



Small-molecule screening using a human primary cell model of HIV latency identifies compounds that reverse latency without cellular activation

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The development of highly active antiretroviral therapy (HAART) to treat individuals infected with HIV-1 has dramatically improved patient outcomes, but HAART still fails to cure the infection. The latent viral reservoir in resting CD4⁺ T cells is a major barrier to virus eradication. Elimination of this reservoir requires reactivation of the latent virus. However, strategies for reactivating HIV-1 through nonspecific T cell activation have clinically unacceptable toxicities. We describe here the development of what we believe to be a novel in vitro model of HIV-1 latency that we used to search for compounds that can reverse latency. Human primary CD4⁺ T cells were transduced with the prosurvival molecule Bcl-2, and the resulting cells were shown to recapitulate the quiescent state of resting CD4⁺ T cells in vivo. Using this model system, we screened small-molecule libraries and identified a compound that reactivated latent HIV-1 without inducing global T cell activation, 5-hydroxynaphthalene-1,4-dione (5HN). Unlike previously described latency-reversing agents, 5HN activated latent HIV-1 through ROS and NF- κ B without affecting nuclear factor of activated T cells (NFAT) and PKC, demonstrating that TCR pathways can be dissected and utilized to purge latent virus. Our study expands the number of classes of latency-reversing therapeutics and demonstrates the utility of this in vitro model for finding strategies to eradicate HIV-1 infection.

Introduction

Highly active antiretroviral therapy (HAART) can suppress HIV-1 levels in plasma to below the limit of detection of clinical assays (<50 copies/ml) and reduce the morbidity and mortality of HIV-1 infection. However, HAART alone fails to cure the infection. In particular, HAART leaves latent integrated proviruses unaffected (1, 2). Latent viral genomes reside in a small pool of infected resting memory CD4⁺ T cells that constitute a stable viral reservoir. In these cells, the provirus remains transcriptionally silent as long as the host cells are in a quiescent state (3–5). This allows the virus to evade host immune surveillance and rebound quickly following discontinuation of HAART. The remarkable stability of the latent viral reservoir necessitates lifelong HAART (6). Given the potential for toxicity and resistance, elimination of the latent reservoir has recently been proposed as a goal worthy of a major scientific effort (7).

Novel therapies targeting the latent reservoir generally involve reactivation of latent virus (2, 7–13). Expression of viral genes renders infected cells susceptible to viral cytopathic effects and immune clearance. Along with HAART, this reactivation strategy could ultimately purge latent virus from infected individuals. Latent viruses respond to T cell activation signals (10, 12, 14–17). However, initial attempts to deplete the latent reservoir through TCR stimulation using anti-CD3 antibodies proved toxic (18, 19).

The toxicity likely resulted from nonspecific T cell activation and release of proinflammatory cytokines. Therefore, an ideal treatment should reactivate latent HIV-1 but avoid global T cell activation.

In the search for activators of latent HIV-1, an important tool would be an in vitro model that mimics the latent state of HIV-1 in primary resting CD4⁺ T cells and allows for high-throughput screening. Some in vitro latency models have been established in transformed T cell lines (20–22). Although useful, these cell-line models are fundamentally different from the resting CD4⁺ T cells because of their proliferating nature and aberrant signaling pathways. Some primary cell models have been recently developed in thymocytes or CD4⁺ T cells (23–26). However, a model that yields cells in a truly quiescent state in quantities sufficient for high-throughput screening is lacking. Given that HIV-1 preferentially infects activated CD4⁺ T cells and that latent viral genomes are mainly found in resting memory CD4⁺ T cells (27, 28), it is likely that HIV-1 latency is established when infected lymphoblasts transit back to a resting state and persist as memory T cells. The low frequency of the latently infected cells in vivo (5) indicates that the establishment of latency is inefficient. This is due in part to the fact that only a small fraction of lymphoblasts normally survive to become memory cells. Viral cytopathic effects and host immune clearance may also contribute to the loss of infected cells prior to the establishment of latency. The inefficiency with which latency is established in vivo makes the development of an in vitro model especially challenging.

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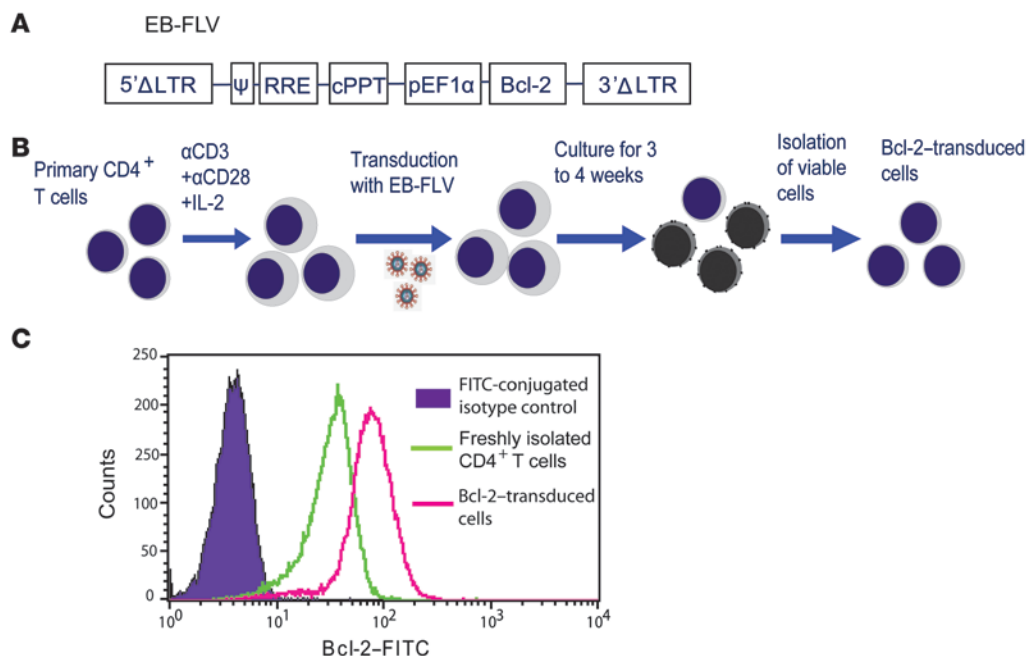


Figure 1

Generation of the Bcl-2-transduced primary CD4⁺ T cells. **(A)** The structure of the lentiviral vector carrying the human Bcl-2 gene (EB-FLV). The U3 region of 3' LTR is deleted (3'ΔLTR) for self inactivation. The expression of human Bcl-2 is driven by EF1α promoter. Ψ, packaging signal; RRE, rev responsive element; cPPT, central polypurine tract; pEF1α, EF1α promoter. **(B)** Strategy to generate Bcl-2-transduced primary CD4⁺ T cells. Primary CD4⁺ T cells from normal donors were activated and transduced with the Bcl-2-expressing lentiviral vector. Viable cells were isolated after 3 to 4 weeks of culture in the absence of TCR stimulants or cytokines. **(C)** Intracellular staining for Bcl-2 with FITC-conjugated anti-Bcl-2 antibody in freshly isolated CD4⁺ T cells and Bcl-2-transduced cells. The Bcl-2-transduced cells were maintained in culture without cytokines for 4 weeks following transduction. Freshly isolated CD4⁺ T cells stained with FITC-conjugated isotype control antibodies served as a negative control (purple).

Certain cytokines play a role in T cell survival (29). IL-7 controls the generation and maintenance of memory CD4⁺ T cells in vivo (30, 31). However, IL-7 also reactivates latent HIV-1 (12, 32), and thus using IL-7 to promote the establishment of latency in vitro has been difficult. The antiapoptotic protein Bcl-2 is a downstream effector of IL-7 signaling and plays an essential role in counteracting the proapoptotic effects of cytokine withdrawal and maintaining the survival of resting memory CD4⁺ T cells (29, 33, 34). The prosurvival effect via Bcl-2 is the key homeostatic function of IL-7. Overexpression of Bcl-2 largely restores peripheral T cell homeostasis in the absence of IL-7 receptor (35, 36), indicating that the prosurvival ability of IL-7 can be decoupled from the proliferative and metabolic effects of IL-7 (37, 38).

Here, we report a strategy for generation of an in vitro HIV-1 latency model in primary CD4⁺ T cells that takes advantage of the prosurvival effects of Bcl-2. We developed a method for establishing latent HIV-1 infection in the Bcl-2-transduced primary human CD4⁺ T cells. We screened libraries of small molecules and identified what we believe to be a novel compound that efficiently reactivates latent HIV-1 but does not induce global T cell activation. Our study reveals a new strategy for reactivating latent HIV-1 and illustrates the application of this in vitro primary cell model in finding novel agents for the cure of HIV-1 infection.

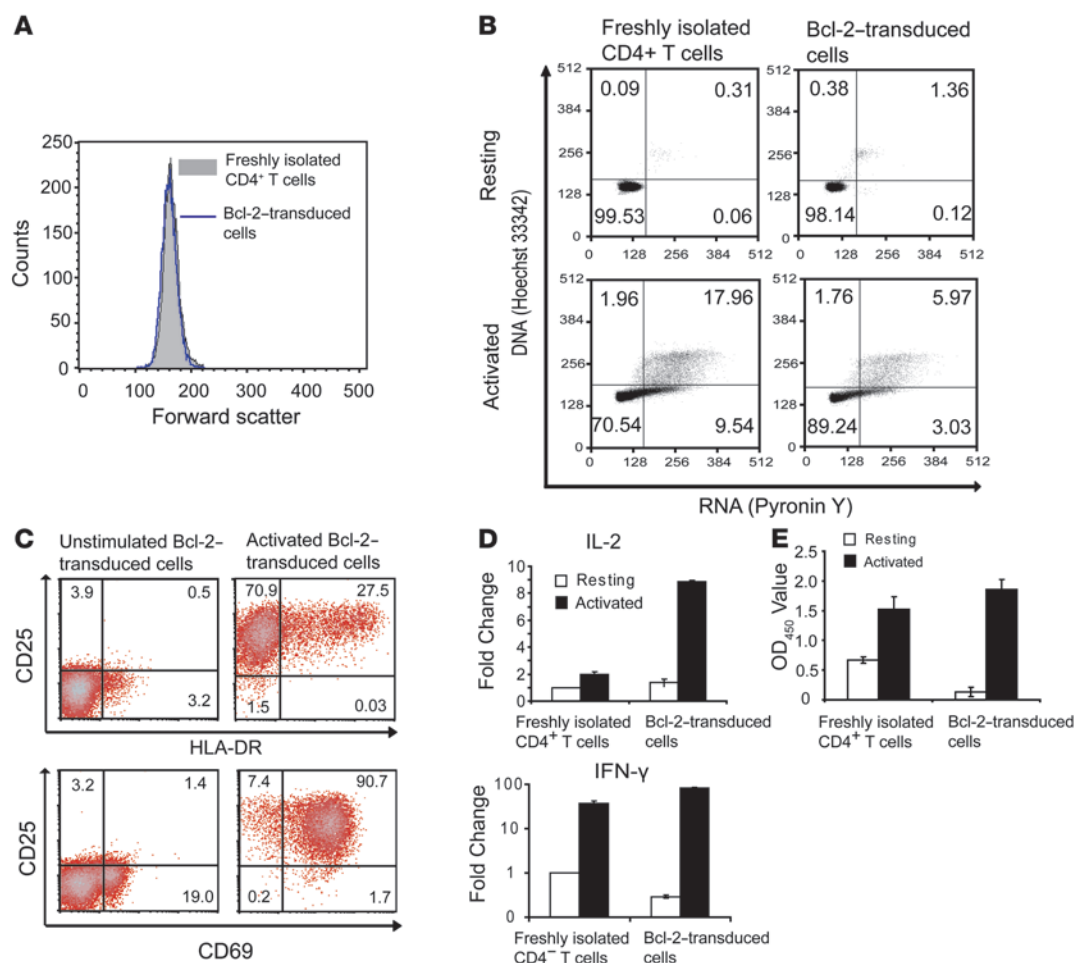
Results

Transduction of primary human CD4⁺ T cells with Bcl-2. To allow long-term in vitro survival, we transduced primary CD4⁺ T cells with Bcl-2 using a lentiviral vector, EB-FLV, in which Bcl-2 expression is

driven by the constitutively active promoter of elongation factor 1α (EF1α) (Figure 1, A and B). Freshly isolated primary human CD4⁺ T cells were costimulated with anti-CD3 and anti-CD28 and then transduced with the Bcl-2-expressing lentiviral vector. Transduced cells were maintained in the absence of TCR stimulants and exogenous cytokines for more than 3 weeks. Viable cells were then isolated using Ficoll-Hypaque density gradient centrifugation. As shown in Supplemental Figure 1A (supplemental material available online with this article; doi:10.1172/JCI39199DS1), more than 80% of the activated cells are lost during the first 3 weeks of cytokine withdrawal. However, the remaining viable cells have greatly improved survival (see below).

Activated primary T cells die quickly in culture without trophic cytokines (29). Therefore, only Bcl-2-transduced cells survive under the above culture conditions. Bcl-2 was overexpressed in the vast majority of viable cells present after 4 weeks of culture (Figure 1C). In control experiments, cells transduced with the control lentiviral vector that did not express Bcl-2 died within 2 weeks. To further confirm the longevity of the Bcl-2-transduced cells and the stability of Bcl-2 expression, we quantified viability and Bcl-2 expression weekly in cultures of isolated Bcl-2-transduced cells maintained without supplemental cytokines. We found less than 20% cell loss (Supplemental Figure 1B) and stable Bcl-2 expression (Supplemental Figure 1C) over a 4-week follow-up period.

Bcl-2-transduced cells reach a quiescent state similar to that of freshly isolated primary resting CD4⁺ T cells. Because resting memory CD4⁺ T cells are a major reservoir for latent HIV-1 in vivo, an in vitro

**Figure 2**

Characterization of resting Bcl-2-transduced cells. **(A)** Flow cytometric measurement of cell size. The forward scatter profile of Bcl-2-transduced cells coincided with that of freshly isolated resting CD4⁺ T cells. **(B)** Cell-cycle analysis in resting and activated freshly isolated CD4⁺ T cells, Bcl-2-transduced cells, and latently infected Bcl-2-transduced cells. Data are plotted with DNA staining (Hoechst 33342) on the y axis versus RNA staining (pyronin Y) on the x axis. Cells were either left in a resting state or activated with anti-CD3 and anti-CD28 antibodies for 2 days. The percentage of cells in each quadrant is indicated. **(C)** The expression of the activation markers CD25, CD69, and HLA-DR on resting and activated Bcl-2-transduced cells. The percentage of cells in each quadrant is indicated. **(D)** Levels of IL-2 and IFN- γ mRNA in resting and activated freshly isolated and Bcl-2-transduced CD4⁺ T cells. The levels of IL-2 and IFN- γ mRNA were quantified using real-time RT-PCR and normalized to the ubiquitin mRNA levels. The fold change was relative to that observed in the freshly isolated resting CD4⁺ T cells. **(E)** Levels of nuclear NF- κ B p65 in resting and activated freshly isolated and Bcl-2-transduced CD4⁺ T cells. The nuclear NF- κ B p65 was quantified by an ELISA-based assay and normalized to the total protein concentration of each nuclear extract. Results shown are relative OD₄₅₀ values. Data in **D** and **E** are mean \pm SD of triplicate samples from 1 of 2 independent experiments, all of which produced similar results.

latency model should consist of infected cells in a similarly quiescent state. After 4 weeks in culture without TCR stimulation or cytokines, surviving Bcl-2-transduced cells were characterized with regard to properties unique to primary resting T cells. These include small cell size, absence of cell proliferation and cytokine production, and lack of activation markers.

Resting Bcl-2-transduced cells exhibited small cell size (Figure 2A) and scanty cytoplasm (Supplemental Figure 2A). Using DNA/RNA staining (39–41), we found that the majority of resting Bcl-2-transduced cells were in the G₀/G_{1a} phase of the cell cycle, as is the case with freshly isolated primary resting CD4⁺ T cells (Figure 2B). Both cell populations readily entered the cell cycle following activation with anti-CD3 and anti-CD28 (Figure 2B). Surface expression of classic T cell activation markers such

as CD25, CD69, and HLA-DR was very low on resting Bcl-2-transduced CD4⁺ T cells (Figure 2C). Less than 5% of these cells expressed CD25, and expression of HLA-DR was similarly low. Approximately 15%–20% of these cells had low but detectable levels of CD69. However, following 3 days of activation by anti-CD3 and anti-CD28, more than 90% of cells expressed high levels of CD25 and CD69 and more than 25% of cells expressed HLA-DR (Figure 2C). The resting status of the Bcl-2-transduced CD4⁺ T cells was further confirmed by measurement of cytokine mRNA levels using real-time RT-PCR (Figure 2D). Both the IL-2 and IFN- γ transcripts were present only at very low levels in the resting Bcl-2-transduced cells, while transcript levels were increased 8-fold and 90-fold, respectively, following activation by anti-CD3 and anti-CD28.

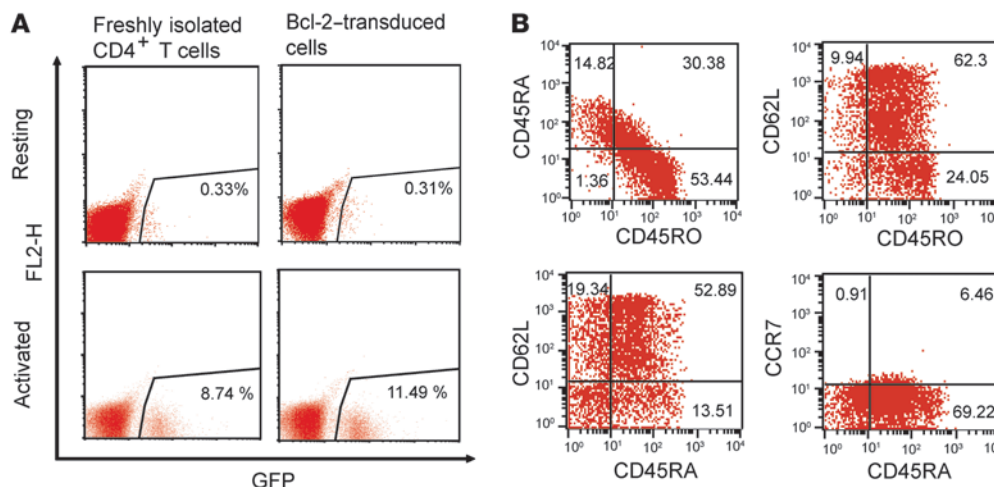


Figure 3

Resting Bcl-2-transduced cells exhibit resistance to HIV-1 infection and phenotypes of T_{EM}. (A) Susceptibility of resting and activated freshly isolated and Bcl-2-transduced CD4⁺ cells to HIV-1 infection. Cells were infected with reporter virus pNL4-3-Δ6-drEGFP pseudotyped with HIV-1-X4 envelope. The number in each plot indicates the percentage of infected (GFP-positive) cells. (B) The expression of CD45RA, CD45RO, CCR7, and CD62L on resting Bcl-2-transduced cells. The percentage of cells in each quadrant is indicated.

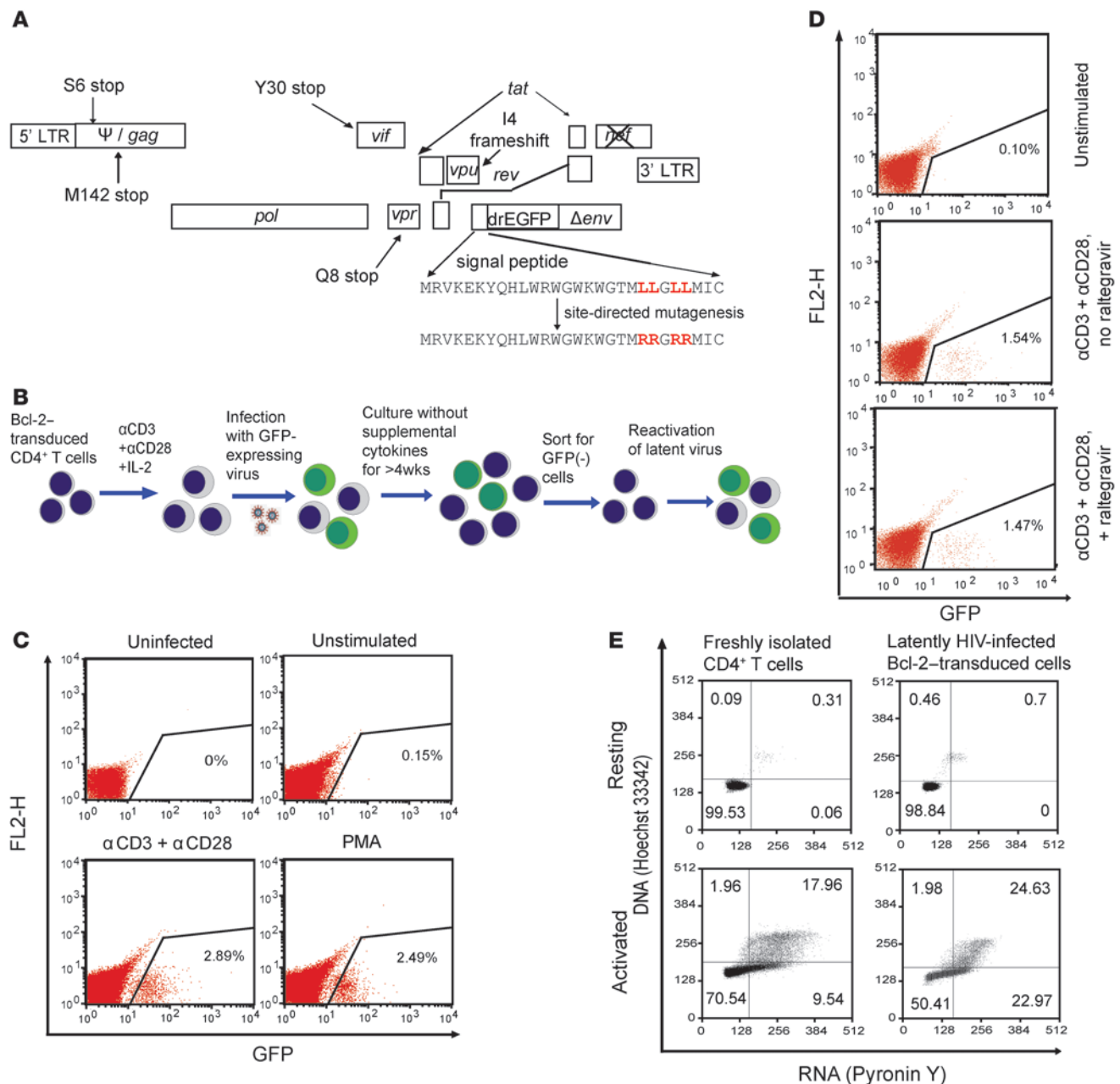
The host transcription factor NF-κB is critical for HIV-1 replication (14–16, 42). Lack of NF-κB activation in resting CD4⁺ T cells is a major factor in HIV-1 latency (43). We therefore examined the activation state of NF-κB in resting Bcl-2-transduced cells by ELISA measurement of the nuclear levels of NF-κB p65. As Bcl-2-transduced cells returned to a quiescent state, nuclear levels of NF-κB fell to below those seen in freshly isolated resting CD4⁺ T cells (Figure 2E). Upon stimulation with anti-CD3 and anti-CD28, nuclear NF-κB was readily induced to high levels equivalent to those of activated primary CD4⁺ T cells (Figure 2E).

An additional characteristic of resting CD4⁺ T cells is their resistance to productive HIV-1 infection. Using a recombinant HIV-1 vector capable of expressing GFP, we showed that resting Bcl-2-transduced CD4⁺ T cells were resistant to HIV-1 infection regardless of whether the viruses were pseudotyped with an X4 HIV-1 envelope (Figure 3A) or VSV-G (Supplemental Figure 2B). However, T cell activation overcame this resistance (Figure 3A and Supplemental Figure 2B). Taken together, the above data demonstrate that the Bcl-2-transduced cells reach a resting state similar to that of primary resting CD4⁺ T cells while retaining the capability to be fully activated.

Because latent HIV-1 primarily resides in memory CD4⁺ T cells (44), we further characterized Bcl-2-transduced cells for expression of memory T cell markers. Memory T cells are CD45RO positive. Central memory T cells (T_{CM}) constitutively express CCR7 and CD62L, while effector memory T cells (T_{EM}) show heterogeneous expression of CD62L but not CCR7 (45). A large portion of Bcl-2-transduced cells (~85%) expressed CD45RO (Figure 3B). Interestingly, approximately 30% of cells expressed both CD45RA and CD45RO. Only a few cells (~6.5%) expressed CCR7. Therefore, we concluded that resting Bcl-2-transduced cells are more like T_{EM} than T_{CM}. Of note, a recent study reported that CD4⁺ T_{CM} and transition memory T cells (T_{TM}) are 2 major cellular reservoirs for HIV-1 in vivo (46). Determining whether the latently infected cells generated in our model exhibit properties similar to those of T_{TM} cells requires further investigation.

Establishment of HIV-1 latency in Bcl-2-transduced resting CD4⁺ T cells. Latently infected cells are rare in vivo (47) because most infected cells die from viral cytopathic effects or host cytolytic responses before they can revert to a resting state in which HIV-1 gene expression is shut off. Preliminary results confirmed the previously reported cytopathic effects of HIV-1 gene products (48, 49). To increase the efficiency with which HIV-1 latency was established, we mutated the *gag*, *vif*, *vpr*, *vpu*, and *nef* genes of the reference strain NL4-3. Point mutations were introduced to produce premature stop codons in each gene except for *nef*, which was truncated (Figure 4A). Our primary goal was to study the reactivation of latent HIV-1, and thus the *rev* and *tat* genes were left unchanged because their products are crucial for the expression of viral genes. To keep splicing sites intact and mimic the processing of viral transcripts, the overall genome structure of HIV-1 was preserved. Destabilized EGFP was introduced to track the viral infection. Compared with NL4-3, this modified viral vector, pNL4-3-Δ6-drEGFP, gave dramatically improved viability of infected Bcl-2-transduced cells. As shown below, this approach made it possible to obtain large numbers of latently infected primary resting CD4⁺ T cells that could be used to study upregulation of HIV-1 gene expression. The ultimate yield of latently infected cells was much lower (approximately 5- to 10-fold) when Bcl-2-transduced cells were infected with an HIV-1 construct in which all the reading frames except *env* were open. For this reason, subsequent experiments were performed with the NL4-3Δ6-drEGFP virus.

To establish HIV-1 latency, Bcl-2-transduced cells were activated and then infected with pseudotyped NL4-3-Δ6-drEGFP. Because the *env* gene is disrupted by insertion of EGFP, an X4 envelope was provided in *trans* to achieve single-round infection. The infection rate, based on the percentage of GFP⁺ cells 3 days after infection, was 5% to 10%. Following more than 4 weeks of culture in the absence of cytokines and other activating stimuli, approximately 20%–30% of cells that initially expressed GFP became GFP negative. We then isolated the GFP-negative cells by sorting. To determine whether latency had been established, the GFP-negative cells were

**Figure 4**

Establishment of in vitro HIV-1 latency in resting Bcl-2-transduced CD4⁺ T cells. **(A)** Genome structure of the reporter virus NL4-3-Δ6-drEGFP. It contains a truncated *nef* and premature stop codons in the ORFs of *gag*, *vif*, *vpr*, and *vpu* that alter the indicated amino acids shown in the standard single-letter code. A portion of *env* was replaced with destabilized EGFP, and the signal peptide of *env* was mutated to allow the destabilized EGFP to remain in the cytoplasm. The red letters indicate the mutated amino acids in the signal peptide. **(B)** Strategy for generating latently infected Bcl-2-transduced cells. **(C)** Detection of latently infected cells in the sorted GFP-negative population. The sorted GFP-negative cells were activated with anti-CD3 and anti-CD28 or PMA for 2 days and then analyzed by flow cytometry to quantify the number of GFP-positive cells. FL2-H, red fluorescence channel. **(D)** Latently infected cells contain integrated viral genomes. Latently infected Bcl-2-transduced cells were left untreated (upper panel) or were pretreated with either medium alone (middle panel) or 1 μM raltegravir (lower panel) for 1 day and then activated with anti-CD3 and anti-CD28 monoclonal antibodies for 2 days. Cells were analyzed using flow cytometry. **(E)** Cell-cycle status of latently infected cells was determined using Hoechst 33342/pyronin Y staining for DNA/RNA. The controls for the resting and activated cells are the same as in Figure 2B. The percentage of cells in each quadrant is indicated.

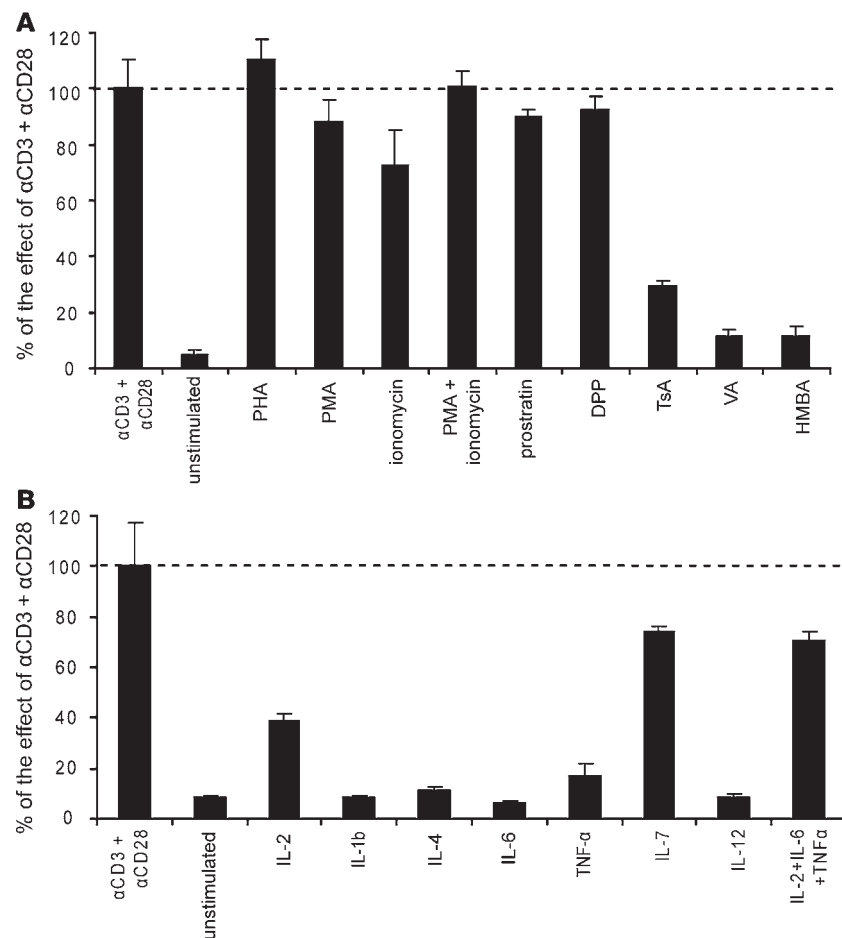


Figure 5

Response of latently infected Bcl-2-transduced CD4⁺ T cells to small molecules and cytokines known to reactivate latent HIV-1. Latently infected, Bcl-2-transduced cells were treated with known small-molecule activators (**A**) or cytokines (**B**). Bars represent the percentage of GFP-positive cells normalized to the response to anti-CD3 plus anti-CD28 antibodies. Data are mean \pm SD of triplicate samples from 1 of 2 independent experiments. The concentrations of small molecules and cytokines used for the experiments are as follows: phytohemagglutinin (PHA) (1 μ g/ml), PMA (10 ng/ml), ionomycin (1 μ M), prostratin (1 μ M), DPP (1 μ M), TsA (200 nM), VA (5 mM), HMBA (5 mM), IL-2 (100 U/ml), IL-1b (5 ng/ml), IL-4 (3 ng/ml), IL-6 (5 ng/ml), TNF- α (10 ng/ml), IL-7 (10 ng/ml), and IL-12 (10 ng/ml). For a positive control, cells were activated with 2.5 μ g/ml anti-CD3 and 1 μ g/ml anti-CD28.

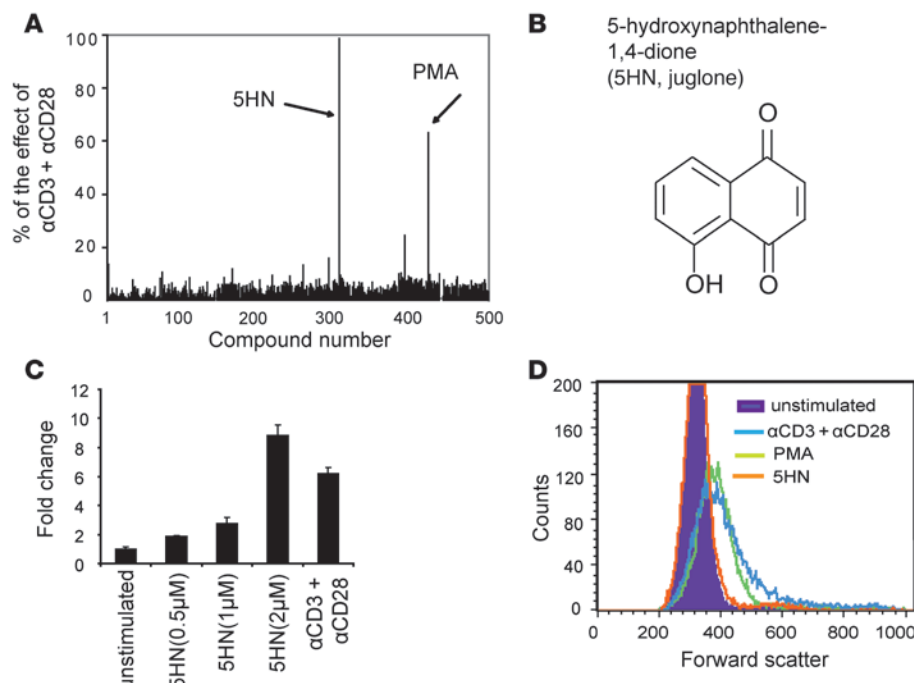
activated and analyzed for GFP expression by flow cytometry. Of the sorted GFP-negative cells, 1% to 3% could be induced to express GFP by activating stimuli such as anti-CD3 plus anti-CD28 or PMA (Figure 4, B and C). In control infected cultures that were not stimulated, the fraction of GFP-positive cells remained less than 0.2%. Taken together, these results indicate that latent infection was established in this system. We could further increase the frequency of latently infected cells by enriching the GFP-positive cells via sorting following the initial infection (Supplemental Figure 3A). The frequency of latently infected cells using this strategy could reach 15%–50% (Supplemental Figure 3, A and B). Although this enrichment increased the frequency of latently infected cells, it did not increase their absolute number. Our preliminary data showed that populations containing 1%–3% latently infected cells generated without enrichment were adequate for high-throughput screening. We therefore performed subsequent experiments without enrichment (Figure 4B).

An important feature of the stable reservoir for HIV-1 in vivo is that the viral genome is integrated into a host-cell chromosome (27, 47). To prove that the latently infected cells generated in this system contain an integrated form of the viral genome, we activated the cells in the presence of an HIV-1 integrase inhibitor, raltegravir. The concentration used was sufficient to block 99% of integration events (50). Raltegravir did not block the reactivation of latent HIV-1 (Figure 4D). This result confirms that this in vitro system is a model for postintegration latency.

Even after prolonged culture in the absence of activating stimuli, a small fraction of GFP-negative cells still expressed low levels of activation markers such as CD25, CD69, and HLA-DR. To exclude the possibility that latent HIV-1 might reside in the cells with some extent of activation, we removed these CD25⁺, CD69⁺, or HLA-DR⁺ cells by negative depletion. A very similar frequency of cells with inducible GFP expression was found in the CD25[−]CD69[−]HLA-DR[−] cells, excluding the possibility that latent HIV-1 resided in the cells with low levels of activation. We also evaluated the cell-cycle status of latently infected cells using DNA/RNA staining. As shown in Figure 4E, the majority of latently infected cells were in G₀/G_{1a} phase. Taken together, these observations demonstrate the establishment of HIV-1 latency in vitro in resting Bcl-2-transduced primary CD4⁺ T cells.

To investigate whether there was any residual integrated provirus that did not respond to T cell activation signals in this system, we sorted the GFP[−] cells following activation of latently infected cells by anti-CD3 and anti-CD28 for 3 days. The frequency of cells with proviral DNA among the GFP[−] cells determined using real-time PCR was similar to the percentage of GFP⁺ cells following activation. This suggests that approximately 50% of integrated proviruses were unresponsive to T cell activation (refer to Supplemental Material for details). Whether these viruses can be reactivated by subsequent rounds of activation requires future exploration.

The signaling pathways leading to the reactivation of latent HIV-1 remain intact in this in vitro model. To validate that the signaling pathways

**Figure 6**

Screening of small-molecule libraries identifies 5HN as a candidate activator. **(A)** Summary of screening results from JHDL. The results were expressed as the percentage of GFP-positive cells after normalization to the response to anti-CD3 plus anti-CD28. For simplicity, only 500 drugs including the hits PMA and 5HN are shown. **(B)** Chemical structure of 5HN. **(C)** Effects of 5HN, PMA, and anti-CD3 plus anti-CD28 on the size of latently infected resting CD4⁺ T cells. Cell size was measured by flow cytometry using the forward scatter. **(D)** Effect of 5HN on the transcription of HIV-1. Latently infected Bcl-2-transduced cells were left unstimulated or were stimulated with the indicated concentrations of 5HN or anti-CD3 plus anti-CD28 antibodies. The levels of viral mRNA were quantified using real-time RT-PCR and were normalized to the β -actin mRNA levels. The fold change is shown relative to that observed in the unstimulated samples. Data are mean \pm SD of triplicate samples from 1 of 2 independent experiments, all of which produced similar results.

known to induce reactivation of latent HIV-1 remain intact in this in vitro model, we tested known HIV-1 activators, including TCR agonists, mitogens, cytokines, and small molecules alone or in combination. Representative flow cytometry plots are shown in Supplemental Figure 4, A and B. The results are summarized in Figure 5, A and B. PMA, prostratin, and 12-deoxyphorbol 13-phenylacetate (DPP), known activators of PKC, all strongly activated latent HIV-1. Ionomycin, an activator of nuclear factor of activated T cells (NFAT), reactivated a smaller fraction of latent HIV-1. Interestingly, valproic acid (VA), a known histone deacetylase (HDAC) inhibitor and HIV-1 activator (13), barely activated latent virus, even at concentrations as high as 5 mM. However, trichostatin A (TSA), a more potent HDAC inhibitor, activated a greater fraction of the cells at a concentration of 200 nM. Hexamethylene bisacetamide (HMB), which activates latent HIV-1 in some cell line systems through regulation of active and inactive forms of positive transcription elongation factor b (pTEFb) (51, 52), only caused slight activation of latent HIV-1 at a concentration of 5 mM in our assay.

We also tested some cytokines that are known to activate latent HIV-1 (12, 53). Cytokines were used at concentrations previously reported to induce activation of latent HIV-1 in various primary cell systems. IL-7 alone strongly induced reactivation of latent HIV-1 as previously reported (12, 32), while TNF- α , a well-known activator of latent HIV-1 in transformed T cell lines (16, 21), activated only a small fraction of latent virus (Figure 5B). Consistent with this observation, previous studies using the resting CD4⁺ T cells isolated from patients on HAART also revealed the low activity of TNF- α in reactivation of latent HIV-1 (53). However, a combination of IL-2, IL-6, and TNF- α dramatically increased the reactivation of latent HIV-1 in our system. This response is also similar to that reported in resting CD4⁺ T cells isolated from patients on HAART (53).

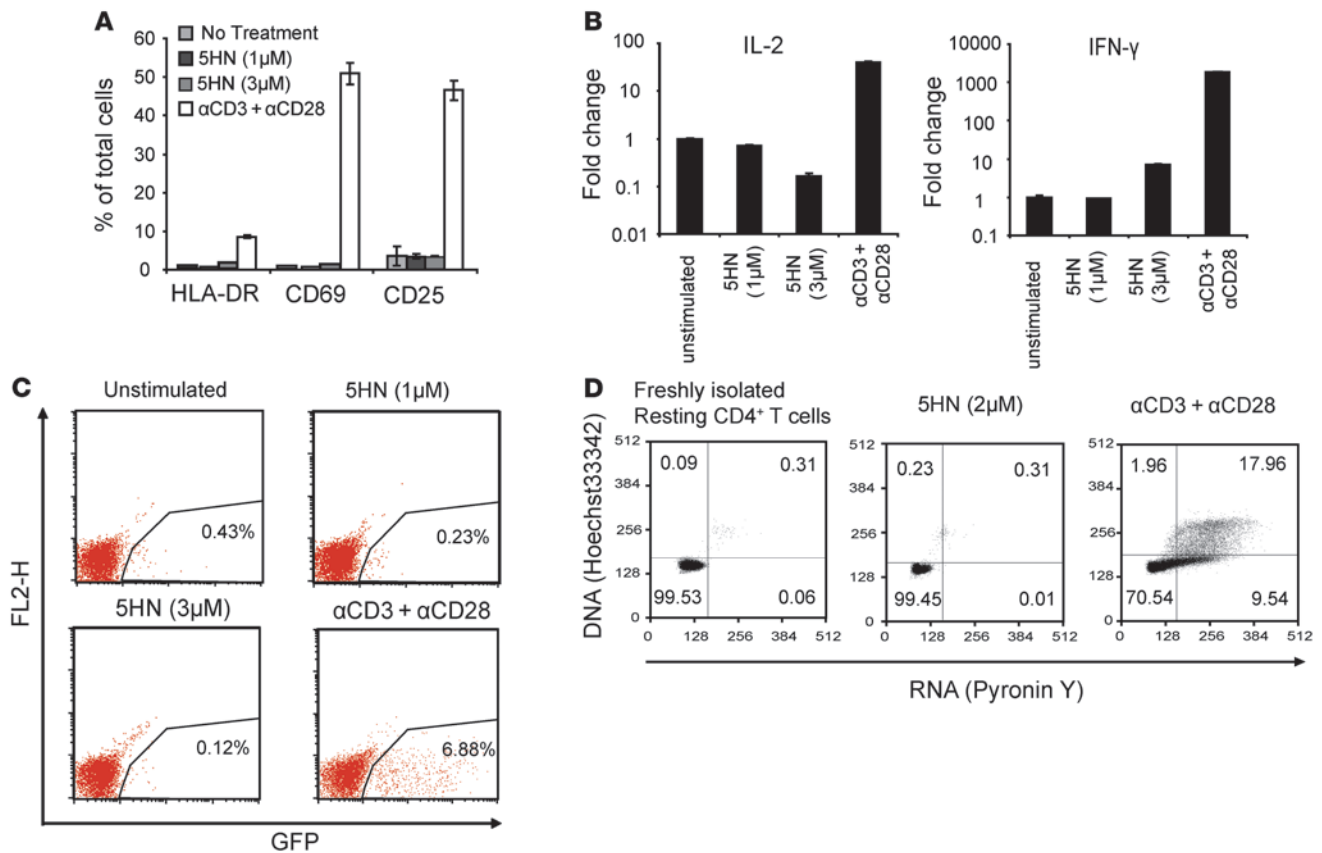
Overall, the responses of the latently infected cells generated in this system to known activators were very similar to those previously reported in other primary cell systems or in resting CD4⁺

T cells isolated from patients on HAART (10, 12, 53, 54). These results strongly suggest that this in vitro HIV-1 latency model can mimic the latently infected CD4⁺ T cells present in vivo.

Screening of small-molecule libraries for compounds that reactivate latent HIV-1. Using the system described above, we screened a library of 2000 drugs and natural products and another set of more than 2400 compounds from the Johns Hopkins Drug Library (JHDL) (55). Representative results are shown in Figure 6A. In total, 9 and 8 compounds that could activate latent HIV-1 were discovered in the former and latter libraries, respectively. For further study, we selected 1 of these compounds, 5-hydroxynaphthalene-1,4-dione (5HN), because it was detected in both libraries and displayed the highest capacity to reactivate latent HIV-1. 5HN is a natural quinone (Figure 6B) found in the leaves, roots, and bark of the black walnut tree. 5HN was comparable to the combination of anti-CD3 and anti-CD28 antibodies in its ability to reactivate latent HIV-1 (Figure 6A and Supplemental Figure 5A).

To confirm the effect of 5HN on latent HIV-1, we used real-time RT-PCR to examine levels of HIV-1 transcripts before and after stimulation with 5HN. Viral transcription was induced up to 8.8-fold in a dose-dependent manner by 5HN (Figure 6C). The ED₅₀ of 5HN in reactivating latent HIV-1 was 0.5 μ M (Supplemental Figure 5B), while the LD₅₀ measurements for freshly isolated and Bcl-2-transduced primary CD4⁺ T cells were 9.9 μ M and 7.7 μ M, respectively (Supplemental Figure 5C). These results indicate the narrow therapeutic range of 5HN.

We also examined the effect of 5HN in a transformed cell line model of HIV-1 latency, the J-Lat system (22). We measured the expression of viral marker GFP and viral transcripts. 5HN also strongly activated latent HIV-1 in J-Lat cells (Supplemental Figure 6, A and B). Interestingly, although the potency of 5HN to reactivate latent HIV-1 was comparable to that of PMA in our primary cell model, 5HN was less effective than PMA in J-Lat cells. These results highlight the importance of using primary cells to screen for compounds that target latent HIV-1.

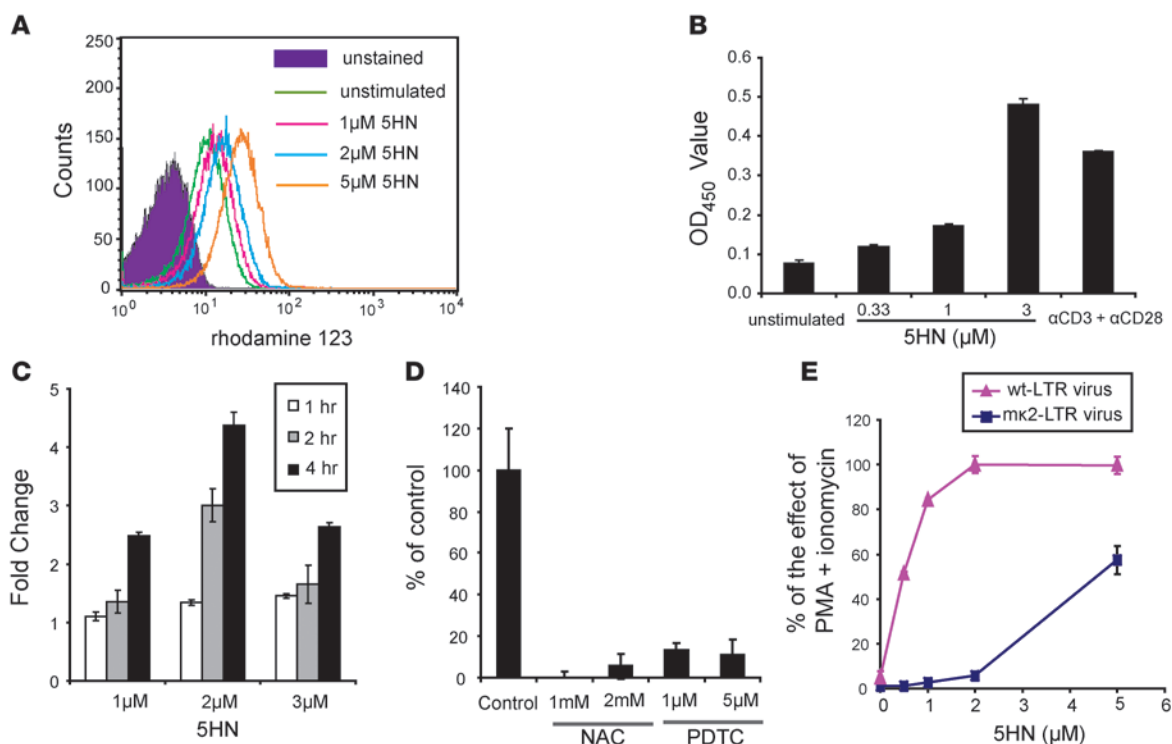
**Figure 7**

5HN does not activate CD4⁺ T cells. **(A)** Effects of 5HN on the expression of activation markers in primary resting CD4⁺ T cells. Freshly isolated CD4⁺ T cells were treated with the indicated concentrations of 5HN or with anti-CD3 plus anti-CD28 antibodies for 3 days. Expression of activation markers was quantified by flow cytometry. The values indicated the percentage of cells expressing individual markers. Data are mean \pm SD of triplicate samples from 1 of 2 independent experiments. **(B)** Effects of 5HN on transcription of IL-2 and IFN- γ genes. Resting Bcl-2-transduced CD4⁺ T cells were left unstimulated or were stimulated with 5HN or anti-CD3/anti-CD28. Levels of IL-2 and IFN- γ transcripts in total cellular RNA were quantified by real-time RT-PCR and normalized to β -actin mRNA levels. The fold change relative to unstimulated samples is shown. Data are mean \pm SD of triplicate samples from 1 of 2 independent experiments. **(C)** Effect of 5HN on the susceptibility of freshly isolated resting CD4⁺ T cells to HIV-1 infection. Cells were incubated with medium alone, 5HN, or anti-CD3/anti-CD28 antibodies for 3 days and were then infected with reporter virus NL4-3- Δ E-GFP. The number in each plot indicates the percentage of GFP-positive cells quantified by flow cytometry. **(D)** The effects of 5HN on the proliferation of latently infected cells. Cell proliferation was determined using Hoechst 33342/pyronin Y staining for DNA/RNA. The controls for the resting and activated cells are the same as in Figure 2B. The percentage of cells in each quadrant is indicated.

5HN does not cause global T cell activation. Agents that induce latent HIV-1 by causing global T cell activation are likely to be too toxic for clinical use. Therefore, we determined whether 5HN activates T cells. Unlike cells stimulated with PMA or anti-CD3 and anti-CD28 antibodies, the 5HN-treated latently infected cells retained the same small size as untreated cells as determined by flow cytometric analysis (Figure 6D). We further showed that 5HN does not cause upregulation of classic T cell activation markers such as CD69, CD25, or HLA-DR on freshly isolated resting CD4⁺ T cells (Figure 7A). Since the toxicity induced by T cell-activating agents is likely to be due to cytokine release, we determined whether 5HN induced production of T cell cytokines. IL-2 expression was not induced by 5HN, while IFN- γ transcripts were upregulated by approximately 10-fold. However, this level of IFN- γ activation is much less than that induced by costimulation with anti-CD3 and anti-CD28, which caused a greater than 1000-fold upregulation (Figure 7B). Importantly, at concentrations that induce reactivation of latent HIV-1, 5HN did not render uninfected resting CD4⁺

T cells susceptible to HIV-1 infection (Figure 7C). Finally, 5HN did not stimulate the proliferation of CD4⁺ T cells. This was evidenced by measurement of DNA/RNA staining (Figure 7D) or the dilution of CFSE in the 5HN-treated cells (Supplemental Figure 7). The above results demonstrate that 5HN did not cause global T cell activation at concentrations that activated latent HIV-1.

ROS are involved in the reactivation of latent HIV-1 by 5HN. 5HN is a quinone that can be reduced to a semiquinone radical by enzymes such as NADPH oxidoreductase. Under aerobic conditions, the semiquinone radical then generates superoxide anion (O₂⁻) and hydrogen peroxide (H₂O₂), which induce oxidative stress (56, 57). Because ROS can indirectly activate the host transcription factor NF- κ B that can in turn activate latent HIV-1 (58, 59), we hypothesized that induction of oxidative stress by 5HN may play a role in the reactivation of latent virus. To test this hypothesis, we first assessed the effects of 5HN on the production of ROS in freshly isolated primary resting CD4⁺ T cells using dihydrorhodamine 123 (DHR123). DHR123, a nonfluorogenic dye, is oxidized

**Figure 8**

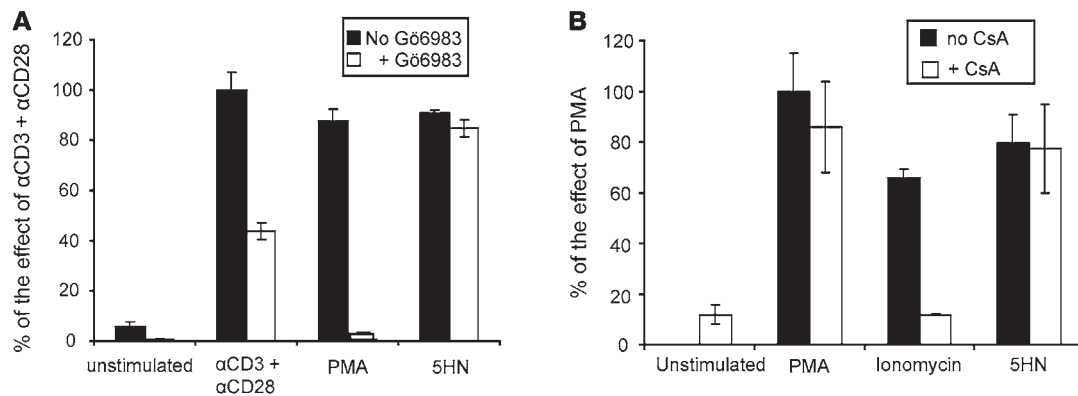
5HN activates latent HIV-1 via ROS and NF- κ B. (A) ROS generation in primary resting CD4⁺ T cells. Cells were incubated with DHR123 prior to 5HN treatment. DHR123 conversion, an indicator of intracellular ROS, was evaluated by flow cytometry. (B) 5HN activates NF- κ B in primary CD4⁺ T cells. Cells were left untreated, treated with 5HN, or stimulated with anti-CD3/anti-CD28 antibodies. The nuclear NF- κ B p65 was quantified and normalized to the total protein concentration of each sample. Results are relative OD₄₅₀ values. (C) Effects of 5HN on I κ B α transcription. Freshly isolated CD4⁺ T cells were left unstimulated or were stimulated with 5HN for different periods of time. I κ B α transcripts in total cellular RNA were quantified by real-time RT-PCR and normalized to β -actin mRNA levels. The fold change is relative to unstimulated samples. (D) Antioxidants NAC and PDTC block 5HN reactivation of latent HIV-1. Latently infected cells were treated with NAC or PDTC 1 hour prior to 5HN treatment. The percentage of the GFP-positive cells was measured using flow cytometry and normalized to that of cells receiving 5HN without prior treatment. (E) NF- κ B is involved in the reactivation of latent HIV-1 by 5HN. Bcl-2-transduced cells latently infected with NL4-3- Δ 6-drEGFP or mk2-NL4-3- Δ 6-drEGFP were treated with 5HN. GFP-positive cells were measured using flow cytometry and normalized to the maximal percentage of GFP-positive cells in each population following treatment with PMA plus ionomycin. Data in B–E are mean \pm SD of triplicate samples from 1 of 2 independent experiments, all of which produced similar results.

intracellularly by ROS into the fluorescent dye rhodamine 123 (60). Production of ROS in primary CD4⁺ T cells was detected within 2 hours of addition of concentrations of 5HN known to reactivate latent HIV-1 (Figure 8A). Because ROS can indirectly activate NF- κ B, we next assessed the activation of NF- κ B by measuring the nuclear levels of NF- κ B using an ELISA-based assay. 5HN activated NF- κ B in a dose-dependent manner (Figure 8B). To ensure that 5HN activates NF- κ B in primary CD4⁺ T cells, we also quantified the transcripts of I κ B α , an NF- κ B-responsive gene, and showed that 5HN stimulates the expression of I κ B α (Figure 8C). Antioxidants like N-acetylcysteine (NAC) or pyrrolidine dithiocarbamate (PDTC) suppress the effects of ROS on the activation of NF- κ B (59). Consistent with this observation, preincubation of the latently infected cells with NAC or PDTC dramatically suppressed the stimulatory effect of 5HN on the expression of latent HIV-1 (Figure 8D). This result supports the role of ROS in the activation of latent HIV-1 by 5HN.

To further confirm that NF- κ B is required for the activation of latent HIV-1 by 5HN, we mutated the 2 tandem NF- κ B-binding sites in the enhancer region of 3' LTR of pNL4-3- Δ 6-drEGFP

(mk2-LTR- NL4-3- Δ 6-drEGFP). Following reverse transcription and integration into the genome of infected cells, the 5' LTR of integrated reporter virus is derived from the 3' LTR that contains mutated NF- κ B-binding sites (61). The response to 5HN in the cells latently infected with mk2-LTR NL4-3- Δ 6-drEGFP was much lower than that in cells infected with wild-type NL4-3- Δ 6-drEGFP (Figure 8E), indicating the essential role of NF- κ B in the signaling pathways downstream of 5HN. Interestingly, at higher concentrations of 5HN (5 μ M), there was partial activation of the mk2-LTR NL4-3- Δ 6-drEGFP reporter virus, suggesting the possibility of NF- κ B-independent pathways for the reactivation of latent HIV-1 by 5HN.

PKC θ is a master regulator in TCR signaling pathways (62). Activation of PKC leads to activation of key transcription factors in the immune response, including NF- κ B and activator protein 1 (AP-1). Because 5HN activates latent HIV-1 via NF- κ B, we determined whether the effects of 5HN depend on PKC. Using the pan PKC inhibitor Gö6983, we found that inhibition of PKC completely suppressed PMA-induced activation of latent HIV-1 and reduced by approximately 50% the response to anti-CD3 and anti-CD28 costimulation.

**Figure 9**

5HN activates latent HIV-1 independent of PKC and NFAT. **(A)** Effect of a PKC inhibitor on the reactivation of latent HIV-1 by 5HN. Latently infected Bcl-2-transduced cells were incubated with PKC inhibitor G66983 1 hour prior to the treatment with each activator. Cells were collected and analyzed for GFP-positive cells after 2 days of incubation. The results were normalized to the effect of anti-CD3 plus anti-CD28 costimulation. Data are mean \pm SD of triplicate samples from 1 of 2 independent experiments, all of which produced similar results. **(B)** Effect of CsA on the reactivation of latent HIV-1 by 5HN. Latently infected Bcl-2-transduced cells were incubated with CsA 1 hour prior to the treatment with each activator. Cells were collected and analyzed for GFP-positive cells after 2 days of incubation. The results were normalized to the effect of PMA treatment. Data are mean \pm SD of triplicate samples from 1 of 3 independent experiments.

However, it almost had no effect on the activation of latent HIV-1 by 5HN. This suggests that 5HN acts downstream of PKC in the NF- κ B signaling pathway leading to the activation of latent HIV-1 (Figure 9A). NFAT, another essential transcription factor for T cell activation, is also known to promote HIV-1 gene expression (63). To exclude the possibility that NFAT plays a role downstream of 5HN, we showed that cyclosporin A (CsA), an inhibitor of the NFAT signaling pathway, does not suppress the effect of 5HN on the reactivation of latent HIV-1, while it inhibits the effect of ionomycin (Figure 9B).

5HN is also known to inhibit Pin1, a peptidyl-prolyl isomerase that plays an important role in transcription regulation (64, 65). To determine whether inhibition of Pin1 is involved in reactivation of latent HIV-1, we knocked down Pin1 expression in the latently infected Bcl-2-transduced cells using Pin1-specific RNAi. Down-regulation of Pin1 did not activate latent HIV-1 (data not shown). We also demonstrated that fredericamycin A, a Pin1 inhibitor (66), failed to induce latent HIV-1 in our in vitro primary cell model (data not shown). Together, these results suggest that inhibition of Pin1 by 5HN is not responsible for reactivation of latent HIV-1.

Discussion

The latent reservoir for HIV-1 in resting memory CD4⁺ T cells remains a major barrier to virus eradication. The lack of a convenient in vitro latency model in primary resting CD4⁺ T cells has hampered progress in exploring ways to overcome HIV-1 latency. In this study, we took advantage of the genetically modified primary CD4⁺ T cells to establish an in vitro HIV-1 latency system. We demonstrated that Bcl-2-transduced cells can reach a profoundly quiescent state in which HIV-1 latency can be established. More importantly, using this model, we performed small-molecule screening and identified 5HN as an activator of latent HIV-1 that, unlike previously described activators, does not induce global T cell activation. These results illustrate the application of this approach to the discovery of novel agents for the eradication of latent HIV-1.

Overexpression of Bcl-2 allows the primary CD4⁺ T cells to survive in a quiescent state, a key step to establishing HIV-1 latency in vitro. Although overexpression of Bcl-2 may alter the physiology of

CD4⁺ T cells, our extensive analysis revealed that the Bcl-2-transduced cells are similar to freshly isolated CD4⁺ T cells in both the activated and resting states. Bcl-2 is a downstream antiapoptotic effector of IL-7, an essential cytokine in maintaining the survival of resting naive and memory T cells in vivo. Prior studies have shown that the Bcl-2 transgene can partly compensate for deprivation of IL-7, indicating the important role of Bcl-2 in the homeostasis of resting T cells (35, 36). Because IL-7 also activates latent HIV-1 (12, 32), decoupling Bcl-2 from other IL-7 signaling pathways provided us an opportunity to measure the HIV-1 LTR activity in cells that are in a profoundly quiescent state.

Another concern is that a modified HIV-1 vector was used instead of wild-type virus. Although the modifications increased the yield of latently infected cells, there is the possibility that modified HIV-1 may behave differently than wild-type virus. We have preserved portions of the HIV-1 sequence relevant to the regulation of viral gene expression (LTR, *tat*, and *rev*). Therefore, this model is most useful in studying the upregulation of HIV-1 gene expression in latently infected cells, as we have done here. We have shown that the signaling pathways that lead to reactivation of latent HIV-1 are well preserved in this in vitro model. We tested an array of small molecules and cytokines and observed responses very similar to those seen in other primary cell models and latently infected CD4⁺ T cells from patients on HAART (10, 12, 53, 54). This result justifies the use of this model in investigating the reactivation of latent HIV-1. In order to increase the yield of latently infected cells, we inactivated genes whose products have been associated with cytopathicity. For this reason, the model may not be ideal for studying the fate of cells reactivated from latency or the efficiency with which latency is established. Importantly, we have recently shown that latently infected cells can be obtained using the same approach with a vector in which all of the HIV-1 ORFs except *env* are functional (L. Shan et al., unpublished observations). However, as expected, the yield of latently infected cells is substantially lower.

Although several in vitro HIV-1 latency models in primary CD4⁺ T cells have been described recently, certain factors limit their utility in high-throughput screening for activators of latent HIV-1.



Several studies have demonstrated that HIV-1 can establish latency by direct infection of resting CD4⁺ T cells in vitro (40, 67, 68). This strategy does have some advantages because it avoids the activation of T cells and utilizes wild-type HIV-1. However, the efficiency with which latency is established may be lower than that of the activation-dependent pathway described here, and this system is still subject to the short life span of resting CD4⁺ T cells in an in vitro culture system. Bosque et al. infected activated CD4⁺ T cells and maintained them in the presence of IL-2 (25). Because IL-2 also activates latent HIV-1, it is uncertain whether IL-2 disturbs the quiescent state of these cells. Some models were created by infection of thymocytes with HIV-1 (24, 69). However, the majority of the resulting cells were resting naive CD4⁺ T cells rather than memory CD4⁺ T cells, which harbor latent HIV-1 in vivo. Marini et al. used low doses of IL-7 to generate and maintain memory CD4⁺ T cells in vitro up to 2 months and established viral latency among these cells (26). Although interesting, this model only yields approximately 20% viable cells and therefore may not produce enough latently infected cells for high-throughput screening. In addition, IL-7 can activate latent HIV-1 (12). This may also confound the results of studies examining the reactivation of latent HIV-1 by other agents. Therefore, our primary cell HIV-1 latency model is the only one in which cells are maintained in vitro in the absence of activating cytokines.

The system described here can serve as an efficient platform to screen for activators of latent HIV-1 that do not induce global T cell activation. Bcl-2 transduction allows us to generate sufficient numbers of latently infected cells for high-throughput screening. In addition, the resting phenotype of the resulting cells recapitulates the quiescent state of primary resting CD4⁺ T cells. By flow cytometry analysis, we can easily detect changes in parameters that measure T cell activation, for instance, cell size. We illustrated the potential application of this in vitro system by identifying 5HN as an activator of latent HIV-1 that does not induce global T cell activation. 5HN can strongly reactivate latent HIV-1 with an efficiency similar to that of TCR stimulants. High reactivation efficiency is essential for eradicating HIV-1 because even a small number of viruses released from any residual latently infected cells can rebound exponentially after discontinuation of HAART.

Our data suggest that 5HN activates latent HIV-1 through the ROS and NF- κ B signaling pathways. The role of ROS in the activation of NF- κ B was first described by Schreck et al. (59). Some investigators have proposed a central role for ROS in mediating the ability of extrinsic stimuli to activate NF- κ B, although even after more than a decade of studies, there is still uncertainty about the target protein that senses ROS and transduces the signals to activate NF- κ B (58). The potential toxicity of ROS also creates concern about the therapeutic application of 5HN. Previous studies revealed differential cellular responses to the different levels of ROS. In response to low levels of ROS, cells upregulate the antioxidant genes, while high levels of ROS induce apoptosis. Interestingly, intermediate levels can stimulate some inflammatory genes, such as NF- κ B and AP-1 (58). This may explain why 5HN has a narrow therapeutic window. In addition, 5HN is chemically reactive and affects several cellular proteins (70). Although we showed that 5HN activates latent HIV-1 independent of Pin1, it is possible that other mechanisms are involved. Further studies are required to determine whether we can decouple the toxicity of 5HN from its ability to activate latent HIV-1. One intriguing finding is that 5HN activates latent HIV-1 without causing nonspecific T cell activa-

tion. Given that NF- κ B is a key transcription factor in controlling innate and adaptive immunity, it is surprising that 5HN stimulates latent HIV-1 by activating NF- κ B without inducing global T cell activation. This can be explained by our observation that 5HN activates latent HIV-1 independent of NFAT and PKC θ . PKC θ is a master regulator of T cell activation and activates multiple signaling pathways, including the NF- κ B, AP-1, and ERK1/2 pathways (62). The importance of NFAT proteins in T cell activation is also well documented (71). Bypassing PKC θ and NFAT may allow 5HN to avoid full-blown T cell activation. This finding also suggests that reactivation of latent HIV-1 does not necessarily require T cell activation and that the pathways leading to the activation of latent HIV-1 can be separated from those involving in T cell activation.

Because of the high cost and potential toxicities of long-term HAART and the disappointing results from the clinical trials of HIV-1 vaccines and microbicides (72, 73), there is still a pressing need for pursuing the goal of eradication. To cure HIV-1 infection is exceptionally challenging and will likely require combining HAART with agents that can purge latent virus. The identification of 5HN not only expands the number of classes of latency-reversing agents but also demonstrates the possibility of utilizing pathway(s) further downstream of TCR stimulation to avoid global T cell activation. Although the toxicities of 5HN raise concerns for its clinical application, this is a proof of concept for this approach to finding novel strategies to reactivate latent HIV-1 without inducing global T cell activation.

Methods

Reagents and cell lines. Monoclonal anti-CD3 (catalog no. 555336) and anti-CD28 (catalog no. 555725) antibodies were from BD Biosciences. VA, HMBA, PMA, 5HN, NAC, TsA, and DHR123 were from Sigma-Aldrich. Prostratin and DPP were from LC Laboratories. IL-1b, IL-2, IL-4, IL-6, IL-7, IL-12, and TNF- α were from R&D Systems. PDTC was from EMD. Fredericamycin A was a gift from Ben Shen (University of Wisconsin Madison, Madison, Wisconsin, USA). J-Lat full-length clone 10.6 was obtained through NIH AIDS Research and Reference Reagent Program (contributed by Eric Verdin).

5HN was dissolved in DMSO at a concentration of 10 mM and then was aliquotted and stored at -20°C for up to 2 months.

Plasmids and vector construction. The lentiviral vector FURW was a gift from Linzhao Cheng (Johns Hopkins University School of Medicine). Bcl-2 cDNA was a gift from M. Hardwick (Johns Hopkins University School of Medicine). To generate the Bcl-2 expression vector EB-FLV, Bcl-2 was first amplified by PCR from cDNA using the primers containing the *KpnI* and *EcoRI* recognition sites and then subcloned downstream of EF1 α promoter in the vector pEF1/Myc-His-A (Invitrogen). The DNA sequence containing the EF1 α promoter and Bcl-2 was then cloned into Zero-Blunt TOPO cloning vector PCR4Blunt-TOPO (Invitrogen), verified by direct sequencing, and subsequently subcloned into FURW using the *PacI* and *SalI* restriction sites.

The reporter viral vector pNL4-3- Δ 6-drEGFP was derived from the vector pNL4-3- Δ E-GFP, which contains an HIV-1 genome with a portion of envelope replaced with EGFP (74). Instead of EGFP, pNL4-3- Δ 6-drEGFP contains destabilized EGFP, in which the PEST sequence cloned from pZGreen-DR (Clontech) was ligated in frame to the 3' end of EGFP. To allow the destabilized EGFP to remain in the cytoplasm, the signal peptide of the envelope in pNL4-3- Δ 6-drEGFP was mutated using QuickChange II XL site-directed mutagenesis kit (Stratagene) with primer pairs 5'-GGGGCACCATTGCGCCGTGGGACATCGATGATCTGTAGTG and 5'-CACTACAGATCATCGATGTCCCACGGCGCATGGTGCCCC. Premature stop codons were introduced into the *gag*, *vif*, *vpr*, and *vpu* genes of pNL4-3- Δ 6-drEGFP sequentially using QuickChange II XL site-directed



mutagenesis kit. The nucleotide positions and primer sequences used for mutagenesis are shown in Supplemental Table 1. The *nef* gene was also truncated. To generate pmk2-LTR- NL4-3-Δ6-drEGFP, the tandem NF-κB-binding sites (GGGACTTTCC) in the 3' LTR of pNL4-3-Δ6-drEGFP were both replaced with the sequence of CTCACTTTCC. All the mutations were confirmed by sequencing. A packaging vector pC-Help (75) was a gift from Jakob Reiser (Louisiana State University Health Sciences Center, New Orleans, Louisiana, USA).

Virus stock production. The Bcl-2 lentiviral vectors were generated by cotransfecting HEK293T cells with a plasmid encoding EB-FLV, VSV-G envelope (pVSVG), and a packaging vector pC-Help using Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions. Supernatants were harvested after 72 hours, spun at 335 g, and filtered through a 0.22-μm membrane to clear cell debris. Virus was pelleted at 100,000 g with 10% volume of 20% sucrose in the bottom for 2 hours at 4°C. The recombinant CXCR4-tropic HIV-1 pseudoviruses were generated by the same procedure using a plasmid encoding the HIV-1 envelope (pCXCR4), pC-Help, and pNL4-3-Δ6-drEGFP.

Cell culture and generation of latently HIV-1-infected Bcl-2-transduced cells. This study was approved by the Johns Hopkins Institutional Review Board. Healthy adult blood donors provided informed consent before enrollment. Primary human CD4⁺ T cells were isolated from PBMCs using a Human CD4⁺ T Cells Isolation Kit II (Miltenyi Biotec). To bind anti-CD3 antibodies to plates, 1 ml of antibodies in PBS at 10 μg/ml was incubated in each well of a 6-well plate for 90 minutes at 37°C. Primary CD4⁺ T cells were activated by incubation in these plates with 1 μg/ml anti-CD28 monoclonal antibody, 100 U/ml IL-2, and T cell growth factor-enriched medium. On day 3, the activated CD4⁺ T cells were transduced with EB-FLV at the MOI of 5 to 10 by spinoculation of cells at 1200 g at room temperature for 2 hours. The transduced cells were cultured in IL-2 and T cell growth factor-enriched medium for another 3 days, and the medium was replaced with RPMI 1640 with 10% FBS and 1% penicillin/streptomycin without supplemental cytokines. Following 3–4 weeks of culture in the absence of exogenous cytokines, viable cells were isolated using Ficoll-Hypaque density gradient centrifugation.

To expand Bcl-2-transduced cells, we activated them with plate-bound anti-CD3 antibody and soluble anti-CD28 antibody. We then added 100 U/ml IL-2 every other day for 10–12 days. Usually, the number of cells increased to 5- to 10-fold. To generate latently HIV-1-infected, Bcl-2-transduced cells, we infected activated Bcl-2-transduced cells with reporter virus NL4-3-Δ6-drEGFP at an MOI of less than 0.1. We maintained the infected cells in IL-2 and T cell growth factor-enriched medium for 3 days after infection. We then incubated the infected cells in the RPMI 1640 with 10% FBS and 1% penicillin/streptomycin without exogenous cytokines for more than 1 month. Finally, we isolated the GFP-negative cells to more than 99.9% purity using fluorescence-activated cell sorting.

RNA extraction and real-time RT-PCR. We isolated total cellular RNA using RNeasy Mini Kit (QIAGEN). We performed RT reactions using SuperScript III Reverse Transcriptase (Invitrogen) with random primers (Invitrogen). IFN-γ and IL-2 transcripts were measured using TaqMan Gene Expression Assays on an ABI 7300 Real-Time PCR System (Applied Biosystems assays Hs00174143_ml and Hs00174114_ml, respectively). For an internal control, ubiquitin transcripts (GenBank accession number M26880) were also measured by real-time RT-PCR with SYBR Green PCR Master Mix (Applied Biosystems) and a pair of primers: 5'-ATTTGGGTGCGAGTTCTTG-3' and 5'-TGCCTTGACATTCTCGATGGT-3' (76). In the 5HN treatment experiment, β-actin was used as an internal control because 5HN activates ubiquitin. β-actin was measured using TaqMan Gene Expression Assay with the primers and probes of ACTB

(part number 433762F). Control reactions with no template or without reverse transcriptase were negative.

Measurement of NF-κB activation. Nuclear extracts were prepared using a Nuclear Extract Kit (Active Motif) according to the manufacturer's instructions. Protein concentration of nuclear extract was determined using DC-protein assay (Bio-Rad) following the manufacturer's instructions. NF-κB activation was measured by the TransAM NF-κB p65 ELISA kit (catalog no. 40097; Active Motif). This kit provides a 96-well plate with immobilized oligonucleotides containing the NF-κB-binding sites (5'-GGGACTTTCA-3') derived from HIV-1 LTR. Nuclear extract was added to each well, and NF-κB that bound to the oligonucleotides was detected by an NF-κB p65 antibody and a secondary antibody conjugated with HRP. The levels of NF-κB activation, as bound NF-κB p65, were measured in triplicate on a Vmax kinetic microplate reader (Molecular Devices) at OD₄₅₀.

Compound libraries. The compound libraries tested were The Spectrum Collection (MicroSource Discovery Inc.) and JHDL. The 2000 compounds in The Spectrum Collection consist of FDA-approved compounds, natural products, and other bioactive compounds. A list of the compounds is available at the MicroSource Discovery website (www.msdiscovery.com/spectrum.html). The compounds in JHDL are primarily FDA-approved compounds (55).

Measurement of the reactivation of latent HIV-1 from the latently infected Bcl-2-transduced cells by activators. Fifty thousand latently infected cells were resuspended in 200 μl of medium (RPMI 1640 with 10% FBS and 1% penicillin/streptomycin) and were cultured in round-bottom 96-well plates. The cells were then treated with the indicated concentrations of activators. As a positive control, cells were treated with 2.5 μg/ml anti-CD3 and 1 μg/ml anti-CD28 monoclonal antibodies. After 40–48 hours at 37°C, reactivation of latent HIV-1 was determined by quantifying the percentage of GFP⁺ cells using the standard mode of the High Throughput Sampler (HTS) in FACSCalibur and analyzed using Plate Manager (BD Biosciences). The percentage of GFP⁺ cells was normalized based on the response to anti-CD3 plus anti-CD28 antibodies.

Screen of JHDL. For high-throughput screening, 2.5×10^4 latently infected cells were resuspended in 200 μl of medium in round-bottom 96-well plates. Each compound was added at 10 μM. Each plate contained cells treated with either 10 ng/ml PMA or 2.5 μg/ml anti-CD3 plus 1 μg/ml anti-CD28 antibodies as positive controls. After 40–48 hours at 37°C, reactivation of latent HIV-1 was determined as above.

Detection of intracellular ROS. Primary CD4⁺ T cells isolated from healthy donors or Bcl-2-transduced cells were resuspended in RPMI 1640 with 10% FBS and 1% penicillin/streptomycin at 10^6 cells/ml. 5×10^5 cells/well in 500 μl medium were plated in 48-well plates. DHR123 was dissolved in DMSO at a concentration of 10 mM and was stored at -20°C. Before assay, stock solutions were diluted in PBS to 100 μM and added at the indicated concentrations 30 minutes prior to 5HN treatment. Samples were collected and analyzed at 30, 60, and 120 minutes after addition of 5HN. Conversion of DHR123 to rhodamine 123 by ROS was quantified as increasing green fluorescence using a FACSCalibur (BD Biosciences).

Measurement of intracellular HIV-1 transcripts. Total cellular RNA was isolated from 1×10^6 latently infected Bcl-2-transduced cells or 0.5×10^5 J-Lat cells using RNeasy Mini Kit (QIAGEN). mRNA was purified using Dynabeads oligo (dT)₂₅ beads (Invitrogen). The bound mRNA was used directly for RT reactions using SuperScript III Reverse Transcriptase (Invitrogen). HIV-1 transcripts were measured by real-time RT-PCR with SYBR Green PCR Master Mix (Applied Biosystems) and the primer pairs that detect single-spliced viral transcripts (5'-GGGTCTCTCTG-GTTAGACCAGATCTGAGCC-3', and 5'-CTCCGCTTCTTCTGCCAT) (77). For an internal control, β-actin was measured using a TaqMan Gene



Expression Assay. Control reactions with no template or without the addition of RT were negative.

Statistics. Activation experiments were performed in triplicate cultures. Data are presented as the arithmetic mean \pm SD for a representative experiment drawn from 2 or 3 experiments, all of which provided similar results.

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- Blankson, J.N., Persaud, D., and Siliciano, R.F. 2002. The challenge of viral reservoirs in HIV-1 infection. *Annu. Rev. Med.* **53**:557–593.
- Geeraert, L., Kraus, G., and Pomerantz, R.J. 2008. Hide-and-Seek: The Challenge of Viral Persistence in HIV-1 Infection. *Annu. Rev. Med.* **59**:487–501.
- Finzi, D., et al. 1997. Identification of a reservoir for HIV-1 in patients on highly active antiretroviral therapy. *Science*. **278**:1295–1300.
- Wong, J.K., et al. 1997. Recovery of replication-competent HIV despite prolonged suppression of plasma viremia. *Science*. **278**:1291–1295.
- Chun, T.W., et al. 1997. Presence of an inducible HIV-1 latent reservoir during highly active antiretroviral therapy. *Proc. Natl. Acad. Sci. U. S. A.* **94**:13193–13197.
- Siliciano, J.D., et al. 2003. Long-term follow-up studies confirm the stability of the latent reservoir for HIV-1 in resting CD4⁺ T cells. *Nat. Med.* **9**:727–728.
- Richman, D.D., et al. 2009. The challenge of finding a cure for HIV infection. *Science*. **323**:1304–1307.
- Margolis, D.M., and Archin, N.M. 2006. Attacking HIV provirus: therapeutic strategies to disrupt persistent infection. *Infect. Disord. Drug Targets*. **6**:369–376.
- Chun, T.W., et al. 1999. Effect of interleukin-2 on the pool of latently infected, resting CD4⁺ T cells in HIV-1-infected patients receiving highly active anti-retroviral therapy. *Nat. Med.* **5**:651–655.
- Kulkosky, J., et al. 2001. Prostratin: activation of latent HIV-1 expression suggests a potential inductive adjuvant therapy for HAART. *Blood*. **98**:3006–3015.
- Brooks, D.G., et al. 2003. Molecular characterization, reactivation, and depletion of latent HIV. *Immunity*. **19**:413–423.
- Wang, F.X., et al. 2005. IL-7 is a potent and proviral strain-specific inducer of latent HIV-1 cellular reservoirs of infected individuals on virally suppressive HAART. *J. Clin. Invest.* **115**:128–137.
- Lehrman, G., et al. 2005. Depletion of latent HIV-1 infection in vivo: a proof-of-concept study. *Lancet*. **366**:549–555.
- Nabel, G., and Baltimore, D. 1987. An inducible transcription factor activates expression of human immunodeficiency virus in T cells. *Nature*. **326**:711–713.
- Tong-Starksen, S.E., Luciw, P.A., and Peterlin, B.M. 1987. Human immunodeficiency virus long terminal repeat responds to T-cell activation signals. *Proc. Natl. Acad. Sci. U. S. A.* **84**:6845–6849.
- Duh, E.J., Maury, W.J., Folks, T.M., Fauci, A.S., and Rabson, A.B. 1989. Tumor necrosis factor alpha activates human immunodeficiency virus type 1 through induction of nuclear factor binding to the NF-kappa B sites in the long terminal repeat. *Proc. Natl. Acad. Sci. U. S. A.* **86**:5974–5978.
- Rabson, A.B., and Lin, H.C. 2000. NF-kappa B and HIV: linking viral and immune activation. *Adv. Pharmacol.* **48**:161–207.
- Prins, J.M., et al. 1999. Immuno-activation with anti-CD3 and recombinant human IL-2 in HIV-1-infected patients on potent antiretroviral therapy. *AIDS*. **13**:2405–2410.
- van Praag, R.M., et al. 2001. OKT3 and IL-2 treatment for purging of the latent HIV-1 reservoir in vivo results in selective long-lasting CD4⁺ T cell depletion. *J. Clin. Immunol.* **21**:218–226.
- Folks, T.M., Justement, J., Kinter, A., Dinarello, C.A., and Fauci, A.S. 1987. Cytokine-induced expression of HIV-1 in a chronically infected promonocyte cell line. *Science*. **238**:800–802.
- Folks, T.M., et al. 1989. Tumor necrosis factor alpha induces expression of human immunodeficiency virus in a chronically infected T-cell clone. *Proc. Natl. Acad. Sci. U. S. A.* **86**:2365–2368.
- Jordan, A., Bisgrove, D., and Verdin, E. 2003. HIV reproducibly establishes a latent infection after acute infection of T cells in vitro. *EMBO J.* **22**:1868–1877.
- Sahu, G.K., et al. 2006. A novel in vitro system to generate and study latently HIV-infected long-lived normal CD4⁺ T-lymphocytes. *Virology*. **355**:127–137.
- Burke, B., et al. 2007. Primary cell model for activation-inducible human immunodeficiency virus. *J. Virol.* **81**:7424–7434.
- Bosque, A., and Planellas, V. 2009. Induction of HIV-1 latency and reactivation in primary memory CD4⁺ T cells. *Blood*. **113**:58–65.
- Marini, A., Harper, J.M., and Romero, F. 2008. An in vitro system to model the establishment and reactivation of HIV-1 latency. *J. Immunol.* **181**:7713–7720.
- Chun, T.W., et al. 1995. In vivo fate of HIV-1-infected T cells: quantitative analysis of the transition to stable latency. *Nat. Med.* **1**:1284–1290.
- Pierson, T., McArthur, J., and Siliciano, R.F. 2000. Reservoirs for HIV-1: mechanisms for viral persistence in the presence of antiviral immune responses and antiretroviral therapy. *Annu. Rev. Immunol.* **18**:665–708.
- Marrack, P., and Kappler, J. 2004. Control of T cell viability. *Annu. Rev. Immunol.* **22**:765–787.
- Kondrack, R.M., et al. 2003. Interleukin 7 regulates the survival and generation of memory CD4 cells. *J. Exp. Med.* **198**:1797–1806.
- Li, J., Huston, G., and Swain, S.L. 2003. IL-7 promotes the transition of CD4 effectors to persistent memory cells. *J. Exp. Med.* **198**:1807–1815.
- Scripture-Adams, D.D., Brooks, D.G., Korin, Y.D., and Zack, J.A. 2002. Interleukin-7 induces expression of latent human immunodeficiency virus type 1 with minimal effects on T-cell phenotype. *J. Virol.* **76**:13077–13082.
- Khaled, A.R., and Durum, S.K. 2003. Death and Baxes: mechanisms of lymphotrophic cytokines. *Immunol. Rev.* **193**:48–57.
- Van, P.L., Peterson, D.A., and Abbas, A.K. 1998. The Fas/Fas ligand pathway and Bcl-2 regulate T cell responses to model self and foreign antigens. *Immunity*. **8**:265–274.
- Akashi, K., Kondo, M., von Freeden-Jeffry, U., Murray, R., and Weissman, I.L. 1997. Bcl-2 rescues T lymphopoiesis in interleukin-7 receptor-deficient mice. *Cell*. **89**:1033–1041.
- Maraskovsky, E., et al. 1997. Bcl-2 can rescue T lymphocyte development in interleukin-7 receptor-deficient mice but not in mutant rag-1^{-/-} mice. *Cell*. **89**:1011–1019.
- Kittipattarin, C., and Khaled, A.R. 2007. Interlinking interleukin-7. *Cytokine*. **39**:75–83.
- Rathmell, J.C., Farkash, E.A., Gao, W., and Thompson, C.B. 2001. IL-7 enhances the survival and maintains the size of naive T cells. *J. Immunol.* **167**:6869–6876.
- Korin, Y.D., and Zack, J.A. 1999. Nonproductive human immunodeficiency virus type 1 infection in nucleoside-treated G0 lymphocytes. *J. Virol.* **73**:6526–6532.
- Swiggard, W.J., et al. 2005. Human immunodeficiency virus type 1 can establish latent infection in resting CD4⁺ T cells in the absence of activating stimuli. *J. Virol.* **79**:14179–14188.
- Shapiro, H.M. 1981. Flow cytometric estimation of DNA and RNA content in intact cells stained with Hoechst 33342 and pyronin Y. *Cytometry*. **2**:143–150.
- Bohnlein, E., et al. 1988. The same inducible nuclear proteins regulates mitogen activation of both the interleukin-2 receptor-alpha gene and type 1 HIV. *Cell*. **53**:827–836.
- Lassen, K., Han, Y., Zhou, Y., Siliciano, J., and Siliciano, R.F. 2004. The multifactorial nature of HIV-1 latency. *Trends Mol. Med.* **10**:525–531.
- Pierson, T., et al. 2000. Characterization of chemokine receptor utilization of viruses in the latent reservoir for human immunodeficiency virus type 1. *J. Virol.* **74**:7824–7833.
- Sallusto, F., Geginat, J., and Lanzavecchia, A. 2004. Central memory and effector memory T cell subsets: function, generation, and maintenance. *Annu. Rev. Immunol.* **22**:745–763.
- Chomont, N., et al. 2009. HIV reservoir size and persistence are driven by T cell survival and homeostatic proliferation. *Nat. Med.* Online publication ahead of print. doi:10.1038/nm.1972.
- Chun, T.W., et al. 1997. Quantification of latent tissue reservoirs and total body viral load in HIV-1 infection. *Nature*. **387**:183–188.
- Sylwester, A., Murphy, S., Shurt, D., and Soll, D.R. 1997. HIV-induced T cell syncytia are self-perpetuating and the primary cause of T cell death in culture. *J. Immunol.* **158**:3996–4007.
- Sakai, K., Dimas, J., and Lenardo, M.J. 2006. The Vif and Vpr accessory proteins independently cause HIV-1-induced T cell cytopathicity and cell cycle arrest. *Proc. Natl. Acad. Sci. U. S. A.* **103**:3369–3374.
- Shen, L., et al. 2008. Dose-response curve slope sets class-specific limits on inhibitory potential of anti-HIV drugs. *Nat. Med.* **14**:762–766.
- Contreras, X., Barboric, M., Lenasi, T., and Peterlin, B.M. 2007. HMB4 releases P-TEFb from HEXIM1 and 7SK snRNA via PI3K/Akt and activates HIV transcription. *PLoS Pathog.* **3**:1459–1469.
- Choudhary, S.K., Archin, N.M., and Margolis, D.M. 2008. Hexamethylbisacetamide and disruption of human immunodeficiency virus type 1 latency in CD4⁺ T cells. *J. Infect. Dis.* **197**:1162–1170.
- Chun, T.W., Engel, D., Mizell, S.B., Ehler, L.A., and Fauci, A.S. 1998. Induction of HIV-1 replication in latently infected CD4⁺ T cells using a combination of cytokines. *J. Exp. Med.* **188**:83–91.



54. Brooks, D.G., Arlen, P.A., Gao, L., Kitchen, C.M., and Zack, J.A. 2003. Identification of T cell-signaling pathways that stimulate latent HIV in primary cells. *Proc. Natl. Acad. Sci. U. S. A.* **100**:12955–12960.
55. Chong, C.R., Chen, X., Shi, L., Liu, J.O., and Sullivan, D.J., Jr. 2006. A clinical drug library screen identifies astemizole as an antimalarial agent. *Nat. Chem. Biol.* **2**:415–416.
56. Hassan, H.M., and Fridovich, I. 1979. Intracellular production of superoxide radical and of hydrogen peroxide by redox active compounds. *Arch. Biochem. Biophys.* **196**:385–395.
57. Blum, J., and Fridovich, I. 1983. Superoxide, hydrogen peroxide, and oxygen toxicity in two free-living nematode species. *Arch. Biochem. Biophys.* **222**:35–43.
58. Gloire, G., Legrand-Poels, S., and Piette, J. 2006. NF-kappaB activation by reactive oxygen species: fifteen years later. *Biochem. Pharmacol.* **72**:1493–1505.
59. Schreck, R., Rieber, P., and Baeuerle, P.A. 1991. Reactive oxygen intermediates as apparently widely used messengers in the activation of the NF-kappa B transcription factor and HIV-1. *EMBO J.* **10**:2247–2258.
60. Emmendorffer, A., Hecht, M., Lohmann-Matthes, M.L., and Roesler, J. 1990. A fast and easy method to determine the production of reactive oxygen intermediates by human and murine phagocytes using dihydrorhodamine 123. *J. Immunol. Methods.* **131**:269–275.
61. Chen, B.K., Feinberg, M.B., and Baltimore, D. 1997. The kappaB sites in the human immunodeficiency virus type 1 long terminal repeat enhance virus replication yet are not absolutely required for viral growth. *J. Virol.* **71**:5495–5504.
62. Isakov, N., and Altman, A. 2002. Protein kinase C(theta) in T cell activation. *Annu. Rev. Immunol.* **20**:761–794.
63. Kinoshita, S., et al. 1997. The T cell activation factor NF-ATc positively regulates HIV-1 replication and gene expression in T cells. *Immunity.* **6**:235–244.
64. Lu, K.P., Hanes, S.D., and Hunter, T. 1996. A human peptidyl-prolyl isomerase essential for regulation of mitosis. *Nature.* **380**:544–547.
65. Hennig, L., et al. 1998. Selective inactivation of parvulin-like peptidyl-prolyl cis/trans isomerases by juglone. *Biochemistry.* **37**:5953–5960.
66. Lu, K.P., Fischer, G., and Pintex Pharmaceuticals Inc. 2004. Methods of inhibiting Pin1-associated states using a fredericamycin A compound. World Intellectual Property Organization. Patent WO/2004/002429, filed June 30, 2003, and issued January 8, 2004.
67. Dai, J., et al. 2009. Human immunodeficiency virus integrates directly into naive resting CD4+ T cells but enters naive cells less efficiently than memory cells. *J. Virol.* **83**:4528–4537.
68. Vatakis, D.N., Kim, S., Kim, N., Chow, S.A., and Zack, J.A. 2009. Human immunodeficiency virus integration efficiency and site selection in quiescent CD4+ T cells. *J. Virol.* **83**:6222–6233.
69. Brooks, D.G., et al. 2001. Generation of HIV latency during thymopoiesis. *Nat. Med.* **7**:459–464.
70. Fila, C., Metz, C., and van der, S.P. 2008. Juglone inactivates cysteine-rich proteins required for progression through mitosis. *J. Biol. Chem.* **283**:21714–21724.
71. Macian, F. 2005. NFAT proteins: key regulators of T-cell development and function. *Nat. Rev. Immunol.* **5**:472–484.
72. Cohen, J. 2008. AIDS research. Microbicide fails to protect against HIV. *Science.* **319**:1026–1027.
73. Ledford, H. 2007. HIV vaccine may raise risk. *Nature.* **450**:325.
74. Zhang, H., et al. 2004. Novel single-cell-level phenotypic assay for residual drug susceptibility and reduced replication capacity of drug-resistant human immunodeficiency virus type 1. *J. Virol.* **78**:1718–1729.
75. Mochizuki, H., Schwartz, J.P., Tanaka, K., Brady, R.O., and Reiser, J. 1998. High-titer human immunodeficiency virus type 1-based vector systems for gene delivery into nondividing cells. *J. Virol.* **72**:8873–8883.
76. Vandesompele, J., et al. 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol.* **3**:RESEARCH0034.
77. Lassen, K.G., Bailey, J.R., and Siliciano, R.F. 2004. Analysis of human immunodeficiency virus type 1 transcriptional elongation in resting CD4+ T cells in vivo. *J. Virol.* **78**:9105–9114.