



# Generation of HIV latency during thymopoiesis

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The use of combination antiretroviral therapy results in a substantial reduction in viremia, a rebound of CD4<sup>+</sup> T cells and increased survival for HIV-infected individuals. However, this treatment does not result in the total eradication of HIV. Rather, the virus is thought to remain latent in a subset of cells, where it avoids elimination by the immune system. In this state the virus is capable of reactivation of productive infection following cessation of therapy. These latently infected cells are very few in number and it has thus been difficult to determine their origin and to study the molecular nature of the latent viral genome. HIV replication is linked to cellular gene transcription and requires target cell activation. Therefore, should an activated, infected cell become transcriptionally inactive prior to cytopathic effects, the viral genome might be maintained in a latent state. We used the SCID-hu (Thy/Liv) mouse model to establish that activation-inducible HIV can be generated at high frequency during thymopoiesis, a process where previously activated cells mature towards quiescence. Moreover, we showed that these cells can be exported into the periphery where the virus remains latent until T-cell receptor stimulation, indicating that the thymus might be a source of latent HIV in humans.

Highly active antiretroviral therapy (HAART) has succeeded in lowering HIV levels in most patients, in some cases to undetectable levels<sup>1,2</sup>; however, this therapy alone cannot completely eradicate the virus<sup>3-5</sup>. The reasons for this inefficiency are not entirely clear, but it is thought that the resurgence in viral replication seen following cessation of HAART originates from activation of cells latently infected with HIV. Although the size of the latent reservoir is small<sup>6</sup> (~1 per  $1 \times 10^6$  cells), it is thought to be stable for up to 60 years during HAART (ref. 7) or until antigenic stimulation of the infected cell. This latent reservoir is generated during acute infection and is maintained regardless of CD4<sup>+</sup> T-cell counts, plasma viral load or duration of HAART treatment<sup>3-5,8</sup>. Reactivable virus was recently identified in HIV-infected children as young as four months of age<sup>9</sup>. Furthermore, persistent viral replication during HAART (refs. 10-12) could lead to constant replenishment of this reservoir, making its eradication an even more formidable task.

There are currently two models to explain how HIV latency is maintained *in vivo*. The first, a labile preintegration latency, occurs when HIV infects a quiescent T cell, but due to the lack of cellular activation, is unable to complete reverse transcription and integrate into the host genome<sup>13,14</sup>. The virus in these cells can later be induced to integrate and express RNA, but this type of infection is usually abortive. The second model, a stable postintegration latency, is hypothesized to exist in previously activated CD4<sup>+</sup> T cells. This infected cell, having resisted viral and immunological cytopathic effects, harbors inactive provirus capable of being induced following cell activation. The transition from an activated effector T cell to a quiescent memory T cell is hypothesized to facilitate the tran-

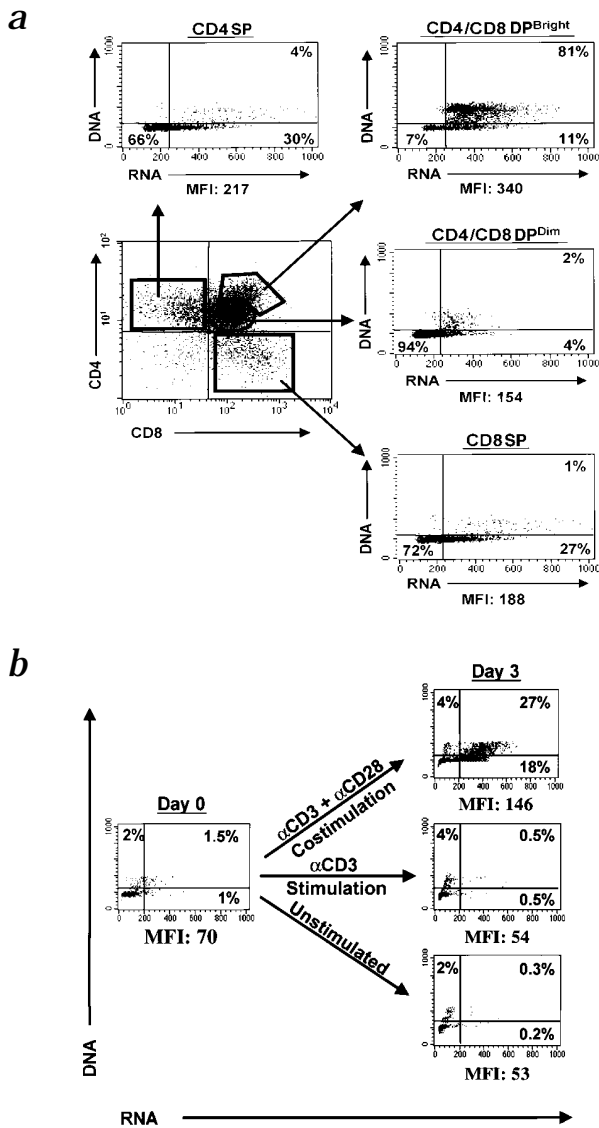
sition to viral latency. Consistent with this mechanism, latent virus was originally isolated from memory T cells *in vivo*<sup>6,15</sup>. Recently, latent virus has also been identified in naive T cells in the peripheral blood of HIV-infected patients<sup>16</sup>. Since naive T cells have not undergone the antigen-induced activation steps necessary for HIV infection and replication, it is likely that the latent virus in this population arose through another mechanism.

During thymopoiesis, immature hematopoietic precursor cells undergo a series of replication, differentiation and selection steps that eventually result in the export of mature CD4<sup>+</sup> and CD8<sup>+</sup> T lymphocytes into the peripheral blood. Immature thymocyte subsets are highly transcriptionally active. As these cells mature they transcribe less cellular RNA (Fig. 1a) until finally becoming quiescent. Most human thymocytes express CD4, the primary receptor for HIV, and also express coreceptors for virus entry<sup>17-19</sup>. Accordingly, infection of these cells is particularly common in fetuses<sup>20</sup>. To determine if this relative loss of cellular transcription during thymopoiesis could influence viral latency, we used the SCID-hu (Thy/Liv) mouse model in which human thymic implants faithfully recapitulate human thymopoiesis and, following HIV infection, demonstrate pathology similar to that seen in infected humans<sup>21-24</sup>. This pathology is manifested by the initial infection and loss of immature CD4/CD8 double positive (DP) cells and the subsequent depletion of the more mature CD4 single positive (SP) subset<sup>25</sup>.

## Identification of latent HIV infection in the thymus

To ascertain whether mature thymocytes could respond to activation signals, we subjected isolated CD4 SP cells to stimula-

**Fig. 1** Activation status of thymocyte subsets. **a**, Thymocytes from a human fetal thymus were simultaneously stained for CD4/CD8 surface expression and DNA/RNA content. The panel from which the arrows originate shows CD4 versus CD8 staining of these thymocytes. DNA/RNA profiles in the surrounding panels are backgated as indicated above each panel. The mean fluorescence intensity (MFI) of RNA expression is indicated for each subset analyzed. The CD4/CD8<sup>Bright</sup> DP subset is highlighted, as these cells are the immediate precursors for the single-positive subsets. **b**, Cell-cycle analysis of CD4 SP thymocytes following stimulation. Mock-infected SCID-hu thymocytes (tissue donor 14) were negatively selected for CD4, cultured for 3 d under the indicated conditions and stained for RNA and DNA content (right panels). The left panel illustrates the cell cycle analysis of uncultured CD4 SP thymocytes immediately following isolation from the Thy/Liv implant. These cells were greater than 90% CD4<sup>+</sup>/CD8<sup>-</sup>. The percentage of cells is indicated for each quadrant. The lower left quadrants of each panel indicate cells in the G<sub>0</sub> or G<sub>1a</sub> phases of the cell cycle while the lower and upper right quadrants represent cells in the G<sub>1b</sub> and S through M phases, respectively. The mean fluorescence intensity of RNA expression is indicated for each subset analyzed. Events in the upper left quadrants are likely bald nuclei formed during the staining procedure.



tion with antibodies specific for CD3 (anti-CD3) alone or to costimulation using antibodies specific for both CD3 and CD28 (anti-CD28) (ref. 26). Costimulation induced RNA expression and DNA synthesis, whereas stimulation with anti-CD3 alone only had minimal effects on cell activation (Fig. 1b). To determine whether latently infected cells are generated during thymopoiesis, we recovered CD4 SP thymocytes from Thy/Liv implants infected with either the CCR5-tropic HIV-1<sub>JR-CSF</sub> or the CXCR4-tropic HIV-1<sub>NL4.3</sub> strains. Throughout all subsequent procedures, cells were cultured in medium containing a viral protease inhibitor to prevent in vitro viral spread<sup>26</sup>, which could confound the results. Costimulation induced increases in viral p24 levels from cells infected with either virus, suggesting that these cells harbored a latent form of HIV-1 (Table 1).

We have previously established that deletion of the viral *vpr* gene has no effect on pathogenesis in the SCID-hu system<sup>25</sup>. So to better define the latent phenotype, in place of the HIV-1<sub>NL4.3</sub> *vpr* gene we inserted the cDNA for murine CD24 (muCD24), generating the pathogenic reporter virus, NL-r-HSAs, which directs the expression of murine CD24 on the surface of productively infected cells<sup>25</sup>. This allows for flow cytometric quantification of cells expressing viral proteins. Costimulation of purified CD4 SP thymocytes resulted in a four-fold increase in the number of cells expressing virus, and an increase in the intensity of reporter expression on the surface of these cells compared with unstimulated controls (Fig. 2a). This was re-

flected in a 10-fold increase of viral p24 in the supernatants of stimulated cells (data not shown), similar to that observed for wild-type virus. Cells harboring reactivatable virus were limited to the mature CD3<sup>+</sup>/CD27<sup>-</sup>/CD4<sup>+</sup> thymocyte subset and were not observed in the immature CD3<sup>-</sup>/CD4<sup>+</sup> subset (data not shown). Although total levels of viral infection varied, this increase was seen in many experiments with Thy/Liv implants derived from different fetal tissue donors (Fig. 2b). When CD8 SP thymocytes were similarly assessed for latent NL-r-HSAs, we observed no discernible increases in reporter or p24 expression upon costimulation (data not shown), indicating that latency in the CD4 SP subset could have resulted from infection of a cell that had already differentiated into the CD4 lineage. However, costimulation of CD8<sup>+</sup> thymocytes from HIV-1<sub>JR-CSF</sub>-infected implants resulted in a 10-fold increase in viral p24 levels (data not shown), whereas virus expression from similarly treated CD4<sup>+</sup> cells increased 25-fold (Table 1). The latent virus in the CD8 SP population likely arose from infection of CD4<sup>+</sup>/CD8<sup>-</sup> precursors; thus the less cytopathic nature of HIV<sub>JR-CSF</sub> (ref. 27) allows some infected CD4<sup>+</sup>/CD8<sup>-</sup> cells to survive differentiation into latently infected single-positive T cells.

**Table 1** Production of p24 *gag* from latently infected thymocytes.

Strain	Condition	p24 (ng/ml)
HIV-1 <sub>NL4.3</sub>	Costimulated	22.5
HIV-1 <sub>NL4.3</sub>	Unstimulated	4.5
HIV-1 <sub>JR-CSF</sub>	Costimulated	34.0
HIV-1 <sub>JR-CSF</sub>	Unstimulated	1.3

HIV-1<sub>NL4.3</sub> (tissue donor 22), HIV-1<sub>JR-CSF</sub> (tissue donor 43) and tissue-donor-matched mock-infected Thy/Liv implants were biopsied 3 wk post-infection. CD8-depleted CD4 SP thymocytes were stimulated for 3 d with activating antibodies to CD3 and to the costimulatory molecule CD28. Viral p24 protein levels (ng/ml) in the culture supernatants were determined by ELISA. No detectable p24 was observed in mock-infected thymocytes.

**Table 2** Levels of HIV integration in latently infected thymocytes.

	cells expressing muCD24 (%)	cells with full-length reverse transcripts (%)	infected cells with integrated provirus (%)
Donor Tissue 20:	12	12	67
Donor Tissue 28:	8	21	80

In 2 experiments (tissue donor 20 and 28) CD4 SP thymocytes isolated from NL-r-HSAs-infected Thy/Liv implants were analyzed on Day 0 by PCR analysis for the presence of full-length HIV reverse transcripts and integrated HIV provirus. CD4 SP thymocytes from the same biopsy were costimulated for 3 d and HIV reporter expression (murine CD24) quantitated by flow cytometry. Levels of integrated provirus were determined by limiting dilution Alu-LTR PCR (ref. 3).

### Characterization of proviral DNA and RNA

We next sought to identify the molecular nature of the viral genome in these latently infected cells. Inclusion of the reverse transcription inhibitor AZT in the medium before and during costimulation did not diminish the reactivation of latent virus (Fig. 2b), indicating that the viral genome was not similar to the incompletely reverse-transcribed and transiently latent form that we have previously reported<sup>13,28</sup>. Results of polymerase chain reaction (PCR) analysis further indicated that the vast majority of viral genomes were fully reverse-transcribed (Table 2). To quantify integrated proviruses, we performed PCR analysis with a primer pair specific for cellular Alu sequences and the viral long terminal repeat<sup>3</sup> (LTR). Following limiting-dilution analysis using this primer pair, we found that approximately 70–80% of full-length reverse transcripts were integrated (Table 2). The number of unintegrated genomes present in these cells was insufficient to account for the number of cells expressing reporter molecules following costimulation. We have previously shown that reverse transcription is aborted in quiescent or anti-CD3-stimulated T cells<sup>26,28</sup> and that infection is non-productive. In contrast, stimulation with anti-CD3 alone induced virus expression from latently infected thymocytes (Fig. 2b) although cellular RNA expression was minimal (Fig. 1b). Together these results

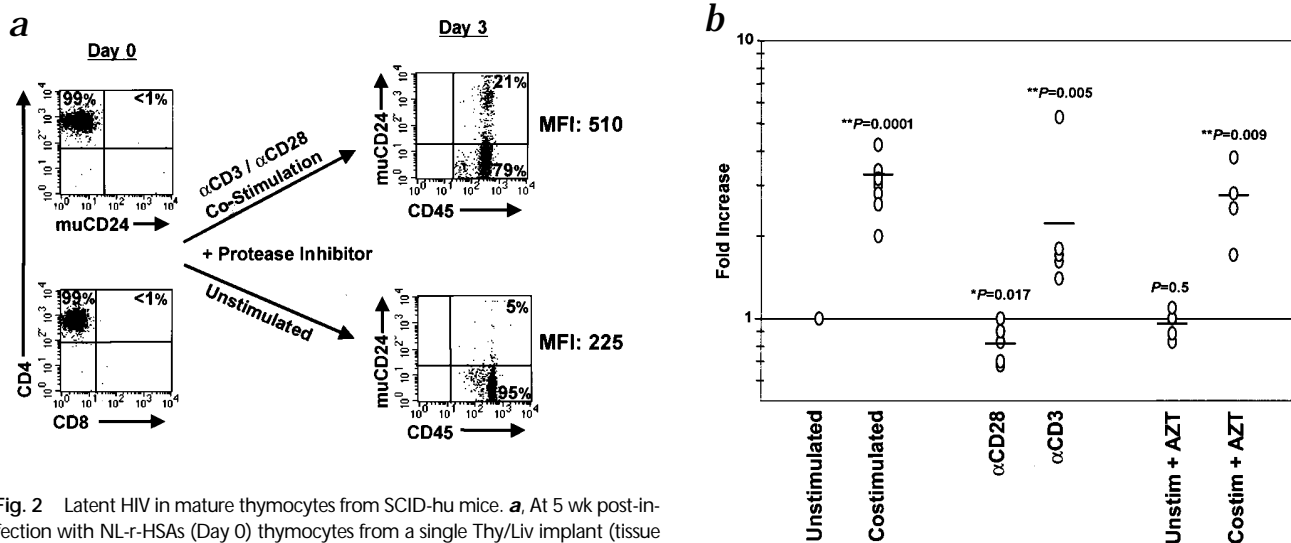
indicate that these CD4 SP thymocytes were infected before entering a state of quiescence, and they harbor an integrated provirus.

We assessed the kinetics of viral RNA expression following activation using RNA-specific PCR analysis to detect unspliced (genomic) and multiply spliced (*tat/rev*) transcripts. When equivalent amounts of cellular RNA were analyzed, unspliced HIV-1 RNA expression increased 24-fold following costimulation (Fig. 3a), beginning as early as day one after activation and peaking at day two, consistent with the calculated half-life of infected cells<sup>1,2</sup>. Stimulation with anti-CD3 alone caused a similar increase in unspliced

HIV-1 RNA (data not shown). There was a less pronounced relative increase of multiply spliced *tat/rev* transcripts following costimulation (Fig. 3a). However, costimulation induced up to a ten-fold increase in total RNA levels, thus the absolute number of these transcripts increased substantially. We also investigated cytokine signals that might lead to activation of HIV expression (Fig. 3b). Of those cytokines tested, the combination of interleukin (IL)-2, IL-6 and tumor necrosis factor (TNF)- $\alpha$ , previously shown by Chun *et al.* to increase expression of latent virus from quiescent human peripheral blood cells<sup>29</sup>, increased HIV expression to the greatest extent. Nonetheless, costimulation alone induced the most robust response.

### Export of latently infected cells from the thymus

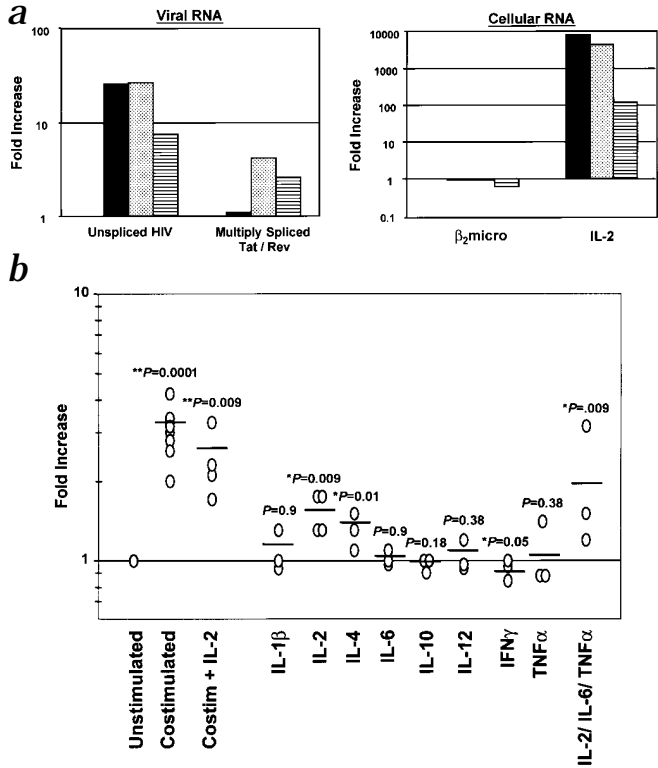
To determine whether latently infected thymocytes could be exported into the periphery, we analyzed HIV production from human lymphocytes derived from peripheral blood and spleens of SCID-hu mice infected with NL-r-HSAs or HIV<sub>JR-CSF</sub>. In addition to protease inhibitor, AZT was added at the onset of all cultures to inhibit infection from extracellular or adhered virions. Due to the high level of endogenous murine CD24 expression in the periphery, we examined viral p24 production in culture supernatants, which increased substantially in the anti-CD3 and



**Fig. 2** Latent HIV in mature thymocytes from SCID-hu mice. **a**, At 5 wk post-infection with NL-r-HSAs (Day 0) thymocytes from a single Thy/Liv implant (tissue donor 187) were stained with mAbs against CD4, CD8, CD45 and murine CD24 (muCD24) then sorted by FACS. The left panels were gated on CD45<sup>+</sup> cells and indicate that greater than 99% of the sorted cells were CD4<sup>+</sup> and muCD24<sup>-</sup> post sort. These thymocytes were then cultured without stimulation or costimulated for 3 d. The right panels show the increase in HIV (muCD24) expression and mean fluorescence intensity resulting from these conditions. **b**, The ability of costimulation, anti-CD28 ( $\alpha$ CD28) or anti-CD3 ( $\alpha$ CD3) alone or costimulation in the presence of AZT to induce HIV from latency. NL-r-HSAs-infected CD4 SP thymocytes

were cultured for 3 d and HIV reactivation was quantified by flow cytometry. The graph displays the fold increase in HIV reporter expression for each condition compared with the unstimulated control, which is set at 1. Each point represents results from a single experiment and the horizontal lines indicate mean values. \*, significance by the Wilcoxon rank sum test (*P* values provided). \*\*, significance also by the more stringent Dunnett's two-tailed multiple comparisons test.

**Fig. 3** Molecular analysis of latent HIV in SCID-hu thymocytes and the effect of cytokines on reactivation. **a**, RNA was collected from NL-r-HSAs-infected, CD4 SP thymocytes (tissue donor 28) on day 0 post harvest, and on day 1 (■), day 2 (▨) and day 3 (▩) following costimulation. RNA concentrations were normalized and RT-PCR was performed to quantify the kinetics of multiply spliced *tat/rev*, unspliced HIV, cellular  $\beta_2$  microglobulin and IL-2 transcripts following cell activation. The graph shows the fold increase of each transcript compared with day 0, which is set at a value of 1. Both graphs are from the same experiment and are representative of multiple experiments. **b**, NL-r-HSAs-infected CD4 SP SCID-hu thymocytes were incubated under the given conditions for 3 d and HIV expression assessed by flow cytometry. \*, significance by the Wilcoxon rank sum test (*P* values provided). \*\*, significance also by the more stringent Dunnnett's two-tailed multiple comparisons test.



costimulated cultures in every experiment (Fig. 4). Thus latently infected cells generated in the thymus after infection with either CCR5- or CXCR4-tropic strains can be exported to the periphery, where viral expression can be induced following T-cell receptor stimulation. PCR analysis of multiple experiments consistently demonstrated high levels of full-length viral DNA in peripheral cells and a relative increase in unspliced HIV transcripts following costimulation of splenocytes—similar to that observed in CD4 SP thymocytes (data not shown).

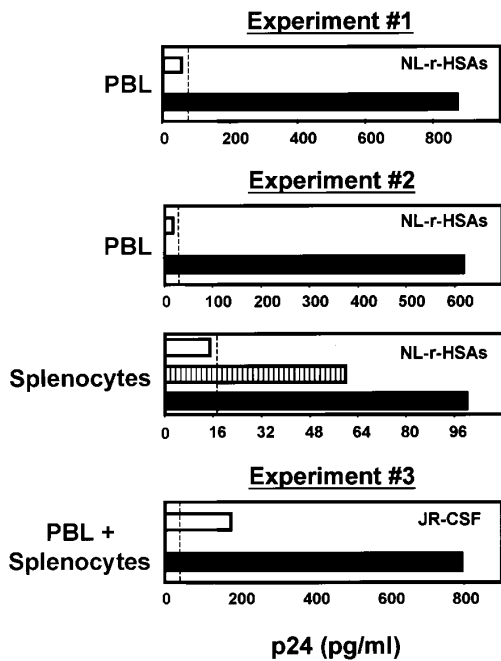
**Discussion**

Our studies indicate that HIV latency can be generated during normal T-cell differentiation, and implicate the resulting decrease in cellular RNA transcription as the mechanism responsible for this phenomenon. These latently infected cells can be induced to express viral proteins in the presence of AZT and a protease inhibitor, and can be exported into the periphery where they remain latently infected. Interestingly, CD4 SP thymocytes that were negative for reporter expression by flow cytometry on day 0 were producing detectable levels of unspliced and multiply spliced HIV transcripts. We cannot yet determine whether the

majority of these cells harbor transcriptionally silent HIV, or whether there is continuous low-level expression of viral RNA in all cells.

For many reasons it is impossible to perform studies of HIV latency in the human thymus similar to those described here. Consequently, we used the SCID-hu mouse, which faithfully replicates human thymopoiesis. Virus loads in this system are higher than those seen in the human thymus, as there is no virus specific host immune response. Nonetheless, the known infection of the human thymus in patients, together with the relatively high efficiency of generation of latently infected cells we observed here, indicate that this mechanism might contribute to the latent pool in HIV-infected patients.

This is the first report to document the mechanism responsible for the generation of HIV latency in primary cells. The decrease in cellular RNA transcription during maturation documented here in the thymus may contribute to the naive CD45RA<sup>+</sup> latent reservoir recently identified in the peripheral blood of HIV-infected individuals<sup>16</sup>. Furthermore, infection of a CD4<sup>+</sup>/CD8<sup>+</sup> thymocyte and subsequent differentiation and export of the infected cell could explain the presence of HIV DNA found in pe-



**Fig. 4** HIV latency in the periphery. Human lymphocytes from peripheral blood or spleen of NL-r-HSAs- or HIV-1<sub>JR-CSF</sub>-infected SCID-hu mice were cultured in the presence of protease inhibitor and AZT and either left unstimulated, □; stimulated with anti-CD3 alone, ▨; or were costimulated, ■. Three days later supernatants were assessed for the presence of p24 gag protein. Dotted lines indicate the level of detection of the ELISA. Experiments 1 and 2 were derived from 2 separate experiments using different mice from tissue donor 30. In experiment 3, PBL and splenocytes from SCID-hu mice (tissue donor 43) were pooled prior to culture. The virus strain used in each experiment is shown. Similar results were obtained with peripheral cells from HIV-1<sub>NL4.3</sub>-infected mice.



peripheral CD8<sup>+</sup> T cells<sup>30</sup>. A similar decrease in cellular transcription likely occurs as activated peripheral T cells revert to the memory phenotype, resulting in a related latency-generating phenomenon. Recent studies using *in situ* hybridization and immunohistochemistry analyses for activation markers have indicated that HIV expression can be detected in non-cycling cells *in vivo*<sup>31</sup>. The initial infection of these cells may have arisen during thymopoiesis or alternatively in the periphery as discussed above. Stimulation by signals through the T-cell receptor alone (as above) or through various cytokines present in the tissues<sup>32</sup> could result in virus expression from latently infected cells in the absence of expression of standard T-cell activation markers. A greater understanding of how cellular activation events can generate and reactivate latent HIV infection may lead to the eradication of this reservoir. Our data indicate that the SCID-hu mouse may be an important model to study events important in viral latency and reactivation, and to evaluate pharmacological agents designed to activate latent virus.

## Methods

**Infection of SCID-hu mice.** SCID-hu mice were prepared by implantation of human fetal liver and thymus tissue as described<sup>21</sup>. Thy/Liv implants were mock-infected with medium or directly injected with viral stocks such that a total of 10 ng viral p24 for HIV-1<sub>NL4-3</sub>, 20 ng of NL-F-HSAs or 50 ng HIV-1<sub>IR-CSF</sub> were introduced. In general 1 ng of p24 contains approximately 100 infectious units. Viral stocks were prepared by electroporation of cloned proviral DNA into CEMX174 cells. p24 *gag* expression was assessed by enzyme-linked immunosorbent assay (ELISA, Coulter, Hialeah, Florida) and was used to quantify viral titers and reactivation from latency.

**Flow cytometry.** Cells from cultures were stained with monoclonal antibodies (mAb) specific for human CD4, CD8, CD45 and murine CD24 directly conjugated to phycoerythrin (PE), peridinin chlorophyll protein (PerCP), allophycocyanin (APC) or fluorescein isothiocyanate (FITC; Coulter), respectively. Samples were analyzed on a FACSCaliber flow cytometer using the Cell Quest Program (Becton-Dickinson, Mountain View, California). Forward- versus side-scatter profiles and 7AAD dead-cell exclusion were used to define the live population. These cells were further gated on the human CD45<sup>+</sup> population to exclude murine cells. Quadrants were set based on isotype controls from mock-infected cells.

**Cell isolation.** Thy/Liv implants were collected 28–40 d post-infection and single-cell suspensions of thymocytes pooled in the presence of 100 ng/ml Indinavir (Merck, West Point, Pennsylvania). Thymocytes from mock-infected implants were isolated and cultured in parallel as negative controls in all experiments. CD4 SP thymocytes were initially purified by sorting on a FACStar<sup>plus</sup> flow cytometer (Becton-Dickinson). Thymocytes were stained with CD4-APC, CD8-FITC, CD45-PerCP and murine CD24-PE and sorted on a FACStar<sup>plus</sup> flow cytometer. Post-sort analysis showed greater than 99% of cells were CD4<sup>+</sup>, CD8<sup>-</sup> and muCD24<sup>-</sup>. In later experiments thymocytes were stained with murine antibodies against human CD8 or CD4 (Becton-Dickinson) and subjected to negative selection by panning with flasks coated with goat antibody against mouse (GAM; Sigma). These cells were then stained with rat antibody against murine CD24 (Pharmingen, San Diego, California) and panned in flasks coated with rabbit antibody against rat (Sigma). Purity was assessed with different antibody clones to prevent protein masking (Coulter). Panning resulted in greater than 99% CD24- and CD8- or CD4-depleted subsets. To obtain human peripheral blood mononuclear cells from SCID-hu mice, blood from 10–15 SCID-hu mice was pooled and cells were isolated over a Ficoll-Paque gradient. Splenocytes from these same mice were pooled and isolated similarly.

**Cell culture.** Cells were cultured in RPMI 1640 supplemented with 10% human AB serum, 100 U/ml of penicillin, 100 µg/ml streptomycin, 2 mM glutamine and 100 nM Indinavir. Cells were costimulated with anti-CD3 monoclonal antibody bound to GAM coated plates and 0.1 µg/ml soluble anti-CD28 (ref. 26). Where indicated cells were pre-incubated and cultured

in the presence of 10 µM AZT (Sigma). Each Cytokine (R&D Systems, Minneapolis, Minnesota) was added to a final concentration of 100 U/ml.

**Cell-cycle analysis.** Cell-cycle analysis of CD4 SP thymocytes from the Thy/Liv implant was performed by flow cytometry as described<sup>26</sup>. Briefly, DNA was visualized with 7AAD and RNA with Pyronin Y (PY). To determine quadrants for the different transitional stages of the cell cycle, purified single-positive thymocytes were either left unstimulated, were co-stimulated, or were co-stimulated in the presence of 3 mM N-acetyl butyric acid or 10 µM aphidicolin, which inhibit the cell cycle at the G<sub>1</sub>a to G<sub>1</sub>b and G<sub>1</sub>b to S-phase transition, respectively. Cell-cycle determination of the different thymocyte subsets from fresh fetal thymic tissue was adapted from a previously described method for four-color cell cycle analysis<sup>33</sup>. Fetal thymic tissue was stained simultaneously for DNA/RNA content and the CD4 and CD8 T-cell surface proteins using mAb CD4-APC and CD8-FITC (Coulter).

**DNA purification and PCR.** Quantitative PCR was performed as previously described<sup>13</sup> with [<sup>32</sup>P]-labeled primer pairs specific for the human β-globin gene, the R/U5 region of the viral LTR (M667/AA55) and adjacent LTR/gag sequences (M667/M661). To assess levels of integrated viral DNA, the limiting dilution Alu-PCR protocol described by Chun *et al.*<sup>3</sup> was modified. The first round of the nested reaction was unchanged. In the second nested PCR amplification instead of using a [<sup>32</sup>P]-labeled probe and Southern hybridization<sup>3</sup>, the primer NI-2 3' was directly [<sup>32</sup>P] labeled and combined with the primer NI-2 5' <sup>3</sup>, PCR Buffer<sup>13</sup>, 3 U Taq Polymerase (Fisher Scientific, Pittsburgh, Pennsylvania) and approximately 1/250 of the first reaction as substrate. The cycling conditions were 28 cycles of 95 °C for 30 s, 63 °C for 30 s and 72 °C for 1 min. PCR products were resolved on a 6% polyacrylamide gel and visualized by autoradiography. The limiting dilution PCR was done in duplicate or triplicate for each sample and the frequency of cells with integrated provirus was statistically analyzed by the method of maximum likelihood<sup>34</sup>.

**RT PCR analysis.** Total RNA was isolated with the RNeasy extraction kit (Qiagen, Chatsworth, California). 200 ng of total RNA for the unspliced HIV transcripts or 600 ng for the multiply spliced transcripts was then reverse transcribed with the GeneAmp ThermoStable rTth Reverse Transcriptase RNA PCR kit (Perkin Elmer, Foster City, California) using the manufacturers protocol. The primer pairs and cycling conditions for the HIV PCR amplifications have been described<sup>35</sup>. Antisense primers to β<sub>2</sub>-microglobulin (β<sub>2</sub>-m, 5'-AGCTACCTGTGGAGCAACCTGC-3') and IL-2 (5'-TCCTCCAGAG-GTTTGTGTTCTTCTTC-3') were each included in the RT reactions. An aliquot of this cDNA was then used as template for the PCR amplifications. To analyze β<sub>2</sub>-m and IL-2 transcription, the [<sup>32</sup>P]-labeled direct primers (β<sub>2</sub>-m, 5'-AAAAGATGAGTATGCCTGCCGTG-3'; IL-2, 5'-ACAGCTACAACTG-GAGCATTATCTGC-3') were each added to separate PCR amplifications. All buffer conditions are as described<sup>13</sup> modified with the addition of chelating buffer (Perkin Elmer). β<sub>2</sub>-m reactions were subjected to 25 cycles at 1 min each of 94 °C denaturation, hybridization at 55 °C, and a 72 °C extension. IL-2 amplifications were carried out by 30 cycles of denaturation at 94 °C for 1 min and 2 min of polymerization at 65 °C.

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