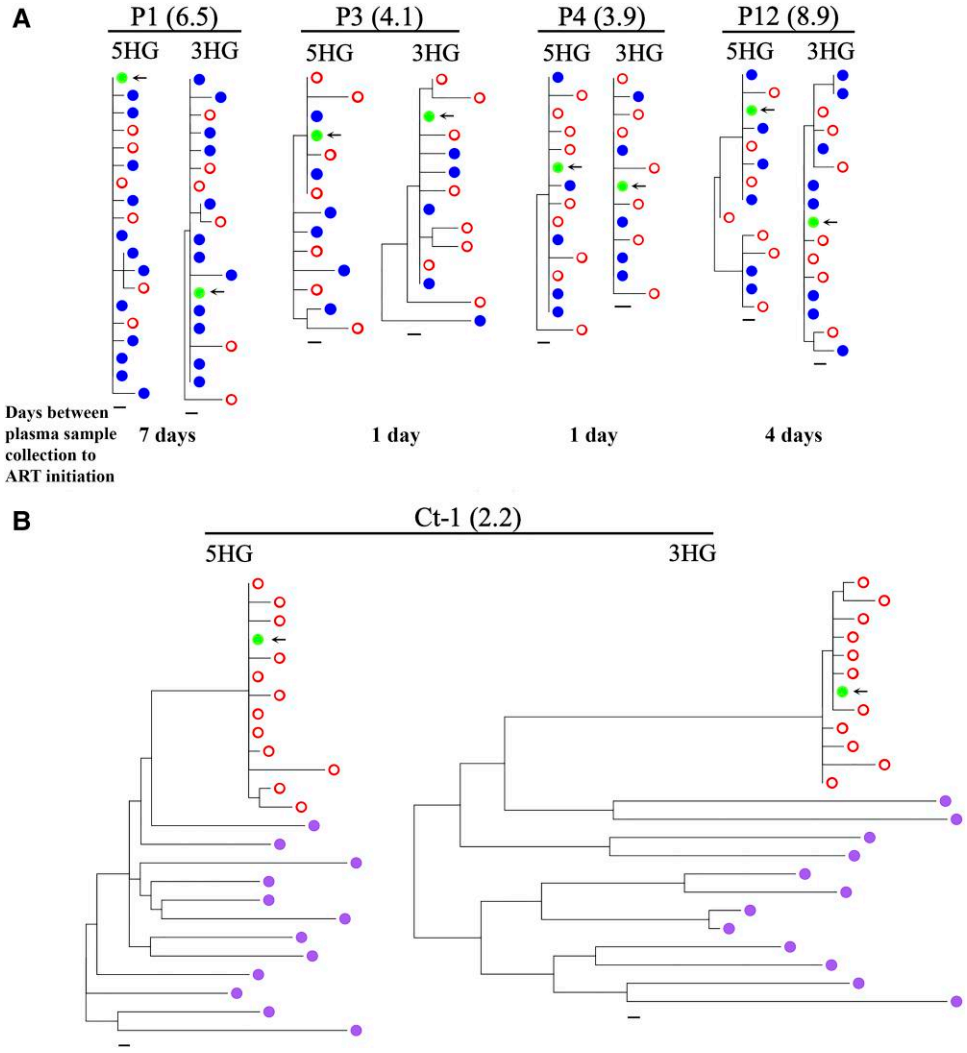

Figure 1. Maximum-likelihood phylogenetic trees of viral RNA–derived 5′- or 3′-half genome sequences. *A*, Phylogenetic trees of participants (P1, P3, P4, and P12) on suppressive therapy. Sequences derived from pretherapy plasma samples are shown in open circles (red), and sequences derived from latent reservoir after years of therapy (culture supernatants from VOA wells) are shown in filled circles (blue). Years of therapy for individual participants are indicated in parentheses. Days between plasma sample collection and ART initiation are indicated. *B*, Phylogenetic tree of an untreated viremic participant, a positive control (Ct-1). Sequences derived from the plasma samples collected at 2 different time points are shown in open circles (first collection) and filled circles (second collection). The interval between 2 time points is shown in parentheses. For phylogenetic trees displayed in (*A*) and (*B*), the 5′-half genome (5HG) represents nucleotides 790 to 5096 covering the *gag-pro-pol* region, and the 3′-half genome (3HG) represents nucleotides 5097 to 9530 covering the rest of the HIV-1 genome. The 3HG ends at the end of the 3′-U3, and nucleotide positions are based on HXB2 numbering. Consensus sequences are indicated by an arrow pointing to the green dot. The scale bars represent 1 nucleotide substitution per 5000 nucleotides. Abbreviations: ART, antiretroviral therapy; VOA, virus outgrowth assay.

VOA sequences would be an indication of viral evolution during suppressive therapy. In the 1 untreated, viremic participant, the average AGD of the second plasma sequence was increased by 8.7-fold for the 5′-half genome and 9.8-fold for the 3′-half genome compared with the average AGD of the baseline plasma sequences, values that were significantly different (Mann-Whitney rank-sum test) (Figure 2). In participants on suppressive therapy, however, the average AGD of VOA sequences was similar to the average AGD of pretherapy plasma sequences, indicating that significant genetic changes were absent in the sequences derived from the latent reservoir after an average of 4.5 years of suppressive therapy (Figure 2). In the 2 cases where there was a significant difference in AGD (Mann-Whitney rank-sum test) between the pretherapy plasma virus and the VOA sequences (P1 and P6), the diversity was less in the VOA sequences, still consistent with a lack of detectable viral replication.

Sequence Evolution Analysis Focused at Sites of Selective Pressure

The previous analysis looked for evidence of sequence evolution that would be apparent when comparing half genome lengths of viral sequence. Emergence of drug resistance mutations and escape mutations from the adaptive immune response is more targeted and potentially a more sensitive way

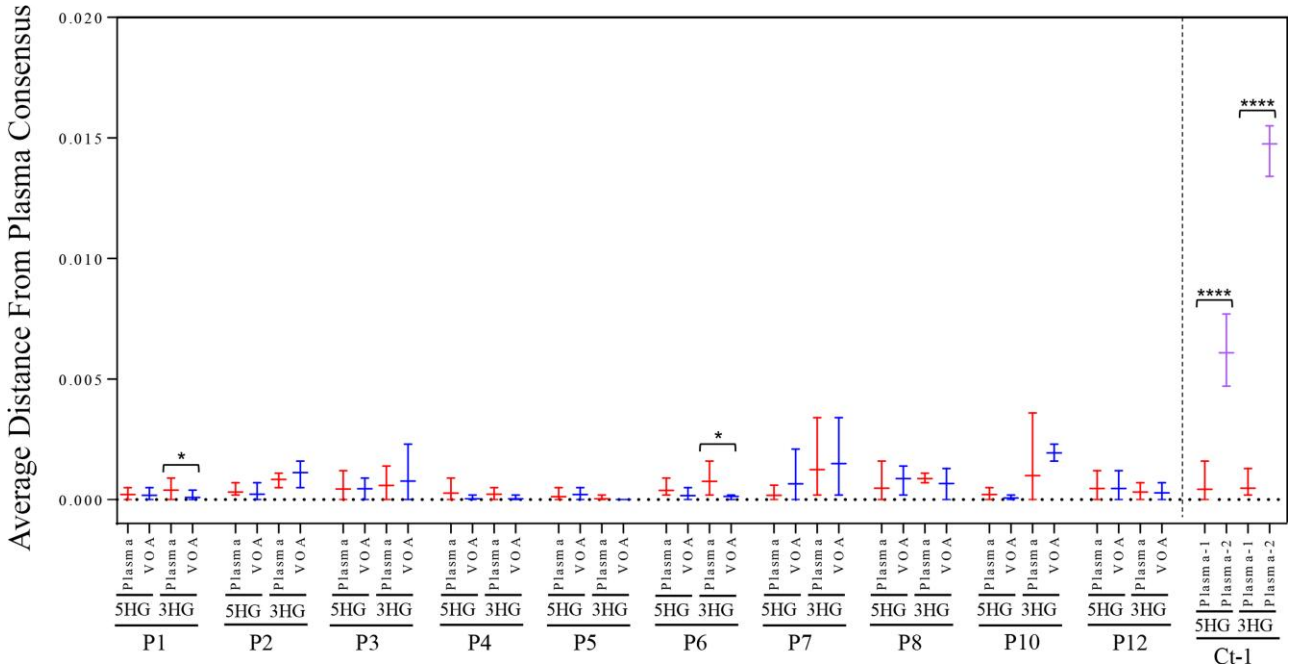

Figure 2. Average distance from plasma consensus sequence. Evolutionary divergence (nucleotide substitutions per site) derived from genetic distance between individual sequences and the plasma consensus sequence was computed in MEGA. Each participant (P1, P2, etc.) on suppresive therapy has two types of sequences (plasma sequences and VOA sequences) labeled on the x axis and also separated for analysis as the 5′-half genome (5HG) and 3′-half genome (3HG). For the viremic control participant (Ct-1), the average distance from plasma consensus from the first and second plasma sample collections are shown in pairs. The level of statistical significance from the Mann-Whitney test is indicated by asterisks. The 5HG represents nucleotides 790 to 5096 on the HXB2 genome, covering the *gag-pro-pol* region, and the 3HG represents nucleotides 5097 to 9530 on HXB2 covering the rest of the HIV-1 genome. Abbreviation: VOA, virus outgrowth assay.

to identify ongoing viral evolution. When 5′-half genome sequences (*gag-pro-pol*) were analyzed for drug resistance mutations, we found only a few sporadic examples of transmitted drug resistance mutations (Supplementary Table 3). No new drug resistance mutations were detected in the viral sequences in the VOA wells (Supplementary Table 3), indicating that if viral replication occurred it did so without fixing drug resistance mutations.

The adaptive immune response can drive the outgrowth of escape mutations within targeted epitopes. We mapped HIV-1 reactive T-cell responses (using HIV-1 subtype B consensus sequence peptides) for 4 participants, shown in Supplementary Figure 2. There was emergence of putative escape mutations in and around reactive T-cell epitopes but no significant differences in diversity between the entry plasma viral sequences and the VOA outgrowth viral sequences at those sites (Supplementary Table 4), consistent with our previous observations [58].

Finally, we considered the selective pressure of an antibody response. The V1-V2 variable region of the viral Env protein is one of the most genetically unstable regions of the viral genome, being the target of type-specific antibody responses. However, when we carried out a phylogenetic analysis of just the V1-V2 region of the *env* gene, we saw no evidence of any sequence diversification in comparing the pretherapy plasma sequences and the VOA viral sequences (Figure 3), while the sequences obtained from the viremic control participant exhibited significant diversification (Mann-Whitney test $P < .001$). Thus, even when considering focused regions of the viral genome under drug and host selective pressure, we found no evidence for viral evolution during the period of suppressive therapy.

DISCUSSION

Development of successful HIV-1 cure strategies requires a clear understanding of the mechanism(s) responsible for the maintenance of the HIV-1 reservoir during suppressive therapy. In this study, we searched for evidence that ongoing viral evolution is a mechanism for the maintenance of the HIV-1 reservoir by comparing HIV-1 populations from plasma at the time of therapy initiation early after infection with those from the latent reservoir after an average of 4.5 years of therapy in 10 participants. For analysis of virus in the reservoir, we focused on inducible, replication-competent virus present in resting CD4+ T cells. We also compared evolutionary divergence observed in participants on therapy with that observed in 1 untreated viremic participant. If ongoing viral replication is a

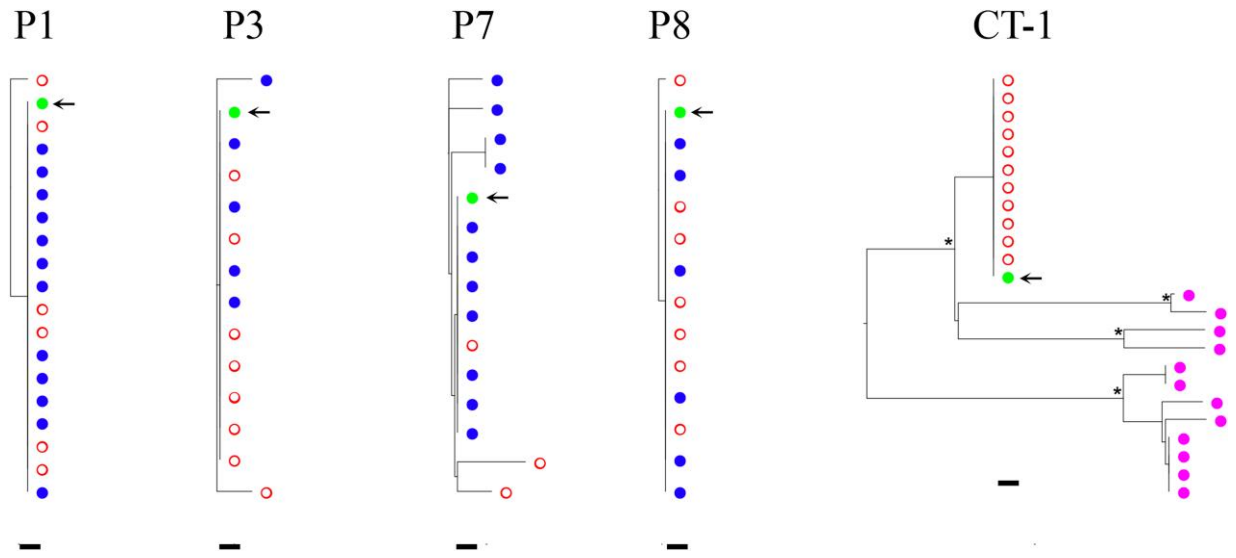

Figure 3. Maximum-likelihood phylogenetic trees of viral RNA at the *env* gene V1-V2 region. Participants P1, P3, P7, and P8 were on suppressive therapy. Sequences derived from pretherapy plasma samples are shown in open circles (red), and sequences derived from latent reservoir after years of therapy (culture supernatants from VOA wells) are shown in filled circles (blue). CT-1 was an untreated viremic participant as the positive control. Sequences derived from the plasma samples collected at 2 different time points are shown in open circles (first collection) and filled circles/purple (second collection). Consensus sequences from the (first) plasma specimens are indicated by an arrow pointing to the green dot. The scale bars represent 1 nt substitution per 200 nts. Asterisks indicate branch points that had >80% bootstrap support. Abbreviation: VOA, virus outgrowth assay.

mechanism for the maintenance of the HIV-1 reservoir during suppressive therapy, we would expect to see some level of evolutionary divergence in participants on therapy, as is observed in untreated viremic participants. Such evolution is easily observed in people with uncontrolled viremia off therapy and can also be seen in elite controllers where the levels of circulating virus are very low [60–63]. When we examined full-length genome sequences by phylogenetic analysis and by computing AGD from the baseline plasma virus consensus sequence, we did not detect significant genetic changes over time, where changes were easily observed in the untreated viremic participant over shorter periods of time. In addition, drug resistance mutations, T-cell escape mutations, and *env* gene variable region diversification were not detected in the participants on suppressive therapy. Therefore, we conclude that our findings do not support the hypothesis of reservoir maintenance due to viral replication in any of the 10 participants on suppressive therapy. These data are consistent with the reservoir originating from cells infected before the initiation of ART, with no further viral replication occurring in the presence of ART.

We employed several relevant criteria in our study design. First, we used viral RNA sequences derived from replication-competent virus in the latent reservoir for phylogenetic analysis to represent viral sequences contributing to HIV-1 evolution. Proviral HIV-1 DNA sequences from participants after years of suppressive therapy have been analyzed to study HIV-1 evolution during therapy and failed to detect any sequence evolution [41–49]; however, most of these proviruses are defective and could not contribute to further replication and potential evolution. Second, unlike other studies in which only a portion of the HIV-1 genome was investigated, we inspected the entire genome to search for genetic changes over time, allowing us to consider different types of selective pressure that could drive evolution. Lastly, to avoid any confounding effects coming from PCR-mediated recombination and misincorporation, we used an end-point dilution PCR method to generate 5′- and 3′-half genomes from plasma viral RNA and end-point dilution of virus outgrowth wells. The use of end-point dilution strategies allows a consensus sequence to be generated for each amplicon, thus removing low-abundance PCR errors. Taken together, our study and most earlier studies [41–49] are consistent in showing a lack of sequence evolution of viral genomes in the latent reservoir.

There are several limitations of this study. First, although we used sequences from accurate end-point dilution sequencing to detect low levels of HIV replication, the sampling depth, that is, the sensitivity of detection, was limited by the number of sequences we obtained. Second, participants in this study were treated with NNRTI- or PI-based ART regimens (Supplementary Table 1). Similar studies focusing on individuals treated with second-generation integrase inhibitors need to be conducted. Third, we analyzed 10 participants who initiated ART with acute/early infection, who were likely to have smaller reservoir sizes than individuals who start ART in chronic infection. Finally, although we looked for evidence of evolution in the *env* variable regions, early treatment is known to blunt the antibody response; for 8 of the participants, we know that 2 did not react to gp120 in

the initial Western blot analysis, 2 had an equivocal reaction, and 4 showed clear reactivity to gp120, although this does not validate selective pressure.

There is strong evidence in support of an alternative model for reservoir maintenance that does not involve viral replication. Much, if not all, viral DNA persists as clonally maintained latently infected CD4+ T cells [64–66]. These CD4+ T-cell clones can include intact viral genomes [12]. These clones can be induced to produce virus in cell culture, and clonally expanded cells can also be observed in some cases to produce low-level viremia in vivo, although this virus does not evolve drug resistance, consistent with virus release but not replication [11, 12, 14, 27, 67]. This alternative model also accounts for the persistence of virus in a latent state in the absence of viral sequence evolution.

Supplementary Data

Supplementary materials are available at *Open Forum Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

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Potential conflicts of interest. The authors report no potential conflicts.

Patient consent. This study was reviewed and approved by the University of North Carolina at Chapel Hill Biomedical Institutional Review Board. Written informed consent was obtained from all participants before sample collection.

Author contributions. S.-K.L., A.S., Y.X., J.W., S.Z., M.G., B.M.H., and N.A. carried out the experiments and analysis of the data, C.G., J.D.K., J.J.E., and D.M. recruited the participants and collected and managed the samples, N.G. and R.S. conceived the study and supervised the work, and S.-K.L., S.Z., and R.S. wrote the manuscript.

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