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Research Article AIDS/HIV

The persistence of latently infected, resting $CD4^+$ T cells is considered to be a major obstacle in preventing the eradication of HIV-1 even in patients who have received effective antiviral therapy for an average duration of 5 years. Although previous studies have suggested that the latent HIV reservoir in the resting $CD4^+$ T cell compartment is virologically quiescent in the absence of activating stimuli, evidence has been mounting to suggest that low levels of ongoing viral replication persist and in turn, prolong the overall half-life of HIV in patients receiving antiviral therapy. Here, we demonstrate the persistence of replication-competent virus in $CD4^+$ T cells in a cohort of patients who had received uninterrupted antiviral therapy for up to 9.1 years that rendered them consistently aviremic throughout that time. Surprisingly, substantially higher levels of HIV proviral DNA were found in activated $CD4^+$ T cells when compared with resting $CD4^+$ T cells in the majority of patients we studied. Phylogenetic analyses revealed evidence for cross infection between the resting and activated $CD4^+$ T cell compartments, suggesting that ongoing reactivation of latently infected, resting $CD4^+$ T cells and spread of virus by activated $CD4^+$ T cells may occur in these patients. Such events may allow continual replenishment of the $CD4^+$ T cell reservoir and resetting of the half-life of the latently infected, resting […]

HIV-infected individuals receiving effective antiviral therapy for extended periods of time continually replenish their viral reservoir

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The persistence of latently infected, resting CD4$^+$ T cells is considered to be a major obstacle in preventing the eradication of HIV-1 even in patients who have received effective antiviral therapy for an average duration of 5 years. Although previous studies have suggested that the latent HIV reservoir in the resting CD4$^+$ T cell compartment is virologically quiescent in the absence of activating stimuli, evidence has been mounting to suggest that low levels of ongoing viral replication persist and in turn, prolong the overall half-life of HIV in patients receiving antiviral therapy. Here, we demonstrate the persistence of replication-competent virus in CD4$^+$ T cells in a cohort of patients who had received uninterrupted antiviral therapy for up to 9.1 years that rendered them consistently aviremic throughout that time. Surprisingly, substantially higher levels of HIV proviral DNA were found in activated CD4$^+$ T cells when compared with resting CD4$^+$ T cells in the majority of patients we studied. Phylogenetic analyses revealed evidence for cross infection between the resting and activated CD4$^+$ T cell compartments, suggesting that ongoing reactivation of latently infected, resting CD4$^+$ T cells and spread of virus by activated CD4$^+$ T cells may occur in these patients. Such events may allow continual replenishment of the CD4$^+$ T cell reservoir and resetting of the half-life of the latently infected, resting CD4$^+$ T cells despite prolonged periods of aviremia.

Introduction

The use of effective antiviral therapy has led to rapid and sustained suppression of HIV replication and has dramatically improved the clinical outcome in a majority of infected individuals (1). However, it has not been possible to eradicate HIV in infected individuals receiving effective antiviral therapy mainly due to the persistence of various viral reservoirs (2–5). Among these, a pool of latently infected cells in the resting CD4$^+$ T cell compartment has been one of the most extensively studied to date and is considered to be a major impediment to HIV eradication (2–4, 6, 7). In this regard, it has been suggested that the extremely long intrinsic half-life of such a latent HIV reservoir makes eradication of the virus all but impossible in infected individuals receiving effective antiviral therapy even after more than 5 years of uninterrupted treatment (5). Of note, HIV that resides in infected, resting CD4$^+$ T cells has been shown to be virologically quiescent and lacking the ability to produce multiply spliced HIV RNA or viral particles (8). However, a number of studies over the past several years have suggested that low levels of ongoing viral replication continue to persist and consequently prolong the overall half-life of HIV in patients receiving antiviral therapy (9–12). These include the persistence of replication-competent virus (2–4), unintegrated proviral DNA, both linear (2) and circularized (13, 14), and cell-associated HIV RNA (6, 7, 11, 15, 16). In addition, other studies have demonstrated that intensification of conventional antiviral regimens in patients who remained aviremic accelerated the decay of the latent HIV reservoir in the resting CD4$^+$ T cell compartment (12) and further suppressed plasma viremia to well below the limit of detection (3.2–23 copies of HIV RNA per ml) (17). These findings suggest that low levels of ongoing HIV replication continue to persist and may contribute to the longevity of the latent viral reservoir by continually fueling new rounds of infection in resting CD4$^+$ T cells. Nonetheless, the source(s) and extent of ongoing viral replication in compartments other than resting CD4$^+$ T cells in patients receiving effective antiviral therapy for extended periods of time (greater than 5 years) have yet to be fully delineated.

In the present study, we investigate the presence and status of residual HIV in patients who had received effective antiviral therapy for up to 9.1 years and examine the underlying mechanisms by which HIV persists in CD4$^+$ T cells of such individuals. We demonstrate that all infected subjects studied carried replication-competent HIV in their CD4$^+$ T cells and the majority of patients harbored considerable levels of HIV proviral DNA in their activated as well as resting CD4$^+$ T cell compartments. Finally, we also provide evidence for cross infection between these 2 compartments, suggesting a possible mechanism by which HIV persists in patients who are receiving clinically effective antiretroviral therapy.

Results

We studied a cohort of patients who had received uninterrupted antiviral therapy for up to 9.1 years (average of 8.3 years) and who were selected on the basis of having maintained undetectable levels of plasma viremia (< 500 or < 50 copies of HIV RNA per ml plasma) for the entire time on therapy prior to enrollment (Table 1 and Figure 1). We first determined the frequency of CD4$^+$ T cells carrying

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Table 1
Profiles of HIV-infected patients

Subject	CD4 count at time of study (cells/μl)	CD8 count at time of study (cells/μl)	Plasma HIV RNA at time of study (copies/ml)[A]	Antiviral therapy at time of study[B]	Duration of therapy (yr)
1	1220	520	< 50	AZT, 3TC, SQV, LPV/r	9.1
2	516	382	< 50	ABC, TDF, NVP	8.3
3	573	406	< 50	ABC, TDF, NVP	8.9
4	330	550	< 50	AZT, 3TC, NFV	7.6
5	610	600	< 50	ABC, 3TC, NVP	8.1
6	980	1170	< 50	3TC, TDF, NVP	8.0
7	520	610	< 50	3TC, TDF, EFV, LPV/r	8.0
8	662	772	< 50	AZT, 3TC, EFV	8.6
9	560	1230	< 50	ABC, 3TC, EFV, LPV/r	8.0
10	605	670	< 50	ABC, TDF, EFV	8.7
11	280	490	< 50	AZT, 3TC, SQV, RTV	8.3
Mean	623	673			8.3

[A]Measured by ultrasensitive bDNA or RT-PCR assay with a detection limit of 50 copies per ml of plasma. [B]Drugs used in antiviral regimens included the nucleoside analogs zidovudine (AZT), lamivudine (3TC), abacavir (ABC), and tenofovir (TDF); the nonnucleoside reverse transcriptase inhibitors efavirenz (EFV) and nevirapine (NVP); and the protease inhibitors lopinavir/ritonavir (LPV/r), nelfinavir (NFV), ritonavir (RTV), and saquinavir (SQV).

replication-competent virus in the study subjects. The persistence of replication-competent virus in resting CD4+ T cells is well documented in patients who have been on effective antiviral therapy for an average duration of 5 years (5). However, such an analysis has not been systematically carried out in patients who have received effective antiviral therapy for longer than 5 years. In all patients examined, purified CD4+ T cells from PBMCs carried replication-competent HIV (median 0.059 infectious units/1 × 10^6 cells, range 0.006–0.322; Figure 2), indicating that none of the study participants had eradicated HIV infection despite having received years of continuous and effective antiviral therapy.

In order to further investigate the presence and status of residual HIV in patients who received effective antiviral therapy for extended periods of time and to delineate the underlying mechanisms by which HIV persists in the CD4+ T cells of such individuals, we measured various virologic markers in highly purified resting and activated CD4+ T cells. Highly purified (>99.0%) FACS-enriched resting ($CD25^-CD69^-HLA\text{-}DR^-$) and activated ($CD25^+CD69^+HLA\text{-}DR^+$) CD4+ T cells were subjected to quantitative real-time PCR specific for detection of HIV DNA. As shown in Figure 3A, HIV proviral DNA was found in both compartments with the frequency of cells carrying HIV proviral DNA in the activated CD4+ T cell compartment (median 980 copies, range 292–3180/1 × 10^6 cells) being significantly higher than in the resting CD4+ T cell compartment (median 589 copies, range 66–1751/1 × 10^6 cells; $P = 0.01$). Thus, in all patients examined, the presence of HIV in resting as well as activated CD4+ T cells was sustained despite extended periods of undetectable HIV plasma viremia.

In order to determine whether the HIV proviral DNA detected in the patients was capable of generating virions, microbead-enriched resting and activated CD4+ T cells were cultured for 24 hours in the absence of any activating stimuli. We have previously demonstrated that resting CD4+ T cells from aviremic patients do not spontaneously release any measurable levels of virus in the absence of activating stimuli (18). Consistent with our previous findings, the resting CD4+ T cells of infected individuals described herein, having received long-term effective antiviral therapy, did not produce any detectable levels of virions during the short incubation period (Figure 3B). However, the activated CD4+ T cells of 6 of 8 patients examined spontaneously released detectable levels of virus, strongly suggesting that the capacity for production of infectious virus persisted in this cellular compartment despite prolonged periods of seemingly effective antiviral drug regimens (Figure 3B). The contribution of virions bound to the surface of activated CD4+ T cells was determined to be negligible as pronase treatment of cells prior to incubation did not alter the copy numbers of HIV in the culture supernatants (data not shown).

Finally, phylogenetic analysis of HIV *env* DNA (C2-V5) isolated from resting and activated CD4+ T cells was performed to investigate whether cross infection occurred between these 2 cellular compartments. HIV *env* was clonally amplified from FACS-sorted resting and activated CD4+ T cell fractions. Phylogenetic (19–22) and cladistic (23) analyses designed to map the history of migra-

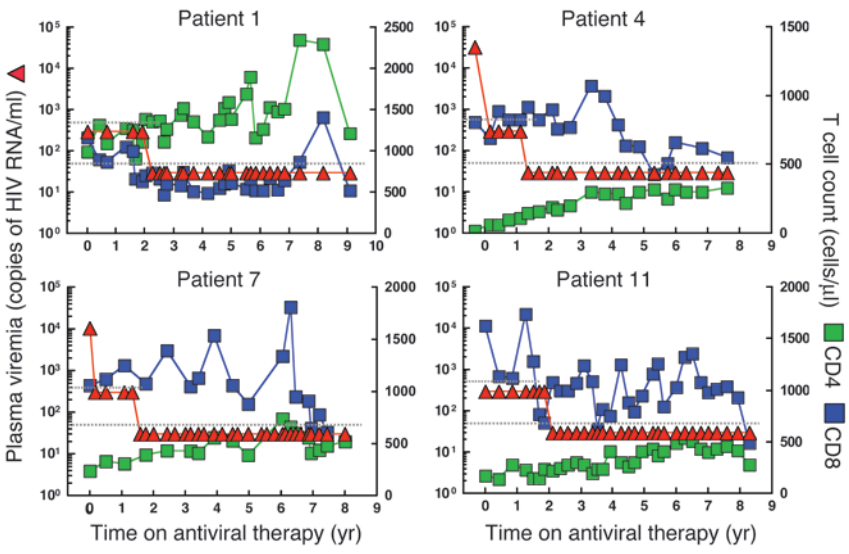

Figure 1
Plasma viremia and CD4+ and CD8+ T cell counts of 4 representative study participants. The dotted lines indicate the limit of detection of plasma viremia (<500 or <50 copies of HIV RNA per ml).

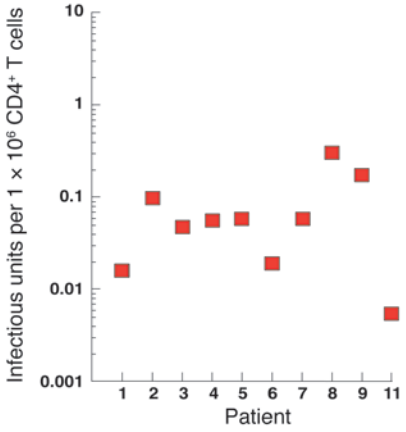

Figure 2
Frequency of replication-competent HIV in CD4+ T cells of infected individuals receiving effective antiviral therapy for prolonged periods of time. The frequency of cells carrying infectious HIV (*y* axis) was assessed by activation of purified CD4+ T cells from the patients studied (*x* axis) with replicates of 1 × 10⁶ CD4+ T cells per well in 12-well plates.

tion onto the branches of maximum-likelihood trees were performed on the sequences obtained (23). As illustrated in Figure 4A, evidence for bidirectional HIV infection between the resting and activated CD4+ T cell compartments was observed in the phylogenetic trees. The calculation of the number of HIV migration events also indicated that viral migration was occurring from activated to resting CD4+ T cells (Figure 4B). Considering that activated CD4+ T cells are the main source of productive HIV replication (24, 25) and our finding that only activated CD4+ T cells produced virions after overnight culture (Figure 3B), these data suggest evidence for spread of infection by productively infected cells. However, our data also suggest migration of HIV from infected, resting CD4+ T cells toward activated CD4+ T cells (Figure 4B), presumably as a result of reactivation of latently infected, resting CD4+ T cells that occurred in infected individuals despite undetectable plasma viremia.

Discussion

In the present study, we investigated the source(s) and the extent of residual HIV replication and examined the underlying mechanisms by which HIV persists in CD4+ T cells of patients who received effective antiviral therapy for extended periods of time. We demonstrated that all infected subjects examined carried replication-competent HIV in their CD4+ T cells despite having received clinically effective antiviral therapy for extended periods of time (8.3 years on average). To our knowledge, this is the first study to examine levels of replication-competent HIV in the CD4+ T cell compartments of patients who have received effective antiviral therapy for such long periods of time (up to 9.1 years). We also demonstrated that, contrary to current dogma, it is the activated CD4+ T cell compartment that harbors the majority of persisting HIV in infected individuals who have had no detectable viremia for extended periods of time as a result of effective antiretroviral therapy. In the years following the first indications of HIV latency in infected individuals (26, 27), the vast majority of studies, if not all, addressing the persistence of HIV in the era of effective antiviral therapy focused heavily on the latent viral reservoir in the resting CD4+ T cell compartment as a major impediment to eradication of HIV (2, 3, 9, 28–31). However, the activated CD4+ T cell compartment has not been fully explored as a potential viral reservoir in infected individuals receiving clinically successful antiviral therapy mainly because it has been believed that such cells are short lived and not expected to harbor virus (25). By demonstrating the presence of HIV in activated CD4+ T cells of maximally suppressed individuals, we thus provide compelling evidence for contribution of this compartment to the continual reseeding of HIV reservoirs. Although mitotic division of activated CD4+ T cells harboring defective virus could account for the higher frequency of cells carrying HIV proviral DNA in activated CD4+ T cell compartments compared with resting CD4+ T cell compartments, the evidence presented herein for spontaneous release of virions and viral migration and the short half-life of activated CD4+ T cells would argue for the persistence of infectious virus in activated CD4+ T cells.

The present study offers a plausible mechanism to explain the persistence of HIV in infected individuals receiving effective antiviral therapy resulting in prolonged periods of aviremia. Our phylogenetic data suggest that latently infected, resting CD4+ T cells may become reactivated, most likely as a result of normal immunologic responses to various recall antigens or routine vaccination (32) and induction of cytokines (33). In turn, virions released during the reactivation process may spread to and infect neighboring resting as well as activated CD4+ T cells; direct cell-to-cell spread in the absence of virion release may also occur. In this regard, the presence of unintegrated HIV DNA in resting CD4+ T cells (2) and circularized HIV DNA in CD4+ T cells (13, 14) has been demonstrated

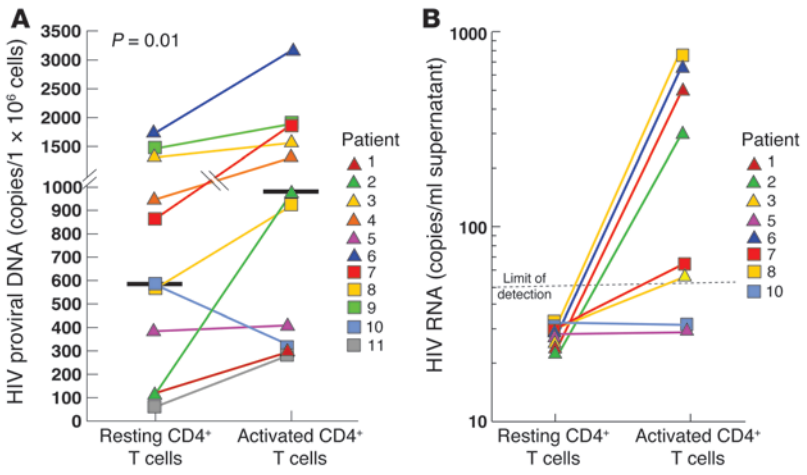

Figure 3
Persistence of HIV in resting and activated CD4+ T cells of infected individuals receiving effective antiviral therapy for prolonged periods of time. (**A**) Frequency of FACS-sorted resting and activated CD4+ T cells carrying HIV proviral DNA. The median values are shown as black bars. (**B**) Levels of cell-free virions released by resting and activated CD4+ T cells in overnight cultures in the absence of activating stimuli. Cell-free supernatants from each culture harvested after 1 day of culture were subjected to the Amplicor HIV-1 test (detection limit of 50 copies of HIV RNA per ml).

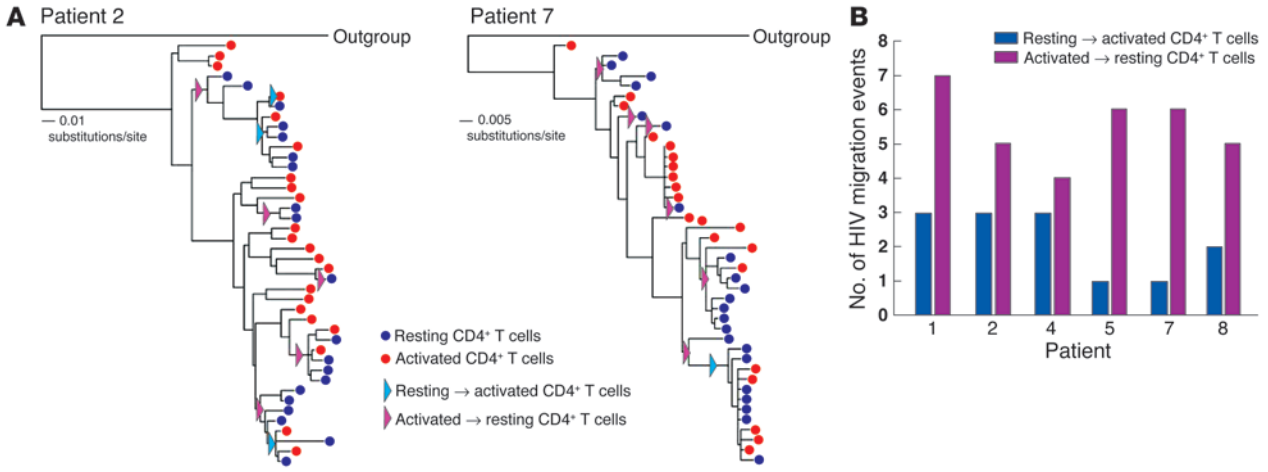

Figure 4

Phylogenetic analysis of HIV *env* DNA and evidence for cross infection between resting and activated CD4+ T cells in patients receiving effective antiviral therapy. (**A**) Phylogenetic trees of HIV *env* sequences in resting (blue circles) and activated (red circles) CD4+ T cells of 2 representative patients. The outgroup sequences were obtained from unrelated HIV-infected patients. The direction of HIV migration from activated to resting (pink arrow) and from resting to activated (blue arrow) CD4+ T cells is shown. The bar indicates genetic distance. (**B**) The number of viral migration events observed on the phylogenetic trees within the resting and activated CD4+ T cell compartments is shown.

in patients receiving effective antiviral therapy, indicating recent infection. Although the half-life of integrated HIV DNA in resting CD4+ T cells has been firmly demonstrated to exceed the previous estimates (5), the reactivation events described above could allow continual replenishment of the CD4+ T cell viral reservoir and reset the overall half-life of HIV in infected patients receiving effective therapy. We also provide evidence for migration of virus from activated to resting CD4+ T cells, although it should be noted that our findings do not exclude other viral reservoirs as sources of infectious HIV. In addition, while these findings were based on cross-sectional phylogenetic analyses of viral DNA, the presence of HIV in activated CD4+ T cells, a population thought to have a relatively short half-life (25), strongly suggests that viral migration events noted in our study were relatively recent and involved infectious virions. Indeed, highly enriched activated CD4+ T cells from the majority of patients we examined spontaneously released virus in the overnight culture in the absence of any activating stimuli, further strengthening the above observation. In addition, the persistence of virus in this cellular compartment may be responsible for prompt rebound of plasma virus upon cessation of effective antiviral therapy in infected individuals (9, 29, 30).

The eradication of HIV by antiretroviral therapy has thus far been elusive. The present study could potentially have a significant impact on the design of future therapeutic strategies aimed at eradicating HIV in patients receiving effective antiviral therapy for extended periods of time. Considering that reactivation of latently infected, resting CD4+ T cells contributes to the persistence of HIV and initiation of new infection cycles, coadministration of an effective and safe reagent that dampens cellular activation could minimize the spread of virions to uninfected bystander cells (34). In addition, given the persistence of HIV in activated CD4+ T cells in patients receiving effective antiviral therapy without detectable plasma viremia, intensification of existing drug regimens or the addition of a new class of drug, such as HIV entry inhibitors, in aviremic patients may be necessary to abrogate the low but detectable levels of ongoing viral replication originating from this cellular compartment.

Methods

Study subjects. Eleven HIV-infected individuals (mean CD4+ T cell count 623 per mm^3 blood, range 280–1220; mean $CD8^+$ T cell count 673 per mm^3 blood, range 382–1230) who were receiving effective antiviral therapy for an average of 8.3 years (range 7.6–9.1) were studied. None of these patients experienced any detectable plasma viremia after initial suppression of HIV replication following the start of antiviral therapy and at the time of study (lower limit of detection of 50 copies HIV RNA/ml). All patients were receiving various antiviral regimens containing at least 1 protease inhibitor and/or 1 nonnucleoside reverse transcriptase inhibitor in addition to 2 reverse transcriptase inhibitors of HIV. Leukapheresis was conducted in accordance with protocols approved by the Institutional Review Boards of the University of Toronto, Ontario, Canada and the National Institute of Allergy and Infectious Diseases, NIH. Each patient signed a consent form that was approved by the above institutional review boards.

Isolation of CD4+ T cells. PBMCs were obtained from leukapheresis by Ficoll-Hypaque density gradient centrifugation. CD4+ T cells were isolated from PBMCs of HIV-infected individuals using a column-based cell separation technique (StemCell Technologies) as previously described (35). In order to isolate resting and activated CD4+ T cells, total CD4+ T cells were labeled with anti-CD3 (APC), CD4 (FITC), CD25 (PE), CD69 (PE), and HLA-DR (PE) antibodies (BD Biosciences). Then, $CD3^+CD4^+CD25^-CD69^-HLA\text{-}DR^-$ (resting) and $CD3^+CD4^+CD25^+CD69^+HLA\text{-}DR^+$ (activated) cells were separated using FACSAria (BD Biosciences) to very high purity (>99.0%).

Quantitative coculture assays. In order to determine the frequency of CD4+ T cells carrying replication-competent HIV, high-input quantitative coculture assays were carried out in which multiple wells containing 1×10^7 CD4+ T cells were subjected to activation as described previously (36).

Quantitative real-time PCR for measurements of HIV DNA. In order to determine the frequency of resting CD4+ T cells carrying HIV proviral DNA in infected individuals, real-time PCR was carried out on genomic DNA isolated from $1–2 \times 10^6$ purified resting or activated CD4+ T cells using the Puregene DNA isolation kit according to the manufacturer's specifications (Gentra Systems). Then 1 μg of DNA was used as template for real-time PCR in an iCycler (Bio-Rad Laboratories). The amplification reac-

tion was carried out in triplicate using 0.5 μM primers, 0.2 μM fluorescent probe, 0.8 mM dNTPs, 5 mM $MgCl_2$, and 2.5 U Platinum Taq Polymerase (Invitrogen Corp.) in 50 μl total volume. The following primers were used: 5′-GGTCTCTCTGGTTAGACCAGAT-3′ (5′ primer) and 5′-CTGCTAGAGATTTTCCACACTG-3′ (3′ primer) along with the fluorescent probe 5′-6FAM-AGTAGTGTGTGCCCGTCTGTT-TAMRA-3′. PCR conditions consisted of a denaturation step at 95°C for 3 minutes followed by 45 cycles of 15 seconds at 95°C and 1 minute at 58°C. Serially diluted ACH-2 DNA was also subjected to the above PCR to obtain standard curves.

Quantitation of cell-free HIV virions. Spontaneous release of HIV virions from 6×10^6 bead-separated resting and activated $CD4^+$ T cells (Miltenyi Biotech) was quantitated following a 16-hour incubation in the absence of any activating stimuli. Culture supernatants were then harvested, and cell-free HIV was quantified using the Amplicor HIV-1 Monitor Test (Version 1.5, detection limit 50 copies/ml; Roche Diagnostics Corp.) according to the manufacturer's instructions.

Sequencing of HIV env isolated from resting and activated $CD4^+$ T cells. HIV DNA was amplified to single copy by limiting dilution PCR. First-round primers were ED5 (5′-ATGGGATCAAAGCCTAAAGCCATGTG-3′, nucleotides 6556 to 6581, HIV-1 HXB2) and ED12 (5′-AGTGCTTCCTGCTGCTCCCAAGAACCCAAG-3′, nucleotides 7822 to 7792, HIV-1 HXB2). Second-round reactions consisted of 1 μl of the first-round product as template and primers DR7 (5′-TCAACTCAACTGCTGTTAAATGGCAGTCTAGC-3′, nucleotides 6989 to 7020, HIV-1 HXB2) and DR8 (5′-CACTTCTCCAATTGTCCCTCATATCTCCTCC-3′, nucleotides 7637 to 7667, HIV-1 HXB2) as previously described (9). The final PCR products (650 base pairs) spanning the C2 to V5 region of the HIV *env* were ligated into the sequencing vector pCR4-TOPO (Invitrogen Corp.), followed by transformation, amplification, and sequencing.

Phylogenetic trees and cladistic analysis of viral migration. HIV *env* sequences isolated from resting and activated $CD4^+$ T cells of 5 patients were aligned using CLUSTAL W (19) followed by manual adjustment where necessary using the program MacClade (version 3; Sinauer Associates Inc.). Likelihood ratio tests (LRT) (20, 21) were performed to establish a maximum-likelihood model of viral evolution that was statistically consistent with the data while making the fewest number of assumptions about the evolution of the sequences themselves (22). Parameters derived from the best-fit model were then applied to the datasets to obtain maximum-likelihood trees using the program PAUP* (version 4.0b10; Sinauer Associates Inc.). In order to obtain maximum-likelihood trees, 10 random addition replicates were performed using the most exhaustive branch-swapping algorithm (tree bisection-reconnection).

A cladistic method (23) was used to investigate whether virologic crosstalk occurred between the resting and activated $CD4^+$ T cell compartments. If no viral crosstalk took place between these 2 cellular compartments, one would expect a single common ancestor for these 2 cellular compartments. The estimated maximum likelihood phylogenetic tree was used to map the number of potential migration events using parsimony (MacClade). Specifically, 2 state characters were created so that sequences derived from activated and resting $CD4^+$ T cells were assigned to 1 and 2, respectively. The number of transitions from state 1 to state 2 or the reverse in each phylogenetic tree was then recorded. If all sequences in 1 cellular compartment were monophyletic, the maximum number of character state changes would equal 1 in one direction and 0 in the other. If, however, sequences from one cellular compartment were more closely related to those from the other cellular compartment, the number of character state changes would increase. As a result, the most parsimonious estimate of the minimum number of viral exchanges between the 2 cellular compartments is predicated on the basis of the estimated maximum likelihood phylogenetic tree.

Statistics. Differences in the levels of HIV-1 proviral DNA in resting and activated $CD4^+$ T cells were tested for significance by the Wilcoxon signed-rank test.

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