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Persistent HIV-1 transcription in CD4$^+$ T cells from ART-suppressed individuals can originate from biologically competent proviruses

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ABSTRACT

HIV-1 is able to persist in the face of potent antiretroviral therapy (ART). A number of strategies are being explored to allow ART-free viral remission or viral eradication. In order to gauge the progress of these strategies, assays with which to measure viral reservoir size and activity are needed. In a large percentage of aviremic individuals on suppressive ART, viral transcripts can be detected in peripheral blood CD4$^+$ T cells. While this cell-associated RNA has been considered as a marker of viral reservoir activity, it is unclear whether cell-associated viral transcripts in aviremic individuals originate from biologically competent proviruses as opposed to being a product of abortive transcription from defective proviruses. We assessed whether cell-associated viral RNA in peripheral blood CD4$^+$ T cells from aviremic individuals on ART originated from biologically competent proviruses. We demonstrate that cell-associated viral RNA transcripts were highly related to viral sequences obtained by *ex vivo* outgrowth. This relationship was also observed when viral transcription in the outgrowth cultures was limited to donor CD4$^+$ T cells. Our study indicates that cell-associated viral RNA warrants further consideration as a viral reservoir surrogate in individuals on suppressive ART.

1. Introduction

Antiretroviral therapy (ART) has improved the prognosis for HIV-1-infected individuals by halting viral replication and disease progression. Furthermore, through prophylactic use, ART has had significant impact on new HIV-1 infections.[1] While ART can indefinitely sustain suppression of plasma viremia, ART interruption leads to a rapid rebound in plasma viral RNA. This points to the existence of viral reservoirs that can sustain HIV-1 persistence in the face of effective ART. As strategies for control and elimination of persistent viral reservoirs are pursued, it is important to understand the mechanisms of viral persistence during effective ART and to identify viral or host surrogates that can inform on the dynamics and size of the viral reservoirs.

A number of standardized assays with which to gauge reservoir size and activity, have been described (for reviews, see Ref. [2,3,4,5,6,7]). The challenge has been in developing reservoir assays that inform on the biologically competent reservoir i.e. that component of the reservoir that contains replication-competent virus capable of initiating viral recrudescence if antiviral suppression is halted.[8] While most reservoir assays are nucleic acid-based, their interpretation is hampered by the fact that most viral nucleic acids within cells from infected individuals on ART is defective-comprising non-functional proviruses that harbor inactivating point mutations and deletions.[9] Viral outgrowth assays have the capacity to assess the replication competent proviruses but are less practical for archival samples of limited availability.

Cell-associated viral transcripts are detectable in CD4 + T cells in most individuals on effective ART.[10,11,12,13,14,15] This component of the reservoir, sometimes referred to as the expressed viral reservoir[16] contains unspliced, genomic viral RNA as well as sub-genomic and multiply spliced RNAs[10] that exhibit different decay rates in individuals after initiating ART.[17] Investigators have attempted to define whether there are associations between cell-associated RNA under ART and viral reservoir measures as well as indicators of host immunopathogenesis. Although there was a strong correlation between proviral DNA copy number and levels of cell-associated RNA in ART-suppressed subjects, cell-associated RNA levels did not correlate with levels of persistent

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viremia on ART[15] which might indicate that cell-associated RNAs in ART-suppressed individuals are not biologically competent for virion production. While that study suggested that ongoing viral transcription did not contribute to residual viremia in ART-suppressed individuals, in such individuals, there was a strong correlation between levels of unspliced viral transcripts in unstimulated PBMCs and inducible virus production *ex vivo*.[18] Although unspliced RNAs are detectable in PBMCs from ART-suppressed individuals, those cells lack spliced messages that encode rev proteins that are required for nuclear export of unspliced mRNAs.[19] Since nuclear export of unsliced RNA is necessary for RNA to reach sites of virus assembly at the plasma membrane, this might account for lack of association between levels of cell-associated RNA and residual viremia on ART. There is some debate as to the immunopathogenic consequences of ongoing viral transcription under ART. Markers of immune inflammation were associated with plasma viral loads pre-ART but not with cell-associated RNA during ART[20] In contrast, higher levels of HIV-1 transcription were associated with increased systemic inflammation but increased viral transcription did not appear to be a result of that systemic inflammation.[21]

While it is unclear whether the presence of cell-associated RNAs reflect reservoir cells exiting latency or whether they reflect reservoir cells in different cell cycle states, levels of cell-associated RNA were shown to predict rates of virologic suppression on ART.[22],[12] More recently, higher levels of cell-associated viral RNA correlated with more rapid viral rebound following ART interruption.[16],[23] While those studies suggest a relationship between levels of cell-associated viral RNA and viral rebound dynamics, the nature of the cell-associated RNA is not well defined. In this study we examined whether cell-associated RNA in PBMCs from ART-suppressed individuals originate from biologically competent proviruses. We demonstrate that virions produced from PBMCs *ex vivo,* either with or without an activation stimulus, are closely related to pre-existing, cell-associated transcripts. This indicates that cell-associated transcripts are competent and templates for production of replication-competent virus. This adds to the evidence supporting cell-associated RNA as a valid marker for reservoir analysis.

2. Materials and methods

2.1. Study population

We studied 14 HIV-1-infected individuals who were recruited at the AIDS Clinical Research Unit of the Miami Center for AIDS Research (CFAR) at the University of Miami Miller School of Medicine. The protocol was approved by the University of Miami Institutional Review Board and the participants provided written consent for participation after an informed consent process. All participants had chronic HIV-1 infection, were on suppressive ART regimens and had undetectable plasma viral loads at the time of recruitment. Viral load was assessed in the sample drawn at recruitment using the COBAS® Ampliprep/ COBAS® Taqman® HIV-1 test kit version 2.0 (Roche Molecular Systems, Inc).

2.2. $CD4^+$ T lymphocytes isolation and cell culture for virus recovery

PBMCs were isolated from 50 to 100 ml of EDTA blood by gradient centrifugation (Ficoll-Paque Plus; GE Healthcare) and $CD4^+$ T lymphocytes were enriched from the PBMC by negative magnetic separation ($CD4^+$ Cell isolation Kit; Miltenyi Biotec). A fraction of these $CD4^+$ T cells was stored following baseline (levels found prior to *ex-vivo* cell activation) cell-associated RNA and DNA measurements. Another fraction was resuspended in RPMI +10% fetal bovine serum (R10) at 2×10^6 cells/ml. In most cases the cells were stimulated for 18 h with 50 ng/ml PMA (SIGMA) and 1 μM ionomycin (SIGMA). After activation, cells were washed with medium, counted and plated in cell culture wells with R10 + 30 U/ml IL-2. Co-cultures were then set up by adding activated uninfected donor $CD4^+$ T cells to each well. In some cases the participant's cells were cultured by themselves i.e. no co-culture with feeder cells. In some experiments, 1 μM nevirapine (NVP) was added to the culture medium to prevent new rounds of viral replication. Cell cultures were monitored for viral production by measuring p24 antigen in the supernatant (Lenti-X p24 Rapid Titer Kit; Clontech). Cells were harvested when p24 was detectable in the supernatant and stored for analysis of cell -associated RNA and DNA as well as viral RNA in culture supernatants.

2.3. Isolation and amplification of cell-associated RNA and DNA

RNA and DNA were isolated from cell pellets using Trizol (Life Technologies) and QIAamp DNA Blood Mini Kit (Qiagen), respectively. For the quantification of unspliced, cell-associated RNA, the RNA was digested with DNase I (Invitrogen) and then retro-transcribed to cDNA using SuperScript First-Strand synthesis system (Invitrogen) with the HIV-1 specific primer LA17/r (5′-TCTCCTTCTAGCCTCCGCTAGTCAA-3′). cDNA was then used in a real-time PCR reaction (qPCR) using primers LA17/r and C1 (5′-TAGACCAGATCTGAGCCTGGGA-3′), plus a Taqman probe (5′-AGCCTCAATAAAGCTTGCCTTGAGTGC-3′). For the phylogenetic analysis of the cell-associated RNA and DNA, the C2V4 region of the *env* gene was amplified by end-point PCR from RNA using the Superscript III One-Step PCR System (Invitrogen) and from DNA using the Phusion High-fidelity DNA polymerase (New England Biolabs). In both cases, the primers used were LA11 (5′-CACAGTACAATGTACACATGGA-3′) and env7b (5′-TTCCTACTTCCTGCCACATGTTTA-3′), which amplify a 565 bp fragment suitable for deep-sequencing analysis.

2.4. Deep-sequencing analysis

Gel purified PCR *env* products were modified with additional overhangs to bind Illumina barcodes and further analyzed with Illumina MiSeq Sequencing, 300x2 PE. Adapter and primer sequences were trimmed with Cutadapt[24] from raw sequences. Raw sequences were filtered with TRIMMOMATIC[25] defining a minimum of 250bp and a 30:20 quality threshold. Resulting paired-end sequences were merged into single sequences using PEAR software[26] and human contaminating sequences were filtered using bowtie against hg18 human reference. Usearch[27] was used to detect chimeric sequences and cluster the resulting dataset at a 98% similarity threshold. All unique sequences supported by less than 0.5% of the sequence reads were removed. MAFFT[28] and FastTree2[29] were used to build maximum likelihood phylogenetic trees with all the sequences from all patients to identify potential cross-contaminating ones. After removal of cross-contaminating sequences, IQ-TREE v. 2.1.0[30] was used to construct a phylogenetic maximum likelihood tree for each subject using the best-fitting evolutionary model chosen according to Bayesian information criteria[31] and ultrafast bootstrap (BB) approximation.[32]

2.5. Compartmentalization test

Distance-based F_{ST} test,[33] that compares the mean pairwise genetic distance between two sequences sampled from different compartments to the mean distance between sequences sampled from the same compartment, and tree-based Slatkin and Madison (SM) test,[34] that determines the minimum number of migration events between the separated populations consistent with the structure of the reconstructed phylogenetic tree, were conducted to detect compartmentalization of viral and proviral populations at baseline compared to sequences from cultures. F_{ST} test was performed with 100 permutations and 1000 bootstrap, while SM was conducted with 1000 permutations.

3. Results

We studied 11 HIV-1-infected individuals with chronic HIV-1

Table 1
Characteristics of study participants at the time of recruitment.

PID	Age	Gender	Baseline viral load (copies/ml)	Years on ART	Years with undetectable VL	Baseline CD4 (cells/ul)	ART drugs
VP24	44	F	<20	NA	NA	811	NA
VP29	37	M	<20	4.2	4.0	649	2 NRTI + 1 boosted PI
VP33	51	F	56	1.7	1.0	296	2 NRTI + 1 NNRTI
VP34	55	M	ND	NA	0.7	265	2 NRTI + 1 INI
VP35	46	F	ND	8.0	8.0 *	344	2 NRTI + 1 boosted PI
VP05	46	M	ND	8.4	7.8	506	2 NRTI + 1 NNRTI
VP06	48	M	<20	3.7	3.2	449	2 NRTI + 1 boosted PI
VP07	42	M	ND	8.0	6.1 *	NA	2 NRTI + 1 boosted PI
VP01	60	M	213	2.5	2.0	913	2 NRTI + 1 NNRTI
VP18	56	M	<20	NA	NA	153	NA
VP20	54	M	<20	3.0	NA	462	2 NRTI + 1 INI

PID: participant Identification; ND: not detected; ART: antiretroviral therapy; NA: not available NRTI: nucleoside reverse transcriptase inhibitor; NNRTI: non-nucleoside reverse transcriptase inhibitor; PI: protease inhibitor; INI: integrase inhibitor; NVP: Nevirapine.
* Undetectable VL with occasional blips.
PMA: Phorbol myristate acetate.

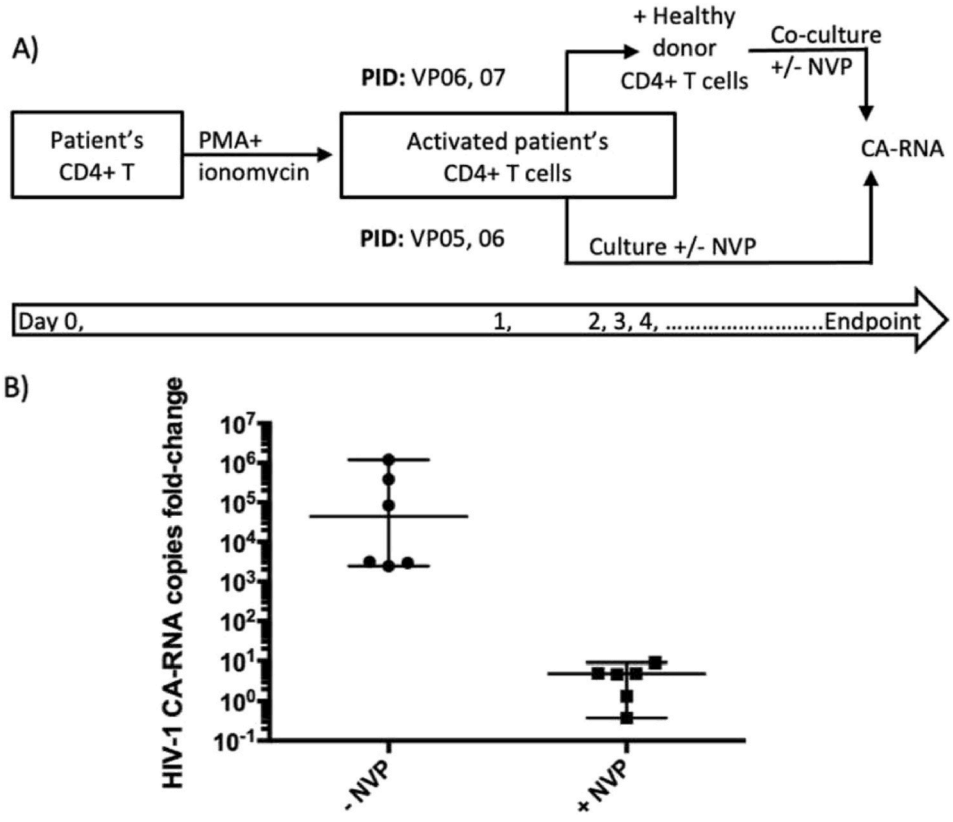

Fig. 1. (A) Experimental design for participants VP 05–07. The cultures and/or co-cultures were set up in the presence and absence of Nevirapine (NVP), which prevents now rounds of viral infection in the culture. At endpoint (+p24 antigen in supernatant of cultures without NVP), CA-RNA copies were quantitated. (B) Fold-change between the number of copies/million cells at endpoint over baseline with or without NVP in the culture.

infection, on ART, who had undetectable HIV-1 viral load in the last blood draw prior to analysis. Eleven subjects were males and the median age was 50 y (37–60 y). The median duration of ART was 4.0 years (y) (1.7–8.4 y) using either 2 nucleoside reverse transcriptase inhibitors (NRTI) plus 1 non-nucleoside reverse transcriptase inhibitor (NNRTI), 2 NRTI plus 1 boosted Protease inhibitor (PI) or 2 NRTI plus 1 Integrase inhibitor (INI) regimens. The median time with undetectable viral load at the time of recruitment (baseline) was 3.6 y (0.7–8.0 y) and their median CD4 count was 449 cells/mm^3 (153–913 cells/mm^3). The individual characteristics of the study subjects at the time of recruitment are shown in Table 1.

Cell-associated RNA was detected in all samples at baseline, with a median of 640 copies/million cells, ranging from 3 to 4401 copies/million cells. This finding prompted the question as to whether this cell-associated RNA present in CD4$^+$ T cells of virologically suppressed individuals was biologically competent. To answer this question, we first assessed if *ex vivo* cell activation was required for virus recovery in cell culture. CD4$^+$ T cells from 5 individuals (PID:VP24, 29, 33, 34, 35) were incubated overnight in the presence or absence of PMA/ionomycin. Patient CD4$^+$ T cells were then co-cultured with healthy donor activated CD4$^+$ T cells and monitored for p24 antigen virus production (Gag p24 ELISA) in the culture supernatants. Cell cultures were kept for an average of 19 days but stopped at 30 days if gag p24 was not detected. Virus was recovered in 5/5 cultures where the patients' CD4$^+$ T cells were activated prior to co-culture with donor CD4$^+$ T cells. In 4 of 5 of these cultures, virus was recovered without the need for initial PMA/

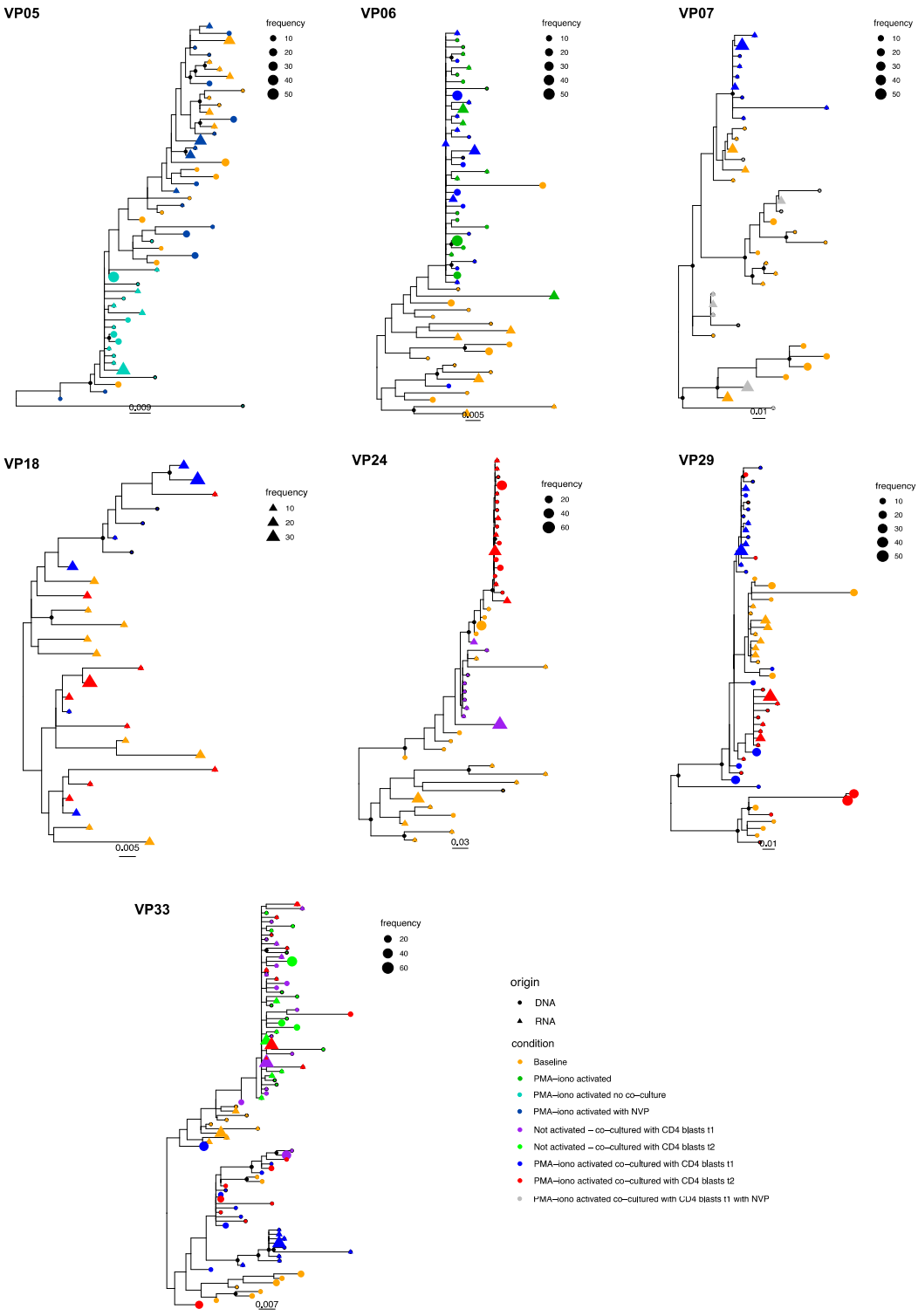

Fig. 2. Maximum likelihood phylogenies of seven patients that show intermixing of RNA baseline sequences with RNA or DNA populations after recovery. Phylogenetic trees are midpoint rooted, tips are colored according to culture condition as detailed in the legend, with baseline sequences given in yellow. Shapes indicate the origin of the sequence: RNA is indicated by triangles and circles denote DNA. Frequency for each sequence is given by the size of the tip shape and a legend is provided for each phylogeny at the right of the tree. Circles at nodes indicate ultrafast bootstrap (BB) support >90%. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Ionomycin -mediated *ex vivo* cell activation. Whenever gag p24 was detected in supernatant, the number of unspliced cell-associated RNA transcripts increased significantly.

We next assessed whether the increase in transcription was due to increased transcription within the original subject $CD4^+$ T cells or whether it was driven by new rounds of infection in these cultures. We wanted to limit the contribution of viral transcripts that emerged following *ex vivo* expansion of reactivated latent proviruses so as to focus only on cell-associated transcripts that existed at baseline. To achieve this, $CD4^+$ T cells from some participants (PID: VP05, 06, 07) underwent

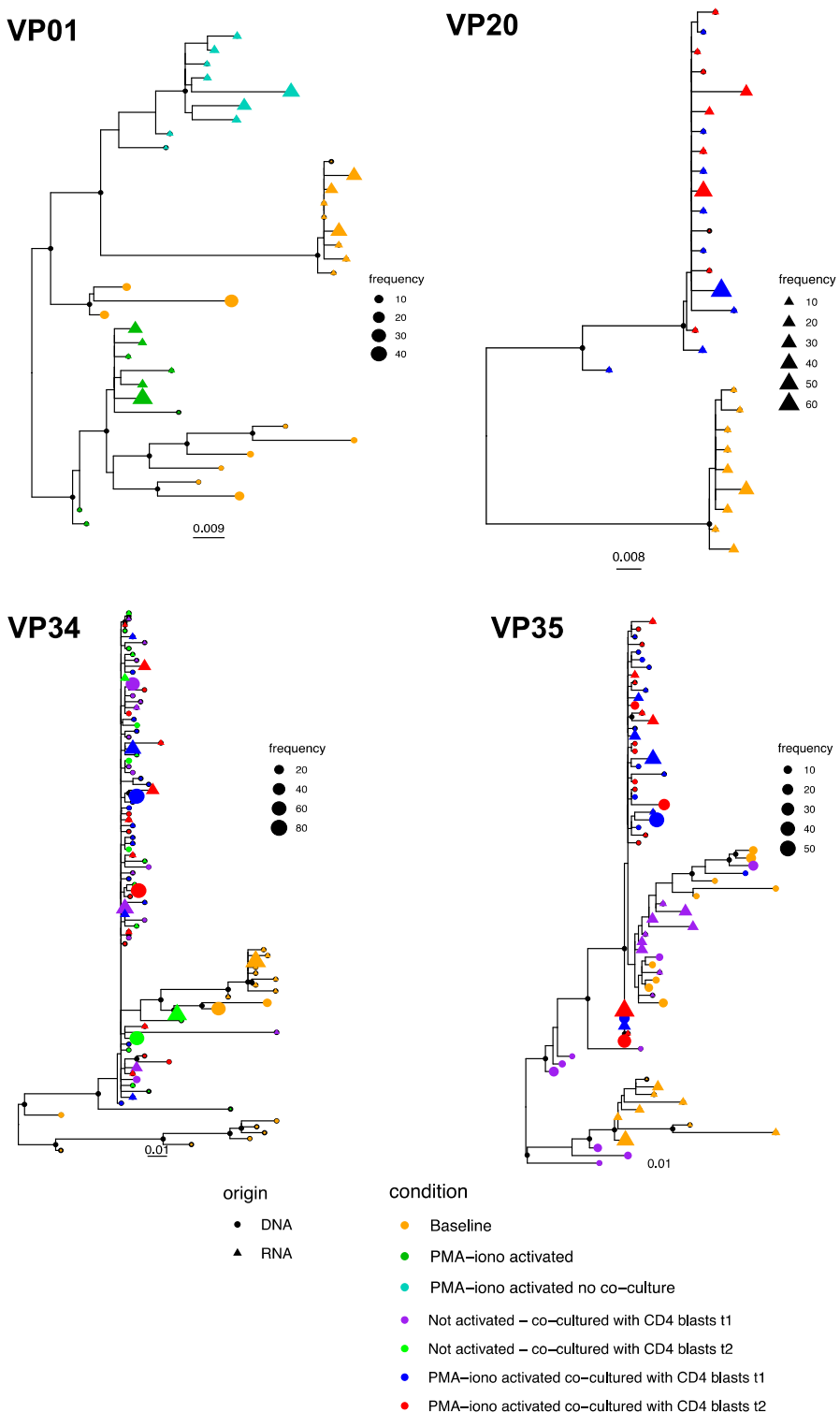

Fig. 3. Maximum likelihood phylogenies of four patients that show compartmentalization of RNA baseline. Phylogenetic trees are midpoint rooted, tips are colored as described in the legend, with baseline sequences indicated in yellow. Shapes indicate the origin of the sequence: triangles denote RNA and circles signify DNA. Frequency for each sequence is given by the size of the tip shape and a legend is provided for each phylogeny at the right of the tree. Circles at nodes indicate ultrafast bootstrap (BB) support >90%. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

PMA/Ionomycin activation in the presence or absence of the reverse transcriptase inhibitor nevirapine (NVP), (Fig. 1a). Inclusion of nevirapine prevents new rounds of infection and limits transcription to the original donor CD4$^+$ T cells, At endpoint (when gag p24 became detectable in the supernatant of cell cultures without NVP), cell-associated RNA was quantitated and the fold-change between the transcript copy number/million cells over the transcript copy number at baseline was calculated (Fig. 1b). Cell-associated HIV transcripts increased significantly after cell culture, both in the presence and in the absence of cocultured, feeder CD4$^+$ T cells (Fig. 1b). By comparison, cell-associated RNA copy number remained constant or slightly decreased compared to baseline when new rounds of infection were halted by inclusion of NVP (Fig. 1b).

In order to assess if the cell-associated RNA found at baseline was biologically competent and capable of yielding virus in culture supernatants, we amplified and sequenced the C2V4 region of the *env* gene

from cell-associated RNA transcripts at baseline, after virus recovery and from proviral DNA from all individuals (PID: VP01, 05, 06, 07, 18, 20, 33, 34, 35, 37). Deep-sequencing and phylogenetic analysis were performed to examine the relationship between the sequences. Phylogenetic analysis showed that there was no cross-contamination between sequences from the participants (Supplemental Fig. S1). Phylogenetic analysis and F_{ST} compartmentalization test showed that in seven out of eleven participants, cell-associated RNA (viral) and DNA (proviral) sequences isolated after virus recovery were closely related to the cell-associated RNA baseline sequences (PID: VP05, VP06, VP07, VP18, VP24, VP29 and VP33) (Fig. 2, Table S1). Strong evidence of compartmentalization (i.e. absence of intermixing) was found for recovered viral population in the remaining four patients (PID: VP01, VP20, VP34, VP35) (Table S1), suggesting that RNA baseline sequences were more distantly related to virus after recovery (Fig. 3). On the contrary, all patients, except for patient VP34, showed intermix of populations after recovery with baseline proviral DNA (Figs. 2 and 3, Table S1). For VP18, proviral DNA was not available. These results show that in some cases, the cell-associated RNA found in $CD4^+$ T cells from aviremic individuals, originates from transcribing, biologically competent proviruses.

4. Discussion

Reservoir research in the cure arena has been developing standardized assays to provide insight into reservoir size and dynamics.[2,3,4,5,6,7] Those studies indicate that the reservoir that persists in infected individuals on suppressive ART regimens is compositionally diverse in terms of viral activity. This either reflects the existence of different cellular reservoirs with distinct states of viral activity or a single reservoir in which viral activity fluctuates. While in its broadest sense the term "viral reservoir" encompasses all virally infected cells, the majority of viral genomes contain inactivating mutations, frameshifts and deletions that render them biologically inactive (i.e. incapable of producing replication competent viruses). Therefore, we use the term "expressed reservoir" to describe that component of the reservoir in which viral components (RNA, proteins) are being expressed either intermittently or persistently. This pool would include cells harboring proviruses that, although expression competent, may be replication incompetent. We use the term "rebound competent reservoir" to describe that component of the reservoir in which the virus is biologically (replication) competent (i.e. it produces viruses capable of fueling viral replication and rebound after a treatment interruption). This pool would include cells with intact, replication competent genomes, that may be expressed or fully latent. Quantitative viral outgrowth assay (Q-VOA) is considered the "Gold Standard" approach with which to gauge the rebound competent reservoir. However, recent studies suggest that viruses generated *ex vivo* in the Q-VOA assay have limited phylogenetic similarity to rebounding plasma viruses following an ATI.[35,36] Therefore, the extent to which current, viral outgrowth-based reservoir assays inform on the size and composition of the rebound competent reservoir, is unclear.

In agreement with previous studies[10,11,12,13,14,15], we detected cell-associated RNA in all virologically suppressed participants included in this study. Cell-associated RNA levels varied from 3 to ~4000 copies/million $CD4^+$ T cells. We found that in seven of 11 individuals, viruses obtained from $CD4^+$ T cells following *ex-vivo* outgrowth, were closely related to the cell-associated-RNA baseline sequences. This indicates that cell-associated RNA in $CD4^+$ T cells from ART-suppressed individuals, are biologically competent. The nature of cell-associated RNA in ART-suppressed individuals is unclear. Latently infected cells may stochastically exit latency, leading to production of viral transcripts. There is also evidence that clonally expanded proviruses can be transcriptionally active and those transcripts resemble viruses that emerge when therapy is interrupted.[37] It is also possible that the continued transcription of some reservoir cells is a consequence of integration site. Integration in genic regions may be favorable to viral gene expression as opposed to integration into non-genic regions that are less conducive to viral transcription. Prior studies have indicated that the levels of cell-associated viral RNA correlated with viral DNA copy number but not with levels of persistent plasma viremia on ART.[15] Instead, persistent viremia under ART originated from clonally expanded proviruses although the transcriptional activity of those proviruses under ART was not assessed.[38] Our analysis does not allow us to conclude whether $CD4^+$ T cells that were transcribing under ART also contained viral proteins. While expression of viral products would be expected to subject cells to host immune clearance, the level of transcription may not be sufficient for accumulation of viral proteins. While the nature of the persistent transcription under ART requires further study, the demonstration that cell-associated RNA levels predict time to viral rebound suggests that this viral marker warrants further consideration as a surrogate of the rebound-competent reservoir.

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Author contributions

M. Stevenson conceived the project. M. Sharkey, M.V, and V.M.A designed the study. M.V., V.M.A., M.N. and C.M performed experiments. M. Stevenson, V.M.A, M.N, R.P and M.S analyzed data. M.N, R.P. C.M and M. Salemi performed phylogeny analysis. C.B. provided patient samples. M. Stevenson, and M.V. wrote the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jve.2021.100053.

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