To characterize newly arising replication of human immunodeficiency virus (HIV) type 1 in vivo at the cellular level, distinct viral RNA species in peripheral blood mononuclear cells (PBMCs) from HIV-1–infected patients were monitored during 2 weeks of structured treatment interruption (STI). HIV-1 RNA encoding tat/rev and PBMC-associated virions were almost completely depleted during antiretroviral therapy and emerged simultaneously after 2 weeks of STI, thus specifically reflecting productive viral infection at the cellular level. The magnitude of these correlates of reappearing cellular viral replication was predicted by during-therapy levels of nef transcripts in PBMCs. Significant rebound of plasma viremia, representing the progeny of a broader range of anatomical compartments, preceded and predicted productive infection in PBMCs. Thus, cellular viral rebound in PBMCs likely was primed before STI by the expression of nef in HIV-1–infected PBMCs that lacked virion production and was subsequently triggered by the plasma viremia that preceded the recurrence of productively infected PBMCs.

Despite their power to suppress HIV-1 replication [1] with subsequent clinical benefit [2–4], current antiretroviral treatment strategies cannot eradicate the virus [5–8]. Both residual HIV-1 replication and viral latency contribute to the persistence of the infection in the face of potent therapy [9, 10]. Evidence of ongoing, low-level HIV-1 replication in a fraction of patients receiving successful potent antiretroviral therapy has been shown by viral sequence evolution, despite virtual suppression of plasma viremia [11–13], by the fact that both stable and decaying residual plasma viremia can be observed during potent antiretroviral therapy [14], and by the observation that the subpopulations of lymphocytes infected with different HIV-1 quasi species show different rates of decay and replenishment in patients receiving potent antiretroviral therapy [10]. However, residual active replication may occur in only a fraction of successfully treated patients [11–13], and its effect on persistence of HIV-1 during therapy may be low [10, 15]. Similarly, persistence of productively infected cells is rare under conditions of potent antiretroviral therapy [16–19].

In contrast, the pool of latently HIV-1–infected cells is a major viral reservoir during therapy [5–7, 18, 20–23]. Hence, HIV-1 DNA persisting in virtually all HIV-1–infected patients, regardless of their treatment, has predominantly been related to nonproductively infected cells [18, 22, 24]. To facilitate differentiation of residual active HIV-1 replication and persistence of latently infected cells, potential markers indicating ongoing HIV-1 replication
and/or productively infected cells have been examined. 2LTR viral DNA episomes (by-products of retroviral cDNA synthesis) were reported to be short-lived and have been proposed as markers for recently infected cells [25]. However, 2LTR episomes are stable in vivo and in cultured HIV-1–infected cells in vitro [26–29] and thus do not specifically indicate new rounds of infection [26, 30]. Conversely, cell-associated HIV-1 RNA [16, 18, 31–34] may be a more-specific surrogate marker of ongoing replication.

Therefore, in the present study, we examined a panel of different HIV-1 RNA species. In addition to plasma levels of HIV-1 RNA, we analyzed expression of 4 cell-associated viral RNA species: intracellular and extracellular unspliced RNA (usRNA), multiply spliced RNA (msRNA) encoding tat/rev, and msRNA encoding nef.

Intracellular usRNA, when residing in the nucleus, is the primary viral transcript from which all other viral RNA species are derived by splicing. After its translocation into the cytoplasm, usRNA serves as mRNA for the Gag and Pol proteins and, when it is located near the cell membrane, is encapsidated into virions [35].

Cell-bound viral RNA encapsidated in virions (extracellular usRNA) has been shown to be significantly associated with productively infected cells in peripheral blood [16] and lymphoid tissue [18]. Similarly, a particular class of msRNA encoding the Tat and Rev proteins has been related to productively infected cells [16, 18, 31, 32, 34, 36]. Since tat mediates high-level viral transcription of the proviral DNA [37, 38] and rev is responsible for efficient translocation of a majority of viral mRNA species into the cytoplasm (where they can be translated) [39], expression of msRNA encoding tat/rev may be viewed as a surrogate for the efficient expression of viral proteins. Nevertheless, each of the latter 2 parameters, msRNA encoding tat/rev and extracellular usRNA, is not an unambiguous sign of productively infected cells per se, since msRNA encoding tat/rev can be observed in cultured latently infected cells [40] and extracellular usRNA may, in part, also represent virions captured by uninfected cells in trans [16, 18].

A second class of msRNA species that encode the Nef protein may, like intracellular usRNA, play a role in all stages of the intracellular viral life cycle. Although nef is not strictly required for viral replication [41], it affects early stages of infection [42–44], the productive phase of infection [45, 46], and the transition from latency to virus production [44, 47] through its pleiotropic effects on the intra- and extracellular environment and on the HIV-1 virion. These distinct viral RNA species were monitored longitudinally during structured treatment interruption (STI), to characterize their appearance during the transition from latent or persistent to productive infection.

We found that the expression of msRNA encoding tat/rev occurred concomitant with the reemergence of peripheral blood mononuclear cell (PBMC)–associated HIV-1 particles. Thus, parallel increases in these 2 types of viral RNA species reflected reappearance of productively infected cells. Furthermore, we demonstrate that, in peripheral blood, rebound of such virus-producing cells after STI is predicted by during-therapy levels of residual nef-encoding transcripts and by the levels of plasma viremia that preceded cellular viral rebound.

**PATIENTS, MATERIALS, AND METHODS**

**Patients and specimens.** Patients were enrolled in the Swiss Spanish Intermittent Therapy Trial (SSITT). Patients provided written, informed consent, in accordance with the guidelines of the Ethics Committee of the University Hospital Zurich. A prospective virological substudy was performed for 28 of the 29 patients enrolled in SSITT at the Zurich center. One patient was excluded because polymerase chain reaction (PCR) assays could not be performed properly, most likely because the patient was infected with HIV-1 subtype A. At baseline (during therapy), plasma viremia of patients was <50 HIV-1 RNA copies/mL (median, <11 HIV-1 RNA copies/mL) for a median of 25 months; the median CD4+ T cell count was 717 cells/μL (table 1). Virological and immunological outcomes of the present study have been reported elsewhere [48–54]. The present article addresses the first 14 days of the study, which included baseline (when patients were receiving successful therapy) and 2 weeks of STI. Blood samples were obtained from all 28 patients on days 0 and 14. For a subset of 14 patients participating in an extended study protocol with frequent blood and PBMC sampling [26, 51, 53], additional blood samples obtained on days 4 and 8 were analyzed. PBMCs and plasma were separated from anticoagulated blood by use of Ficoll-gradient purification (Lymphoprep; Nycomed) and were frozen at −80°C.

**RNA extraction.** RNA was extracted by use of the RNasy mini kit (QIAGEN) [16], and concentrations of extracted total RNA were measured by use of ribogreen fluorescent dye [55]. Nuclease-digested extracts prepared to obtain extracellular usRNA and matched preparations of total cell-associated RNA [16] were monitored for residual cellular RNA by use of realtime reverse-transcriptase (RT)–PCR for glyceraldehyde phosphate dehydrogenase mRNA [56].

**Quantitation of HIV-1 RNA.** Figure 1 depicts the amplification strategies used for quantification of the different classes of HIV-1 RNA. Plasma HIV-1 RNA was quantified by a modification of the Roche Amplicor Monitor test (version 1.5) [26, 51]. The levels of plasma viremia for the analyzed patients have been described elsewhere [26, 51] but are included here for better clarity. Cell-associated usRNA was dissected into intra- and extracellular fractions [16, 18] and was
Table 1. Baseline characteristics of patients before structured treatment interruption (STI).

<table>
<thead>
<tr>
<th>Patient</th>
<th>Antiretroviral therapy received before STI</th>
<th>Plasma HIV-1 RNA level, copies/mL</th>
<th>Duration of virological suppression&lt;sup&gt;a&lt;/sup&gt; before STI, months</th>
<th>CD4&lt;sup&gt;+&lt;/sup&gt; T cell count, cells/μL</th>
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<tr>
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<td>719</td>
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<td>398</td>
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</table>

Median (minimum, maximum) … 11 (3, 47) 25 (11, 36) 717 (272, 1337)

NOTE. AZT, zidovudine; ddI, didanosine; d4T, stavudine; IDV, indinavir; NFV, nelfinavir; RTV, ritonavir; SQV, saquinavir; 3TC, lamivudine.

<sup>a</sup> Virological suppression is defined as a viral load <50 copies/mL.

msRNA was quantified by use of a modification of the HIV-1 Monitor test (Roche) (figure 1B).

msRNA was quantified by duplicate real-time PCR measurements by use of the following primers: ks1 (5′-CTTAGGC-ATCTCCTATGGCAGGAA-3′) [58], ms83 (5′-GGATCTGTCTCTTCTCTCTCCACC-3′), and ms84 (5′-ACAGTCAGACTCATCAAGTTTCTCTATCAAAGCA-3′). For detection, the following dual-labeled fluorescent probes were used with a fluorescein (FAM) moiety at their 5′ ends and a TAMRA moiety at their 3′ ends: ks2-tq (FAM5′-TTCTTTGGCCTGTGGG- TCCC-3′TAMRA) [58] and ms82-tq (FAM5′-TCTTGCTGCCTG- TGCTTCCGTTCTT-3TAMRA). PCR was performed by use of a single-tube system (QIAGEN 1-step RT-PCR; QIAGEN) and an additional “hot-start” using Ampliwax (Applied Biosystems), to separate cDNA synthesis and PCR [56], as follows: aliquots of a lower-phase mix were prepared containing 10 μL of 1× reaction buffer (including 2.5 mmol/L MgCl₂), 4.7 μmol/L amplification primers (msRNA encoding tat/rev:ks1 and ms83, total msRNA:ms84 and ms83), fluorescent probes (msRNA encoding tat/rev:ms82tq [0.94 μmol/L] and ms2tq [1.88 μmol/L] and total msRNA:ks2-tq [1.88 μmol/L]), 2.35 mmol/L MgCl₂, and 47 ng/μL poly-A cRNA. Ampliwax was added to each reaction, and lower phases were sealed by incubation for 5 min at 90°C and cooling to room temperature. Upper-phase mix (30 μL of 1.25× reaction buffer [including 3 mmol/L MgCl₂], 0.25 μmol/L primer ms83, 0.63 mmol/L dNTP, 6.3% (vol/vol) enzyme mix, and 7 μL of template RNA were added. cDNA synthesis and subsequent amplification were performed in a real-time thermocycler (i-cycler; Biorad) as follows: 30 min at 50°C, 15 min at 95°C, and 60 cycles of 10 s at 95°C and 1 min at 60°C, with monitoring of fluorescence at 60°C. An external standard was prepared by 10-fold serial dilutions of synthetic msRNA en-
Figure 1. Polymerase chain reaction (PCR) strategies for measurement of HIV-1 RNA. A, Genetic map of HIV-1 showing viral genes and exonic structure. Exons were numbered according to the nomenclature of Schwartz et al. [57]. PCR primers are indicated by single-headed arrows (5′→3′), and fluorescent probes are indicated by double-headed arrows. Note that amplicons of multiply spliced RNA (msRNA) encoding tat/rev (msRNA-tat/rev) were detected by use of 2 fluorescent probes, whereas amplicons of total msRNA were detected by use of 1 probe. LTR, long-terminal repeat. B, Schematic overview of the extraction protocols to dissect intracellular (intra) and extracellular (extra) unspliced HIV RNA (usRNA) [16]. C, Real-time PCR assays to detect msRNA species. White symbols, signals obtained from amplification of synthetic RNA standards; black symbols, signals obtained from amplifying RNA from patients' peripheral blood mononuclear cells (patients 119, 123, and 126) or RNA from in vitro–infected CD4 cells (viral isolate from patient 118). R² values within each panel indicate correlation coefficients of the standard curves. Equations below panels B and C indicate the calculation of copy nos. of intracellular usRNA as the difference of total and extracellular usRNA (B) and of msRNA encoding nef (msRNA-nef) as the difference of total msRNA and msRNA-tat/rev (C).
coding tat/rev, which could be amplified both in the RT-PCR for total msRNA species and in the assay for RNA species encoding tat/rev [16]. Calibration was performed by use of the i-cycler software (Biorad). Both assays for msRNA were equally sensitive (1–5 copies/PCR), as shown by measurement of diluted synthetic msRNA.

Genotypic analysis of patients. PBMC DNA from participants was genotyped at CCR5 G2455A, Δ32, CCR2 V64I, RANTES G403A, C28G, macrophage inflammatory protein (MIP)–1α T113C, and stromal cell–derived factor (SDF) 1 A3′ and their HLA loci, as described elsewhere [54].

Calculations and statistics. HIV-1 RNA measurements were normalized to the input of total cellular RNA (expressed in copies/10⁶ cell equivalents) [59]. To represent 1 μg of total cellular RNA represents 10⁶ cell equivalents [59]. To represent specimens that were PCR negative in our analysis, mean detection limits of negative PCR tests were used (RNA plasma, 8 copies/mL; extracellular usRNA, 0.7 copies/10⁶ cells; and msRNA encoding nef and msRNA encoding tat/rev, 3.6 copies/10⁶ cells). Copy numbers of intracellular usRNA and msRNA encoding nef were obtained by subtraction of copy numbers obtained from independent PCR assays (figure 1B and 1C). In 11% of the specimens positive for any msRNA (n = 38), msRNA encoding nef could not be calculated, because the difference resulted in a nominally negative value, presumably because of sequence variations on the primer-binding sites used for the PCR of msRNA. These data were excluded from the analysis. Similarly, in 1% of the samples positive for usRNA (n = 82), determinations of intracellular usRNA had to be excluded. Pearson correlation analyses and 2-tailed t tests not adjusted for multiple comparisons were performed on log_{10} transformed values by use of GraphPad Prism software (version 3.03; AMPL Software). STI-induced changes of HIV-1 RNA levels were calculated as follows: ΔRNA = log_{10} (RNA copies at day 14/RNA copies at day 0).

RESULTS

Kinetics of HIV-1 RNA levels after STI. The aim of the present study was to characterize HIV-1 replication in PBMCs after STI and to compare it to viral rebound in plasma. Thus, before and during 2 weeks of STI, we quantified free HIV-1 particles in plasma (plasma HIV-1 RNA), intracellular and extracellular PBMC-associated usRNA species, and msRNA species encoding tat/rev or nef (figure 1). Longitudinal monitoring of these parameters was accomplished for 28 HIV-1–infected patients at baseline (during therapy) and after 2 weeks of STI (figure 2). To obtain exact kinetics of plasma and cellular viral rebound, additional samples were obtained from 14 patients on days 4 and 8 of STI (figure 2, gray symbols and lines).

At baseline, extracellular usRNA (figure 2B) and msRNA encoding tat/rev (figure 2C) were almost completely depleted. Only 8% and 7% of samples, respectively, were PCR positive. The other types of viral RNA species analyzed showed pronounced persistence during therapy: plasma HIV-1 RNA was detected in 46% of samples (at levels <50 copies/mL) (table 1 and figure 2A), intracellular usRNA was detected in all samples (figure 2D), and msRNA encoding nef was detected in 44% of samples (figure 2E).

STI resulted in significant viral rebound in plasma as early as day 4 (figure 2A and further increases on days 8 and 14 of STI. On day 14, 86% of the patients had a substantial increase in plasma viremia, defined here as a more than one-half log_{10} increase over baseline. In contrast, an equivalent viral rebound in PBMCs, in 1 or several of the cell-associated viral RNA species was observed in only 50% of the patients and became significant only at day 14. The increase in intracellular usRNA reached borderline statistical significance (P = .046) and, overall, was low in magnitude (figure 2D). Changes in extracellular usRNA (figure 2B) and msRNA encoding tat/rev (figure 2C or nef (figure 2E) were more pronounced (P = .003).

The kinetics of rebound in msRNA encoding tat/rev and in extracellular usRNA were intriguingly parallel, showing depletion during therapy and significant increase after day 8 of STI (figure 2B and 2C). This observation was substantiated by the finding that changes in msRNA encoding tat/rev and changes in extracellular usRNA between days 0 and 14 were correlated with high significance (r² = 0.72; P < .0001 [Pearson test]). This tight linkage of increases of extracellular usRNA with increases of msRNA encoding tat/rev, a class of viral RNA strictly required for viral production, supports the concept that the pool of cell-associated extracellular virions is a surrogate for productive infection, because it includes a substantial fraction of virions bound to their progenitor cells [16, 18].

Predictors of cellular viral rebound after STI. To identify predictors of viral rebound, during-therapy plasma levels of HIV-1 RNA and intracellular usRNA and msRNA encoding nef, which were detectable at baseline in >40% of patients, were examined for their association with STI-induced increases in the 2 parameters representing cellular viral rebound (i.e., extracellular usRNA and msRNA encoding tat/rev). During-therapy levels of msRNA encoding nef predicted STI-induced changes of extracellular usRNA with high significance (r² = 0.45; P = .003 [Pearson test]) (figure 3A). The association between msRNA encoding nef and changes of msRNA encoding tat/rev was weaker but still significant (r² = 0.31; P = .046 [Pearson test]) (figure 3B).

Baseline plasma levels of HIV-1 RNA and intracellular usRNA failed to predict cellular viral rebound after STI (P > .13). No significant correlation was observed when the different baseline levels of HIV-1 RNA were probed for increases in msRNA encoding nef or in intracellular usRNA (P > .1).

In a further analysis, we investigated whether HIV-1 RNA
levels at days 4 and 8 of STI could predict cellular viral rebound at day 14 of STI. Most of the various parameters tested failed to predict elevations. Only plasma levels of viremia at day 8 predicted increases in extracellular usRNA with high significance ($r^2 = 0.85; P < .0001$ [Pearson test]) (figure 3C), as well as changes of msRNA encoding tat/rev ($r^2 = 0.49; P = .02$ [Pearson test]) (figure 3D). Thus, the substantial rebound of newly produced viral antigens and, likely, infectious viral particles in plasma around day 8 of STI had a significant effect on cellular viral rebound in PBMCs 1 week later.

To determine whether host genetic factors showed a marked influence on cellular viral rebound after STI in the present analysis, the patients were genotypically tested at the following genetic loci, which can influence HIV-1 replication: CCR5 G2455A and Δ32, CCR2 V64I, RANTES G403A and C28G, MIP-1α T113C, and SDF1 3′A. In addition, HLA genotypes were determined. None of the tested genetic polymorphisms showed significant linkage with the magnitude of rebound in extracellular usRNA or msRNA encoding tat/rev (data not shown).

**DISCUSSION**

The present analysis of the kinetics and the magnitude of viral rebound in plasma and PBMCs from patients after short-term STI resulted in 2 major findings. First, the expression of msRNA encoding tat/rev and PBMC-associated viral particles were
Figure 3. Predictors of structured treatment interruption (STI)–induced changes in cellular HIV-1 RNA levels. Paired correlation of expression levels of msRNA (msRNA) encoding nef (msRNA-nef) during therapy (A and B) or levels of HIV RNA in plasma (RNA plasma) on day 8 (C and D), compared with STI-induced changes in extracellular unspliced HIV RNA (usRNA-extra) (A and C) and multiply spliced RNA (msRNA) encoding tat/rev (msRNA-tat/rev) (B and D). Black diamonds, polymerase chain reaction (PCR)–positive measurements of baseline msRNA-nef or day-8 RNA plasma; white diamonds, average detection limits of undetectable measurements on the X-axis. Note that 2 measurements positive for msRNA-nef were below the average detection limit of the PCR used. Closed and broken lines show the calculated linear regression lines and their 95% confidence intervals. Correlation coefficients and \( P \) values calculated from pairs with PCR-positive values on the X-axis are indicated within each panel. Analyses including PCR-negative measurements (represented by detection limits) also resulted in statistically significant correlations (A: \( r = 0.32, P = .003, n = 26 \); B: \( r = 0.16, P = .04, n = 27 \); C: \( r = 0.90, P < .0001, n = 14 \); D: \( r = 0.51, P = .004, n = 14 \)).

shown to be tightly linked and arose from virtually undetectable baseline levels with almost identical kinetics after STI. Since expression of msRNA encoding tat/rev is a prerequisite of viral replication and production of virions is its consequence, the concomitant rebound of these 2 parameters is a specific correlate of newly reappearing productive infection in PBMCs after STI. Second, during-therapy levels of msRNA encoding nef and viral rebound in plasma after 8 days of STI predicted the magnitude of the cellular viral rebound. Thus, the pools both of nonproductively HIV-1–infected cells persisting during therapy and of virions newly produced after STI determined cellular viral rebound in PBMCs.

Short-term STI led to substantial rebound of viral replication both in plasma and PBMCs, as indicated by significant increases in HIV-1 RNA species in both compartments. Of note, the earlier and more frequent rebound in plasma viremia, compared with cellular viral rebound in PBMCs, reconfirms that plasma HIV-1 RNA reflects the viral progeny from not only PBMCs but also from the much larger pool of infected cells in different lymphatic tissues [18, 60] and, potentially, in other viral reservoirs, such as the central nervous system and the genital tract [61–63].

One key parameter that appeared as a specific correlate of cellular viral rebound was an increased amount of cell-associated extracellular usRNA, which was tightly correlated (\( r^2 = 0.72 \) [Pearson test]) to expression of msRNA encoding tat/rev, which has also been perceived as an indicator of productively infected cells [16, 18, 34, 36]. It may be argued that these cell-associated virions reflected HIV-1 particles captured primarily from the plasma. However, on days 4 and 8 of STI, when plasma levels of HIV-1 RNA were already significantly elevated, almost no PBMC samples positive for extracellular usRNA were discernible (figure 2). Furthermore, the kinetics of extracellular usRNA levels mirrored changes in cell-associated HIV-1 RNA species better than the kinetics of HIV-1 RNA in plasma did. Hence, our findings support the concept that extracellular usRNA denotes productive HIV-1 infection at the cellular level [16, 18].

In contrast to extracellular usRNA and msRNA encoding tat/rev, intracellular usRNA and msRNA encoding nef showed complete or partial persistence during therapy. Like residual intracellular usRNA, which has been shown to stem, to a great extent, from latently infected cells in patients receiving effective antiretroviral therapy [16, 18, 23], msRNA encoding nef may also be synthesized in HIV-1–infected cells lacking virion pro-
duction. HIV-1 RNA species encoding nef [32] and usRNA [16, 18, 32, 64] have been shown to be the predominant cellular HIV-1 RNA species in patients receiving effective antiretroviral therapy, whereas HIV-1 RNA species encoding tat/rev were found to be 10–100 times less abundant [16, 18, 32] and frequently depleted [16, 18, 34, 64]. Therefore, tat-independent transcription [65–68] may likely be the source of persisting cell-associated HIV-1 RNA species in patients receiving potent antiretroviral therapy [16, 18]. To further define the role of these residual cellular HIV-1 transcripts in the transition from repressed to active viral replication, the capacity of their baseline levels to predict rebound of productively HIV-1–infected PBMCs after 14 days of STI was probed. Of interest, during-therapy levels of msRNA encoding nef significantly predicted the STI-induced rebound of both mRNA encoding tat/rev and extracellular usRNA.

In addition, rebound of productive infection in PBMCs was significantly associated with the elevated levels of plasma viremia on day 8 of STI. This finding implies that either cellular viral rebound was initiated by new infections via virions from the plasma or newly arising viremia might have acted as a trigger for activation of latently infected T cells [16, 23, 69–72] to a productive phenotype. These 2 hypotheses are not mutually exclusive, and it cannot be discerned conclusively which explanation applies here. Of note, the finding that baseline expression of msRNA encoding nef also predicts the reappearance of productively infected cells is compatible with both views: either the expression of nef derived from HIV-infected macrophages in PBMCs paved the way for cellular rebound by facilitating new productive infection of bystanding resting T cells [43] with newly produced virions from the plasma or the expression of msRNA encoding nef occurred in latently infected cells primed for activation of the integrated provirus to a productive phenotype [44, 47]. Thus, subsequent activation of these latently infected cells by antigenic or cytokine stimuli induced by the high levels of viremia, which preceded and predicted cellular rebound, might have been facilitated.

In summary, we have demonstrated that msRNA encoding tat/rev and extracellular usRNA can serve as cellular markers of productive infection. Furthermore, the present data suggest that during-therapy expression of msRNA encoding nef likely facilitates cellular viral rebound by priming latently infected cells to move into a productive state or by activating resting target cells for productive infection.

Quantification of the cellular correlates of latent, preactivated, and ongoing HIV-1 replication, as performed in the present study, will be helpful in gaining further insights into the mechanisms and the cell types that govern transition from latent to productive HIV-1 infection in vivo. These measurements might also become important in monitoring future attempts to “purge” latent HIV-1 reservoirs, to move toward eradication of the infection or its control in the absence of antiretroviral combination therapy [20, 21].

**SWISS HIV COHORT STUDY**


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