Short Intracellular HIV-1 Transcripts as Biomarkers of Residual Immune Activation in Patients on Antiretroviral Therapy

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ABSTRACT

HIV-1 patients continue to remain at an abnormal immune status despite prolonged combination antiretroviral therapy (cART), which results in an increased risk of non-AIDS-related diseases. Given the growing recognition of the importance of understanding and controlling the residual virus in patients, additional virological markers to monitor infected cells are required. However, viral replication in circulating cells is much poorer than that in lymph nodes, which results in the absence of markers to distinguish these cells from uninfected cells in the blood. In this study, we identified prematurely terminated short HIV-1 transcripts (STs) in peripheral blood mononuclear cells (PBMCs) as an efficient intracellular biomarker to monitor viral activation and immune status in patients with cART-mediated full viral suppression in plasma. STs were detected in PBMCs obtained from both treated and untreated patients. ST levels in untreated patients generally increased with disease progression and decreased after treatment initiation. However, some patients exhibited sustained high levels of ST and low CD4⁺ cell counts despite full viral suppression by treatment. The levels of STs strongly reflected chronic immune activation defined by coexpression of HLA-DR and CD38 on CD8⁺ T cells, rather than circulating proviral load. These observations represent evidence for a relationship between viral persistence and host immune activation, which in turn results in the suboptimal increase in CD4⁺ cells despite suppressive antiretroviral therapy. This cell-based measurement of viral persistence contributes to an improved understanding of the dynamics of viral persistence in cART patients and will guide therapeutic approaches targeting viral reservoirs.

IMPORTANCE

Combination antiretroviral therapy (cART) suppresses HIV-1 load to below the detectable limit in plasma. However, the virus persists, and patients remain at an abnormal immune status, which results in an increased risk of non-AIDS-related complications. To achieve a functional cure for HIV-1 infection, activities of viral reservoirs must be quantified and monitored. However, latently infected cells are difficult to be monitored. Here, we identified prematurely terminated short HIV-1 transcripts (STs) as an efficient biomarker for monitoring viral activation and immune status in patients with cART-mediated full viral suppression in plasma. This cell-based measurement of viral persistence will contribute to our understanding of the impact of residual virus on chronic immune activation in HIV-1 patients during cART.

Combination antiretroviral therapy (cART) suppresses the HIV-1 load below the detectable limit in the plasma and is effective in delaying the progression of AIDS. However, residual viral replication continues at a low level in some patients (1), and patients continue to remain at an abnormal immune status and have an increased risk of non-AIDS-related morbidity and mortality (2–4). Given these persistent problems, there is growing recognition of the importance of developing novel therapeutic strategies to cure HIV-1 infection (5). To achieve this goal, a better understanding of the dynamics of residual virus, as well as the impact of therapeutic interventions on viral reservoirs, is needed. However, to date, no simple, effective method to measure viral persistence in patients has been available.

To determine the size or activity of the viral reservoirs and to monitor viral persistence in cART patients, additional virological markers are required (6). For example, intracellular HIV-1-specific DNA (e.g., provirus and episomal 2-LTR circles [7–10]) and RNA (e.g., unspliced and multiply spliced [11]) have been proposed as effective targets for quantification. In particular, levels of intracellular viral RNA correlate with patients' conditions, such as

rapid progression to AIDS (12–14). Recently, the methods to detect intracellular viral RNA have been improved using seminested quantitative reverse transcription-PCR (qRT-PCR), patient-matched PCR, or transcription-mediated amplification (TMA) (15–17); however, multiple steps are involved in these strategies. Furthermore, cell-associated viral RNA is still difficult to be detected, which could be due to the low percentage of HIV-1-infected cells among the circulating CD4⁺ T cells (18, 19), as well as

Received 20 December 2015 Accepted 25 March 2016

Accepted manuscript posted online 25 March 2016

Citation Ishizaka A, Sato H, Nakamura H, Koga M, Kikuchi T, Hosoya N, Koibuchi T, Nomoto A, Kawana-Tachikawa A, Mizutani T. 2016. Short intracellular HIV-1 transcripts as biomarkers of residual immune activation in patients on antiretroviral therapy. *J Virol* 90:5665–5676. doi:10.1128/JVI.03158-15.

Editor: G. Silvestri

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premature termination of viral transcription. *In vitro* observations demonstrate that insufficient elongation of viral transcripts results in prematurely terminated short transcripts (STs) comprising the first 60 to 70 nucleotides (nt) of HIV-1 RNA (20–23). This termination is caused by tightly closed nucleosome-1 (nuc-1), which is positioned immediately after the transcription initiation site in the 5′ long terminal repeat (LTR) (24, 25). The disruption of nuc-1 is necessary for efficient elongation of viral transcripts (25, 26). Host factors involved in dynamic modifications of chromatin structure, such as the SWI/SNF chromatin remodeling complex, as well as histone deacetylases and histone acetyltransferases (HATs), are known to modify viral elongation (27–29). We previously reported the Brm-type SWI/SNF complex is required for the disruption of nuc-1 and enhances HIV-1 transcriptional elongation (30). Moreover, HIV-1 transcriptional activation is dependent upon host transcription factors. For example, nuclear factor κ B (NF- κ B) and nuclear factor of activated T cells (NFAT) also enhance the elongation events via recruiting HATs and the SWI/SNF complex to the LTR (31, 32). In addition to these host factors, the viral trans-activating protein Tat also influences transcriptional elongation, not only by enhancing phosphorylation of the C-terminal domain of RNA polymerase II (33) but also by recruiting HATs to the HIV-1 LTR (34). However, in circulating resting CD4⁺ T cells, NF- κ B and NFAT are sequestered in the cytoplasm, and the expression of Brm is downregulated (30).

Since viral replication in peripheral blood mononuclear cells (PBMCs) is much poorer than in lymph nodes (35), cell-associated viral transcripts are generally difficult to detect. However, STs have been detected in PBMC samples from symptomatic and asymptomatic patients with AIDS (20). Further, Lassen et al. found that STs are a predominant viral product in purified resting memory CD4⁺ T cells from cART patients with suppressed viral loads in plasma (36), suggesting that STs could be a marker for circulating infected cells. Moreover, because STs are the initial product of viral transcription that are not affected by subsequent processes, such as transcriptional elongation and alternative splicing steps, they may serve as a direct marker for understanding the transcriptional activity of the HIV-1 LTR. However, no studies to date have quantified the expression of STs in individual patients; therefore, the relationship between ST levels and clinical conditions is not known.

In this study, we developed a method to quantify the levels of STs based on a single round of qRT-PCR in PBMCs from HIV-1-infected patients and investigated the relationship between ST levels and the patients' clinical conditions. ST levels were strongly correlated with persistent immune activation, suggesting that transcriptional activation of residual virus in circulating infected cells reflects sustained immune activation during effective cART. Given that persistent immune abnormalities during cART are harmful to patients, because they are thought to help maintain viral reservoirs and cause non-AIDS-related diseases, monitoring STs and intervening with therapy to diminish ST levels will contribute to improving the prognosis of HIV-1 patients.

MATERIALS AND METHODS

Patients and clinical samples. PBMCs were obtained from patients with chronic HIV-1 infection, 164 untreated subjects (median and interquartile range [IQR] values are shown: median HIV-1 RNA load, 26,500 [8,225 to 60,750] copies/ml plasma; median CD4⁺ cell count, 293 [204 to 400] cells/ μ l) and 96 subjects receiving cART for at least 6 months with com-

plete viral suppression (<50 copies/ml; median CD4⁺ cell count [IQR], 399 [335 to 1,265] cells/ μ l; median cART duration [range and IQR], 352 [192 to 4,922 and 272 to 473] days). All participants provided written informed consent, and this study was approved by the institutional review board of the Institute of Medical Science at the University of Tokyo (20-47-210521, 20-31-1120). For PBMC isolation from blood, Ficoll-Hypaque density gradient centrifugation was used and the samples were frozen in liquid nitrogen. For analysis of STs, approximately 0.5×10^6 to 5×10^6 PBMCs (or 3 to 4 ml of the blood) from each patient were used. We did not blind investigators to treatment, but there were no subjective assessments made.

Cell cultures and measurement of viral particles. U937 and CEM cells were obtained from the JCRB Cell Bank (National Institute of Health Sciences, Tokyo, Japan) and the Cell Resource Center for Biomedical Research, Institute of Development, Aging, and Cancer (Tohoku University, Sendai, Japan), respectively. U1 and ACH-2 cells were gifted from T. Watanabe (Institute of Medical Science, University of Tokyo, Tokyo, Japan). U937, CEM, U1, and ACH-2 cells were maintained in RPMI 1640 medium (Wako Chemicals, Tokyo, Japan) containing 10% fetal calf serum at 37°C in an atmosphere containing 5% CO₂. Cells were treated with 10 ng of tumor necrosis factor alpha (TNF- α ; R&D Systems, Minneapolis, MN)/ml. Viral particles were measured using an HIV-1 p24 antigen enzyme-linked immunosorbent assay (ELISA; ZeptoMetrix, Buffalo, NY) according to the manufacturer's instructions.

RNA purification and qRT-PCR analysis. For analysis of STs and elongated transcripts, the fractions of small RNA (<200 nt) and large RNA (>200 nt) were purified from PBMCs or cultured cells using Isogen II (Nippon Gene, Toyama, Japan), followed by treatment with Turbo DNase (Ambion, Austin, TX) in accordance with the manufacturer's instructions. cDNA was synthesized using the miScript II RT kit with attached 5 $\times$ miScript HiFlex Buffer (Qiagen, Valencia, CA) for STs, and using the PrimeScript RT master mix [Perfect Real Time] (TaKaRa Bio, Shiga, Japan) for elongated transcripts. The amplification reactions were performed in a CFX96 real-time PCR detection system (Bio-Rad Laboratories, Hercules, CA) using Premix Ex Taq [Probe qPCR] (TaKaRa Bio). The PCR protocol consisted of an initialization step at 95°C for 30 s and 50 cycles at 95°C for 5 s and 60°C for 10 s. For the detection of STs, the following primers and a fluorescent TaqMan probe were used: forward primer, 5′-GGT TAG ACC AGA TCT GAG CCT G-3′ (nt 10 to 31); reverse primer, 5′-AAT AAA TCA TAA CAG TTA CGC ATG CCG AGG TC-3′; and TaqMan probe, 5′-6FAM-CTA GCT AGC CAG AGA GCT CCC AGG-BHQ1-3′ (nt 28 to 51). The reverse primer was designed to bind to a universal tag sequence on the 5′ end of the cDNA, and contains a short 5′ AT-rich flap (underlined nucleotides) to improve qRT-PCR signals (37). The cutoff value between the ST⁺⁺ and ST⁺ groups were determined according to T cell activation defined by HLA-DR⁺/CD38⁺/CD8⁺ T cells. Given that PBMC samples with the ST values higher than 1.5×10^4 copies/ 10^6 PBMCs showed significantly elevated levels of T cell activation, we considered this value provides a physiological meaning. For the detection of elongated viral transcripts, the following primers and a TaqMan probe were used: forward primer, 5′-TGT GTG CCC GTC TGT TGT GT-3′ (nt 103 to 122); reverse primer, 5′-GCC GAG TCC TGC GTC GAG AG-3′ (nt 229 to 248); and TaqMan probe, 5′-6FAM-CAG TGG CGC CCG AAC AGG GA-BHQ1-3′ (nt 179 to 198).

Nested qRT-PCR of elongated HIV-1 transcripts. cDNA was prepared from large RNA (>200 nt) fraction as described in Materials and Methods. The first round of PCR protocol was consisted of an initialization step at 95°C for 30 s and 15 cycles at 95°C for 5 s and 69°C for 10 s. For the first round of PCR, the following primers were used: forward primer, 5′-TGT GTG CCC GTC TGT TGT GT-3′ (nt 103 to 122); and reverse primer, 5′-GCC GAG TCC TGC GTC GAG AG-3′ (nt 229 to 248). The second round of PCR protocol was consisted of an initialization step at 95°C for 30 s and 40 cycles at 95°C for 5 s and 69°C for 10 s. For the second round of PCR, the following primers and a fluorescent TaqMan probe were used: forward primer, 5′-CTG GTA ACT AGA GAT CCC TCA

FIG 1 HIV-1 ST detection in *in vitro* models for postintegration HIV-1 latency. (A) Secondary structure of the first 110 nt of the HIV-1 genome. (B) Summary of the method to detect STs. (C) Isolation of the large and the small RNA fractions from cell lines and PBMCs. The fractions of large RNA (>200 nt) and small RNA (<200 nt) were purified from U937 and U1 cell lines, as well as PBMCs obtained from a healthy donor. The RNA samples were electrophoresed on a 5 to 20% gradient polyacrylamide gel. The 5.8S rRNA band (~160 nt) and bulk tRNA bands are shown. *In vitro*-transcribed RNA samples were used as RNA length markers. (D) Copy numbers of STs determined in cells chronically infected with HIV-1 (U1 and ACH-2), uninfected parental cells (U937 and CEM), and PBMCs obtained from three healthy controls. (E) ST copy numbers were determined using 10-fold serial dilutions of U1 cells in U937 cells. (F) Changes in the ST levels and particle production in U1 and ACH-2 cells after TNF- α stimulation. Viral particles in the supernatants were assessed using an ELISA to detect HIV-1 p24. HC, healthy control; ND, not detected.

GAC CC-3' (nt 127 to 152); reverse primer, 5'-AGA GAG CTC CTC TGG TTT CCC-3' (nt 212 to 232); and TaqMan probe, 5'-6FAM-CAG TGG CGC CCG AAC AGG GA-BHQ1-3' (nt 179 to 198).

Proviral load analysis. Genomic DNA was extracted from PBMCs using a MagExtractor Genome kit (Toyobo, Osaka, Japan) in accordance with the manufacturer's instructions. Nested qPCR was performed using

Premix *Ex Taq* [Probe qPCR] (TaKaRa Bio). The primary PCR protocol consisted of an initialization step at 95°C for 30 s and 15 cycles at 95°C for 5 s and 60°C for 20 s using the following primer pair: forward primer, 5'-GAC TAG CGG AGG CTA GAA GG-3' (nt 310 to 329) and reverse primer, 5'-CGA ATC GTT CTA GCT CCC TG-3' (nt 451 to 470). The second PCR protocol consisted of an initialization step at 95°C for 30 s and

FIG 2 Increased levels of STs were associated with decreased CD4⁺ cell counts in patients who never received cART. (A) Detection of STs and elongated viral transcripts in PBMCs from 164 patients who never received cART. (B) Comparison of CD4⁺ cell counts among the 164 patients not treated with cART according to ST expression levels in their PBMCs. ST⁺⁺ and ST⁺ refer to high ($\geq 1.5 \times 10^4$ copies/ 10^6 PBMCs) and low ($< 1.5 \times 10^4$ copies/ 10^6 PBMCs) ST levels, respectively. (C) Correlation between the ST levels and CD4⁺ cell counts among the 121 ST-positive (ST⁺⁺ and ST⁺) patients shown in panel B. (D) Comparison of plasma viral loads among the same patients as in panel B. (E) Correlation between the ST levels and plasma viral load among the same patients shown in panel C. One sample from each patient was included in each analysis shown. For panels B and D, $n = 62$ ST⁺⁺ patients, $n = 59$ ST⁺ patients, and $n = 43$ ST⁻ patients. For panels C and E, $n = 121$ ST-positive patients. The Kruskal-Wallis test, followed by Dunn's multiple comparison tests (B and D) and Spearman's rank correlation test (C and E), were performed. Horizontal lines and error bars in panels B and D indicate median and IQR values, respectively. NS, not statistically significant.

50 cycles at 95°C for 5 s and 60°C for 10 s using the following primer pair and a fluorescent TaqMan probe: forward primer, 5'-GAG AGA GAT GGG TGC GAG AG-3' (nt 329 to 348); reverse primer, 5'-CCC TGC TTG CCC ATA CTA TAT G-3' (nt 434 to 455) and TaqMan probe, 5'-6FAM-CGG TTA AGG CCA GGG GGA AAG-TAMRA-3' (nt 395 to 415).

Preparation of standards for qPCR analysis. To generate a template for transcribing RNA standards, a PCR product was generated by amplification of the plasmid pWLG (30) using the primer pairs as follows: 5'-TAA TAC GAC TCA CTA TAG GGG GGT CTC TC-3' and 5'-GGG TTC CCT AGT TAG CCA GAG-3' for STs; and 5'-TAA TAC GAC TCA

CTA TAG GGT GTG TGC CC-3' and 5'-GCC GAG TCC TGC GTC GAG AG-3' for elongated viral transcripts. *In vitro* transcription reactions were performed using the DNA template with MEGAscript T7 reagent (Ambion). Standard curves were generated by synthesizing cDNAs from serially diluted *in vitro* transcripts. For quantification of proviral load, a standard curve was generated using genomic DNA of U1 cells, which harbor two copies of integrated proviruses.

Flow cytometric analysis. Cryopreserved PBMCs were thawed and rested overnight. The following day, the expression of activated (HLA-DR, CD38), exhaustion (PD-1), and senescent (CD57) markers on CD4⁺

Patients with untreated samples

Patients with both treated and untreated samples

FIG 3 Longitudinal change in the ST levels and CD4⁺ cell counts in individual patients before and/or after the initiation of cART. Black and gray lines refer to the ST levels and the corresponding CD4⁺ cell counts, respectively. ST levels lower than the dotted lines are ST⁻. The corresponding viral load (copies/ml plasma) is shown below each time point according to treatment initiation shown in the individual graphs.

and CD8⁺ T cells were assessed by multicolor flow cytometric analysis. The following antibodies were used for the flow cytometric analysis: CD3-Brilliant Violet 510 (catalog no. 317332), CD4-PerCP (catalog no. 317432), CD8-PerCP (catalog no. 344708), CD45RA-APC-Cy7 (catalog no. 304128), CCR7-PE-Cy7 (catalog no. 353226), HLA-DR-APC (catalog no. 307618), CD38-PE (catalog no. 303516), PD-1-FITC (catalog no. 329904), and CD57-Pacific Blue (catalog no. 322316) (BioLegend, San Diego, CA). Dead cells were excluded with the propidium iodide staining (Sigma-Aldrich, St. Louis, MO). Cells to be analyzed were Fc-Blocked with FcR Blocking Reagent (Miltenyi Biotec, Auburn, CA) at 4°C for 10 min. The expression levels of CD45RA, CCR7, HLA-DR, CD38, PD-1, and CD57 were analyzed by comparing to “fluorescence-minus-one” (FMO) controls (38). Samples were analyzed on FACSARIA flow cytometer cell sorter (Becton Dickinson, Heidelberg, Germany), and data were analyzed with FlowJo software (Tree Star, San Carlos, CA).

Statistical analyses. We performed Mann-Whitney and Spearman’s rank correlation tests, as well as the Kruskal-Wallis test, followed by Dunn’s multiple comparison tests. All statistical tests were two sided. We considered *P* values of <0.05 to be statistically significant. No statistical method was used to predetermine sample size, and no samples were excluded from this study. Statistical analyses were performed, and graphics were generated using GraphPad Prism 6.0 software (GraphPad Software, Inc., La Jolla, CA).

RESULTS

Validation of the method to measure STs. Given that prematurely terminated short viral transcripts (STs, Fig. 1A) are predominantly produced by circulating memory CD4⁺ T cells (36), the best-characterized reservoir for HIV-1 (39), STs may indicate residual viral burden with increased accuracy. Therefore, we developed a qRT-PCR method to measure the level of STs (Fig. 1B) and validated its sensitivity and accuracy using cell lines. We purified both the fractions of small RNA (<200 nt) and large RNA (>200 nt) (Fig. 1C). STs were detected in the small RNA fractions obtained from U1 and ACH-2 cells, which served as *in vitro* models for postintegration HIV-1 latency (40, 41), but not in uninfected parental U937 and CEM cells or PBMCs from three healthy controls (Fig. 1D). Diluting U1 cells into U937 cells showed that the ST levels depend on the ratio of U1 cells to U937 cells (Fig. 1E). U1 and ACH-2 cells treated with TNF-α exhibited sustained increases in ST levels and viral particles, indicating that ST expression reflects the transcriptional activity of the LTR in infected cells (Fig. 1F).

ST levels are associated with disease progression in untreated patients. We next quantified ST levels in PBMC samples

FIG 4 High levels of STs were associated with low CD4⁺ cell counts in cART patients without detectable viremia. (A) Detection of STs and elongated viral transcripts in PBMCs from 96 cART patients with undetectable level of viremia (<50 copies/ml plasma). (B and C) Comparison of CD4⁺ cell counts (B) and period of treatment (C) among the 96 cART patients. ST⁺⁺ and ST⁺ refer to high ($\geq 1.5 \times 10^4$ copies/10⁶ PBMCs) and low (< 1.5×10^4 copies/10⁶ PBMCs) ST levels. (D) Correlation between the ST levels and CD4⁺ cell counts among the 54 ST-positive patients shown in panel B. (E) Comparison of the levels of elongated viral transcripts between ST⁺⁺ and ST⁻ patients on cART. Elongated transcripts were measured by nested qRT-PCR. Elongated RNA levels lower than the dotted line are undetected. One sample from each patient was included in each analysis. For panels B and C, $n = 26$ ST⁺⁺ patients, $n = 28$ ST⁺ patients, and $n = 42$ ST⁻ patients. For panel D, $n = 54$ ST-positive patients. For panel E, $n = 13$ ST⁺⁺ patients and $n = 15$ ST⁻ patients. The Kruskal-Wallis test, followed by Dunn's multiple comparison tests (B and C) and the Spearman's rank correlation test (D), were performed. Horizontal lines and error bars indicate median values and the IQR, respectively. NS, not statistically significant.

from 164 patients infected with HIV-1 who never received cART. Using a small amount of blood (3 to 4 ml) from each patient, we purified small and large RNA fractions (Fig. 2A). STs were detected in the small RNA fractions from 121 (73.8%) patients (detection limit, ~ 100 copies/10⁶ PBMCs). In contrast, elongated viral transcripts, which were amplified using a primer pair located between the U5 region of the LTR and the major splice donor site, were detected in the large RNA fractions from only 16 (9.8%) of the same patients (detection limit, ~ 500 copies/10⁶ PBMCs). Although elongated transcripts were detected at higher rates in some previous studies using seminested qRT-PCR and TMA (15, 17), we quantified viral transcripts by a single round of qRT-PCR in order to compare the detection rates of STs and elongated transcripts. These data suggest that, in our comparison of cell-based measurements, STs are a more sensitively detected target for monitoring viral transcription.

We then divided patients into three groups according to ST expression levels (ST⁺⁺, $\geq 1.5 \times 10^4$ copies/10⁶ PBMCs; ST⁺, detected but $< 1.5 \times 10^4$ copies/10⁶ PBMCs; and ST⁻, undetected). Since the detection limit of the standard sample was 100 copies per reaction, PCR signals below which were considered ST⁻. We found that CD4⁺ cell counts of ST⁺⁺ patients were lower than ST⁺ or ST⁻ patients ($P = 0.0075$ and $P = 0.0096$, respectively; Fig. 2B). Among ST-positive patients, the levels of STs and CD4⁺ cell counts demonstrated a negative correlation ($P =$

0.0014, $\rho = -0.2878$, Fig. 2C). In contrast, among the same patients, there was a significant difference in plasma viral load only between the ST⁺⁺ and the ST⁻ groups ($P = 0.0192$, Fig. 2D), and the ST level and plasma viral load showed no correlation ($P = 0.8959$, $\rho = 0.01202$, Fig. 2E). This suggests that the ST levels in PBMCs are not strictly associated with plasma viremia, a large measure of which is derived from other HIV-1 reservoirs, such as lymphoid tissues (42). We assessed longitudinal changes in ST expression in 12 patients before and/or after cART initiation (Fig. 3). Four patients exhibited increasing levels of STs before cART initiation, which were accompanied by a decline in CD4⁺ cell numbers (patients A and E to G). Other patients maintained steady ST levels before cART initiation (patients B to D and H to K). These observations are consistent with the low CD4⁺ cell counts in ST⁺⁺ patients (Fig. 2B). After initiating cART, ST levels generally decreased (patients E to I), although there were some exceptions who maintained constant ST levels after treatment initiation (patients J and K) and some whose ST level was undetectable before treatment initiation (patient L). In combination with the data in Fig. 2, these observations suggest that STs likely reflect increased risk for disease progression in untreated patients.

ST expression correlates with low CD4⁺ cell counts despite cART-mediated full viral suppression. Next, we assessed ST expression in 96 patients who achieved cART-mediated viral suppression to <50 copies/ml plasma (Fig. 4A). Among these pa-

FIG 5 Quantification of proviral load, STs and elongated viral transcripts in 11 patients who donated more than one specimen. Each PBMC sample obtained from these patients was divided into two for separate purification procedures for genomic DNA and the small and large fractions of RNA. Proviral DNA and viral transcripts were measured using nested-qPCR and a single round of qRT-PCR, respectively. Time points according to treatment initiation are shown. No bars in the graph for proviral DNA refer to not determined because of limited sample availability.

tients, 54 (56.3%) were determined to be ST positive, suggesting that our method was sufficiently sensitive to monitor transcriptional activation levels of peripheral infected cells during effective cART. In addition, elongated viral transcripts were detected only in a single patient. This observation is consistent with a previous analysis of circulating resting memory CD4⁺ T cells from cART patients, which identified STs as the predominant viral product in latently infected cells in these patients (36). Among these patients, the CD4⁺ cell counts of the ST⁺⁺ group tended to be lower than those in the other groups, although the differences between the ST⁺ groups were not statistically significant ($P = 0.0516$ and $P = 0.0048$, Fig. 4B). The period of treatment of each group is shown in Fig. 4C. We also observed that there was a negative correlation between ST levels and CD4⁺ cell counts in the ST-positive patients ($P = 0.0039$, $\rho = -0.3865$, Fig. 4D), which consistent with shorter period of treatment of the ST⁺⁺ group (Fig. 4C). Together with the analyses shown in Fig. 2, these observations reveal that high ST levels in PBMCs are associated with low CD4⁺ cell counts in both pre- and post-cART patients.

In several previous reports on cell-associated HIV-1 RNA, elongated transcripts were measured by nested-PCR approaches. To simply compare the levels of STs and elongated transcripts with a more enhanced sensitivity, we performed nested PCR to amplify elongated HIV-1 transcripts on 13 ST⁺⁺ and 15 ST⁻ patients picked from the patients shown in Fig. 4B (Fig. 4E). The limit of quantification was 10 copies per reaction. Using the large RNA fractions (>200 nt), all of the ST⁺⁺ patients were detected elongated transcripts with the median estimate of $3.307 \log_{10}$ copies/10⁶ PBMCs. On the other hand, in the ST⁻ group, elongated transcripts were detected in 13 of the 15 patients and their median level was $2.490 \log_{10}$ copies/10⁶ PBMCs, which was much lower

than that of the ST⁺⁺ patients. Although the sample size was small, this result shows that patients with elevated levels of STs are more likely to express higher levels of elongated viral transcripts, suggesting that STs reflect transactivation of the LTR in cART patients.

ST levels are strongly associated with persistent immune activation in cART patients. To identify factors affecting the expression levels of STs, we quantified proviral load in PBMCs by nested-PCR. Among 11 patients with more than one specimen obtained, seven patients had detectable levels of proviral load in all specimens (patients M to P and patients S to U, Fig. 5), whereas proviral DNA were not detected in the other patients (patients Q, R, V, and W; Fig. 5). However, their ST expression levels were not proportional to the corresponding proviral loads, suggesting that the ST levels do not simply reflect circulating proviral load in patients.

There is growing recognition that low CD4⁺ cell counts, despite suppressive cART, correlate with persistent immune activation (43, 44). Thus, to determine whether ST levels were related to T cell immunophenotypes in patients with plasma viremia (<50 copies/ml), we analyzed the percentages of CD4⁺ and CD8⁺ T cells expressing markers for T cell activation (HLA-DR and CD38), immune exhaustion (PD-1), and senescence (CD57) (Fig. 6A). Although a modest relationship between cell-associated HIV-1 RNA levels and PD-1 expression was previously reported (44), we did not find a correlation between ST levels and PD-1 or CD57 in CD4⁺ or CD8⁺ T cells, nor HLA-DR and CD38 dual expression in CD4⁺ T cells (Table 1). However, we detected an increased frequency of CD8⁺ T cells coexpressing HLA-DR and CD38 in ST⁺⁺ patients ($P = 0.0432$, Table 1 and Fig. 6B), and observed a strong positive correlation between levels of STs and these molecules ($P = 0.0057$, $\rho = 0.4642$, Fig. 6C). The

FIG 6 Sustained high levels of STs were associated with residual immune activation despite cART-mediated full viral suppression. (A) An example gating strategy for flow cytometry analysis of CD8⁺ T cells. Propidium iodide (PI)-negative cells were selected and then gated based upon expression of CD3 and CD8. Total CD8⁺ T cells, defined as the CD3⁺/CD8⁺ population, were divided into four subsets according to CD45RA and CCR7 expression. The populations of the total CD8⁺ T cells and each of the subsets were analyzed for expression of markers for activation (HLA-DR and CD38), exhaustion (PD-1), and senescence (CD57). (B and D) Comparison of the frequency of HLA-DR⁺/CD38⁺ (B) and HLA-DR⁺ (D) cells in total CD8⁺ T cells among 54 cART patients with undetectable levels of viremia (<50 copies/ml plasma). (C and E) Correlation between ST levels and frequency of HLA-DR⁺/CD38⁺ (C) and HLA-DR⁺ (E) cells among total CD8⁺ T cells in the 34 patients shown in panel B. (F to H) Comparison of the frequency of memory (F), effector (G), and naive (H) CD8⁺ T cells expressing HLA-DR in the same patients shown in panel B. For (B, D, and F to H), $n = 18$ ST⁺⁺ patients and $n = 36$ patients consisted of 16 ST⁺ and 20 ST⁻ patients in the ST^{+/+} group. For panels C and E, $n = 34$ ST-positive patients. Mann-Whitney (B, D, and F to H) and Spearman's rank correlation (96 cART patients with undetectable levels of viremia [<50 copies/ml plasma]) tests were performed. Horizontal lines and error bars indicate median and IQR values, respectively.

elevated level of CD8⁺ T cell activation mainly resulted from increased expression of HLA-DR (Fig. 6D and E), which was accounted for by the memory and effector T cell subsets, but not naive T cells (Fig. 6F to H). The comparisons of T cell activation without grouping the ST⁺ and ST⁻ patients are shown in Fig. 7. To characterize patients with persistently high levels of STs and

coexpression of HLA-DR and CD38 in CD8⁺ T cells during effective cART, 37 of the patients were further analyzed to determine the change in frequency of HLA-DR⁺ CD38⁺ CD8⁺ T cells, as well as for ST expression before and after initiation of cART. We found that 21 of the patients were ST⁺⁺ within 1 year before cART initiation, and that nine of them remained ST⁺⁺ after complete

TABLE 1 Correlation between ST levels and CD4⁺ and CD8⁺ T cells

	Median % (IQR)		
Cell type	ST ⁺⁺ (<i>n</i> = 18)	ST ⁺ and ST [−] (<i>n</i> = 36)	<i>P</i> ^a
Total CD4 ⁺ T cells			
PD-1 ⁺	4.74 (3.24–7.04)	3.89 (2.55–7.21)	0.3051
CD57 ⁺	14.15 (11.30–17.18)	12.25 (9.18–19.98)	0.7816
HLA-DR ⁺	21.40 (16.90–31.08)	19.05 (15.13–27.05)	0.2372
CD38 ⁺	63.85 (59.85–74.90)	65.60 (55.30–73.98)	0.9385
HLA-DR ⁺ CD38 ⁺	12.15 (9.71–15.58)	11.60 (8.86–14.65)	0.5095
Total CD8 ⁺ T cells			
PD-1 ⁺	2.29 (1.55–3.17)	2.76 (1.66–4.37)	0.4694
CD57 ⁺	62.15 (47.08–67.90)	50.60 (41.00–61.08)	0.0518
HLA-DR ⁺	48.05 (31.20–58.10)	31.55 (24.90–37.75)	0.0037*
CD38 ⁺	63.45 (53.10–75.95)	69.10 (61.33–75.90)	0.4638
HLA-DR ⁺ CD38 ⁺	29.95 (19.25–39.30)	23.60 (18.13–28.0)	0.0432†

^a *P* values were calculated using the Mann-Whitney test. *, *P* < 0.01; †, *P* < 0.05.

viral suppression during cART (Fig. 8A). We found that the patients of the subgroup classified ST⁺⁺, before and after initiation of cART, exhibited elevated levels of pre-cART CD8⁺ T cell activation (*P* = 0.0269, Fig. 8B), which was mainly derived from an increased frequency in HLA-DR expression (*P* = 0.0148, Fig. 8C), similar to that observed during cART. This finding suggests that

patients with exceptionally elevated levels of pre-cART T cell activation were more likely to exhibit the ST⁺⁺ values during suppressive cART. The correlation between ST levels and persistent immune activation supports the availability of STs as a marker to monitor the activity of residual infected cells in patients.

DISCUSSION

Latently infected cells generally do not make viral particles, which results in the absence of known biological markers to distinguish those cells from uninfected cells. Given a previous finding that STs were detected in circulating resting T cells in successfully treated patients (36), we analyzed the correlation between ST levels in individual patients and their disease conditions. In this study, we identified STs as a novel marker of viral persistence and residual immune activation. Our method based on single-round qRT-PCR, which requires only a small amount of blood (3 to 4 ml per patient), evaluates residual viral activation in PBMCs with high sensitivity and accuracy. We revealed that ST levels in PBMCs are different with each patient, but patients with high ST levels tend to be in poor condition. Given that the levels of STs are most likely to reflect transactivation of the LTR (Fig. 1F and 4E), these results suggest harmful effects of the residual infected cells harboring activated LTR on patients' conditions. Although the expression of STs alone cannot explain some exceptions (e.g., low CD4⁺ cell counts in a few of the ST⁻ patients in Fig. 2B), ST levels generally increased with disease progression in untreated patients and decreased with the initiation of cART. However, we observed that

FIG 7 Comparison of the level of T cell activation among the three groups of the cART patients shown in Fig. 6. (A and B) Frequency of HLA-DR⁺/CD38⁺ (A) and HLA-DR⁺ (B) cells in total CD8⁺ T cells. (C to E) Frequency of memory (C), effector (D), and naive (E) CD8⁺ T cells expressing HLA-DR.

FIG 8 High level of pre-cART immune activation is associated with post-cART ST expression. (A) Change in frequency of HLA-DR and CD38 coexpressing CD8⁺ T cells before and after the initiation of cART. PBMC samples were collected from each patient twice a year before the initiation of cART and after achieving a viral suppression of <50 copies/ml. (B and C) Comparison of the pre-cART T cell activation defined by the frequency of HLA-DR⁺/CD38⁺ (B) and HLA-DR⁺ (C) cells among total CD8⁺ T cells in the same patients. For panels B and C, $n = 9$ pre-cART ST⁺⁺ patients and $n = 12$ patients consisted of 7 ST⁺ and 5 ST⁻ patients in the pre-cART ST^{+/+} group. The Mann-Whitney test was performed (B and C). Horizontal lines and error bars indicate median and IQR values, respectively.

some patients, with elevated ST levels despite cART-mediated full viral suppression, have low CD4⁺ cell counts and consistently high levels of CD8⁺ T cell activation after cART initiation. Thus, this cell-based measurement provides information on therapeutic effects and is distinct from viral load in plasma. Although statistically significant, the correlation between STs and CD4⁺ T cell counts that we found is not strong (Fig. 4D). Given that CD4⁺ cells are responsible for ST expression, the ST values detected in purified CD4⁺ cells will be more accurate than ST values evaluated per number of unsorted PBMCs. Since patients with high ST values tend to have low CD4⁺ cell counts, we would expect ST expression based on the number of CD4⁺ cells would be more strongly correlated with CD4⁺ cell counts and immune activation.

Our findings also show that STs are not correlated with plasma viremia despite frequent lymphocyte trafficking between lymph nodes and blood (45) and are strongly associated with immune activation. This suggests that the elevated levels of STs in circulating cells likely reflect the host immune environment. We also found that patients with higher pretherapy levels of STs and CD8⁺

T cell activation are likely to be ST⁺⁺ during cART. Taken together with previous findings showing that HIV-1 infection leads to progression of irreversible damage to the host immune system (e.g., incomplete restoration of lymphoid tissue structure during cART [46, 47]), the ST⁺⁺ status may be associated with profound damage to the lymphoid tissues. To examine these possibilities, further studies, including analyses using nonhuman primates, are required. The primate models will be of great value for analysis of the relationship between ST expression and low-level viral replication in tissues despite suppressed viremia.

Although a correlation between intracellular viral RNA levels and the frequency of PD-1-expressing CD4⁺ T cells was documented in a previous report (44), we did not observe this relationship in our study. This is possibly due to differences in the viral RNA species that were quantified and the patients selected. However, both studies demonstrate correlations between host immune environment and viral persistence measured using a cell-based approach. This suggests that host immune abnormalities are either the cause or the consequence of viral persistence. Further studies investigating the causal relationship between the two would yield a better understanding of the mechanisms underlying viral persistence, which will in turn contribute to reducing the size of viral reservoirs. For instance, a mechanism may consist of a potential positive feedback interaction between ST expression and general immune activation. General immune activation is accompanied by secretion of inflammatory cytokines and activation of the classical NF- κ B pathway. This host environment enhances transactivation of persistent virus, which results in increased levels of STs. On the other hand, the TAR stem-loop sequence (i.e., ST) was previously shown to activate dsRNA-dependent protein kinase (PKR), the innate immune sensor (48), which in turn activates NF- κ B via phosphorylation of inhibitory I κ B proteins (49). Given that TAR RNA was also reported to be sequestered within exosomes derived from HIV-infected cells *in vitro* (50), ST may be transferred to CD8⁺ T cells via exosome secretion. If this is the case, ST may be a factor maintaining chronic immune activation in cART patients.

With growing recognition of non-AIDS-related health problems in HIV patients, such as development of non-AIDS-defining cancers and cardiovascular disease, several factors were identified to increase risk, including insufficient recovery of CD4⁺ cell numbers and persistent viral and immune activation (2, 51). Of note, Hunt et al. determined that a higher level of pretherapy CD8⁺ T cell activation, defined by coexpression of HLA-DR and CD38, predicts slower CD4⁺ cell recovery during suppressive cART (52). These researchers also reported that high and persistent CD8⁺ T cell activation, despite cART-mediated viral suppression, correlates with increased mortality (52). Taken together with these earlier findings, the high levels of STs in patients reported here are likely associated with poor clinical outcomes. This supports the importance of quantifying the transactivation level of residual virus, for which ST serves as a marker, in addition to the size of the viral reservoir. Although the full extent of the method's clinical value will be determined by future studies, our development of a simple method for monitoring viral persistence is of significant value to future endeavors. ST potentially serves as an additional biomarker to improve our understanding of low-level immune activation not affecting plasma viremia. Further characterization of ST⁺⁺ patients, such as correlations between ST and inflammatory markers (e.g., IL-6, D-dimer, and soluble CD14 [53, 54]), will

reveal the impact of residual viral activation on immune abnormalities. Given that chronic inflammation is suspected to be both a cause and consequence of the activity of the viral reservoir, these approaches would provide evidence that monitoring and reducing the levels of STs may control the activity of the viral reservoir. We believe ST levels may help monitor residual immune activation in individual patients during cART, enabling the development of novel approaches for complete restoration of normal immune status.

ACKNOWLEDGMENTS

We were greatly saddened by the passing of A.N. during the course of this work. He contributed in deeply discussed all aspects of the data together in this study.

We thank A. Iwamoto (Institute of Medical Science, University of Tokyo, Tokyo, Japan), M. Yoshida (Japanese Foundation for Cancer Research, Japan), and T. Matano (National Institute of Infectious Diseases, Tokyo, Japan) for helpful comments. We received the useful advice from J. Ohashi (University of Tokyo, Tokyo, Japan) on statistical analyses and support from H. Iba (Institute of Medical Science, University of Tokyo, Tokyo, Japan). We thank the volunteers who participated in this study and Editage for providing editorial assistance.

T. Koibuchi has received speaker's honoraria from Torii Pharmaceutical Co. Ltd., MSD, ViiV Healthcare K.K., and Janssen Pharmaceutical K.K. A.K.-T. has received research grants from Banyu Life Science Foundation International, ViiV Healthcare, and Janssen Pharmaceutical K.K. None of the honoraria and the grants is directly related to this work. All remaining authors have declared no conflicts of interest.

FUNDING INFORMATION

This work, including the efforts of Ai Kawana-Tachikawa and Taketoshi Mizutani, was funded by Japan Agency for Medical Research and Development (AMED) (15545492). This work, including the efforts of Ai Kawana-Tachikawa, was funded by Japan Society for the Promotion of Science (JSPS) (25293226). This work, including the efforts of Aya Ishizaka, was funded by Japan Society for the Promotion of Science (JSPS) (15K19117). This work, including the efforts of Taketoshi Mizutani, was funded by Strategic Basic Research Programs of Japan Science and Technology Agency (JST) (12000070). This work, including the efforts of Ai Kawana-Tachikawa, was funded by Ministry of Health, Labour and Welfare (MHLW) (H24-AIDS-IPPAN-008). This work, including the efforts of Ai Kawana-Tachikawa, was funded by Ministry of Health, Labour and Welfare (MHLW) (H25-AIDS-IPPAN-007).

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