Silencing of HIV-1 with RNA Interference: A Multiple shRNA Approach

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Double-stranded RNA can induce gene silencing via a process known as RNA interference (RNAi). Previously, we have shown that stable expression of a single shRNA targeting the HIV-1 Nef gene strongly inhibits HIV-1 replication. However, this was not sufficient to maintain inhibition. One of the hallmarks of RNAi, its sequence specificity, presented a way out for the virus, as single nucleotide substitutions in the target region abolished inhibition. For the development of a durable gene therapy that prevents viral escape, we proposed to combine multiple shRNAs against conserved HIV-1 regions. Therefore, we screened 86 different shRNAs targeting highly conserved regions. We identified multiple shRNAs that act as potent inhibitors of virus replication. We show, for the first time, that expression of three different shRNAs from a single lentiviral vector results in similar levels of inhibition per shRNA compared to single shRNA vectors. Thus, their combined expression results in a much stronger inhibition of virus production. Moreover, when we infected cells transduced with a double shRNA viral vector, virus escape was delayed. These results confirm that RNAi has great potential as an antiviral gene therapy approach and support our efforts to develop this strategy for treatment of HIV-1-infected individuals.

Key Words: HIV-1, RNA interference, combination gene therapy, siRNA, shRNA, lentiviral vector

INTRODUCTION

RNA interference (RNAi) is highly effective in inhibiting HIV-1 replication in transient assays with small interfering RNA (siRNA) [1–3]. A more durable inhibition can be achieved when an antiviral short hairpin RNA (shRNA) is expressed in stably transfected or transduced cell lines [4–7]. However, similar to current antiviral drugs used in the clinic, the application of a single inhibitor against HIV-1 is not sufficient to maintain inhibition. Several in vitro studies have shown that HIV-1 can escape from inhibition by mutation of its RNAi target sequence [4,8–11]. In addition, viral escape may result from mutations outside the target region that stabilize a repressive structure in the viral RNA genome [12]. Like highly active antiretroviral therapy, a successful RNAi-based therapy against HIV-1 requires the simultaneous use of multiple shRNAs. To reduce the chance of viral escape further, highly conserved sequences need to be targeted, which should ideally be present in isolates of all HIV-1 subtypes.

The majority of anti-HIV-1 studies to date have focused on transient inhibition, without testing for viral escape and the need to focus on highly conserved target sequences [1,2,8,13]. Most researchers used one of the available design algorithms for selection of siRNAs or shRNAs. We previously used a criterion for accessibility to binding of antisense DNA oligonucleotides for target identification and shRNA design, albeit with little success [4]. None of the selected targets proved to be effective for inhibition of HIV-1 replication. A survey of recent design rules and algorithms indicated to us that, although these rules perhaps increase the chance of finding effective shRNAs, they are likely also to exclude some effective shRNAs. For durable inhibition of HIV-1 replication it is desirable to have multiple effective shRNAs that target most viral isolates of different subtypes. We therefore made and tested 86 shRNA gene constructs targeting the HIV-1 genome. The only selection criterion we used was high conservation of the target sequence. We identified multiple effective shRNAs targeting eight distinct viral genome regions. We validated these shRNAs by confirming sequence-specific inhibition in independent reporter assays. We demonstrate, for the first time, that HIV-1 inhibition is markedly stronger when multiple shRNAs are expressed in a stable cell line from a single lentiviral vector. Moreover, combined expression of two shRNAs could significantly delay viral escape, indicating that in a multiple shRNA gene therapy, when even more shRNAs are combined, viral escape may be prevented.
RESULTS

shRNAs against Conserved Regions of the HIV-1 Genome

We first identified highly conserved sequences within the HIV-1 genome. As a starting point we used the HIV-1 LAI isolate [14], which is the prototype virus strain used in our studies. Every possible 20-mer sequence of the LAI isolate was aligned with 170 complete genome sequences, including all HIV-1 subtypes, present in the Los Alamos National Laboratory database (http://www.hiv.lanl.gov/). We defined conservation as regions in LAI having 100% identity with at least 75% of the HIV-1 genomes. According to this criterion we identified 19 target regions within the HIV-1 genome (Table 1, Fig. 1A). We constructed shRNA expression plasmids (last column in Table 1) with the H1 polymerase III promoter [15] based on a 19-nucleotide target sequence. Since we used 20-mer LAI sequences in our alignment, at least two different shRNAs were designed for each conserved target. For extended targets, more shRNAs were designed. For instance, the Gag target starting at position 1363 comprises 22 nucleotides, and therefore 4 overlapping shRNAs, Gag-3 to -6, were designed. In total, we constructed 86 different shRNA expression plasmids covering all highly conserved HIV-1 sequences.

Inhibition of HIV-1 Production with shRNAs against Leader, Gag, Pol, and Rev/Tat Sequences

We cotransfected each shRNA expression construct with the pLAI molecular clone to score for its ability to inhibit virus production (Fig. 1B). Multiple constructs showed at least 75% inhibition of HIV-1 production, representing eight target regions in the HIV-1 RNA genome. Part of the leader region of HIV-1 is highly permissive for RNAi, with Ldr-2 through Ldr-9 showing moderate to strong inhibition. Interestingly, Pol-44, Pol-45, and Pol-47 are active, whereas the intervening shRNA construct Pol-46 is inactive. A similar trend was observed for Pol-27 and Pol-29, which are active, and Pol-28, which is inactive. Other effective targets were Gag-5 and Gag-6, Pol-1 and Pol-2, Pol-6, Pol-9, R/T-4, and R/T-5. In total, one in four tested shRNAs was highly active against HIV-1. In our screen we included positive and negative shRNA controls for virus inhibition against previously published targets in HIV-1 Nef and firefly luciferase, respectively [4,16]. We performed two independent transfection experiments, one in duplicate and one in triplicate; the latter is shown. We obtained similar results in the two experiments.

Validation of the Effective shRNAs

The results from the large shRNA screen indicate that 21 of the 86 hairpins are potent HIV-1 inhibitors. We assume that the effective shRNAs induce sequence-specific inhibition of HIV-1. To confirm this sequence specificity we designed firefly luciferase reporter constructs in which we cloned the appropriate HIV-1 target sequences in the 3’ untranslated region (Fig. 2A). We cotransfected reporters for Gag-5, Pol-1, Pol-9, and Pol-47 sequences with different shRNA expression plasmids. Only when the shRNA matched the reporter were luciferase levels reduced

<table>
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<tr>
<th>Target gene(s)</th>
<th>Positiona</th>
<th>DNA/RNA motif(s)b</th>
<th>ORFe</th>
<th>Sequenced</th>
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<td>Nef-1 to -9</td>
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a Position in the LAI viral RNA genome.
b DNA/RNA motifs in targets: Ldr, leader; RSS, ribosome slip site; cPPT, central polypurine tract; CTS, central termination sequence; SD, splice donor site; SA, splice acceptor site; RRE, Rev responsive element; PPT, polypurine tract; LTR, long terminal repeat.
c Number of Open Reading Frame.
d Start codons are shown in bold.
(Supplemental Fig. S1). We obtained similar results for other effective shRNAs: Ldr-3, Ldr-9, Pol-6, Pol-29, Pol-45, shR/T-4, and shR/T-5 (data not shown). This confirms that the observed inhibition of HIV-1 production occurs through a sequence-specific mechanism.

Sequence-Specific Inhibition of HIV-1 in Transduced Cell Lines

For our gene therapy approach, we plan to use the third-generation lentiviral vector system [17–19]. We constructed lentiviral vectors containing an expression cassette for the shRNA against Gag-5, Pol-1, or Pol-47 (Fig. 2B). The empty lentiviral vector, JS1, was used as a negative control. We infected 293T cells with the lentiviral vectors at a multiplicity of infection (m.o.i.) of 0.15 and selected transduced cells with live sorting for GFP. First, we confirmed sequence-specific inhibition in each cell line by transfection of different luciferase reporters. Reduced luciferase expression was exclusively observed in cells that were transduced with the corresponding shRNA lentiviral vector (Fig. 2C). We also transfected a control reporter for the shRNA against Pol-6 in these cell lines, which is not targeted by any of the shRNAs. Luciferase expression levels were comparable in all cell lines, including the mock- and empty vector-transduced cell lines (Fig. 2C). The 293T cell line does not support HIV-1 replication, but can be used for virus production upon transfection of the pLAI molecular clone. When we transfected pLAI in the cell lines, only those cells transduced with a shRNA lentiviral vector showed a reduction in HIV-1 production (Fig. 2D).

In contrast to 293T cells, the SupT1 cell line is fully susceptible to HIV-1 replication, which allowed us to test the impact of shRNA expression in a relevant gene therapy setting. We infected SupT1 cells with the lentiviral vectors at an m.o.i. of 0.15 and selected transduced cells. Untransduced cells or cells transduced with the empty vector, JS1, showed massive syncytia formation and cell death 9 days after infection with the HIV-1 LAI strain. In contrast, cells expressing shRNAs against Gag-5, Pol-1, or Pol-47 remained viable and syncytia were not observed. We determined CA-p24 levels in the supernatant as a measure of virus replication (Fig. 3A). We observed a 4–5 log reduction in CA-p24 for the three shRNA constructs. We obtained similar results in transduced peripheral blood mononuclear cells (PBMCs) (Fig. 3B). Combined, these data confirm that our selected shRNAs induce a sequence-specific inhibition of HIV-1 in stably transduced cells, which is sufficient to block viral replication.
Multiple shRNA Expression Results in Additive HIV-1 Inhibition

Toward a multi-shRNA gene therapy, we combined the three shRNA expression cassettes for Gag-5, Pol-1, and Pol-47 in a single lentiviral vector construct (Fig. 4A). As an initial safety test, we determined that transfection of this multiple shRNA expression construct into 293T cells did not induce interferon-β (Supplemental Fig. S2) or PKR.
phosphorylation (results not shown). In contrast, both in vitro-transcribed nef-dsRNA and poly(I:C) induced high amounts of IFN-β mRNA and PKR phosphorylation [20].

Next, we produced the triple viral vector and infected 293T cells at a m.o.i. of 0.15 and selected cell clones. We obtained two cellular clones, Nos. 3 and 5, for which we confirmed the presence of all three expression cassettes with a PCR across the triple cassette (results not shown). With the individual reporter constructs we could distinguish individual shRNA activity in this multiple shRNA setting. We transfected these reporters into the triple shRNA No. 3 cells and the control JS1 No. 1. We observed specific inhibition of the luciferase reporter with the Gag-5, Pol-1, and Pol-47 target, but not with the Pol-6 control (Fig. 4B), demonstrating that the observed inhibition of the reporter constructs in the triple shRNA cell line is sequence-specific.

Finally, we transfected pLAI into the triple shRNA cell clones 3 and 5 and compared virus production in these cells with that from clones transduced with the control JS1 vector or the single Pol-47 vector. In clones expressing three different shRNAs we observed a severely reduced virus production compared to the cell lines expressing only a single shRNA (Fig. 4C). Combined, these data show that when three different shRNAs are expressed from individual promoters in a single vector, each shRNA is active. Their combined activity against the common HIV-1 target results in a much more potent inhibition of HIV-1 production.

A More Durable Inhibition of HIV-1 with Two shRNAs
To test whether multiple shRNAs can prevent HIV-1 escape, we made and compared SupT1 cells with a single shRNA, Gag-5 or Pol-47, and with the two combined, Gag-5 and Pol-47 (Fig. 5A). We used a relatively high amount of virus to force viral escape in six independent infections for each cell line. We observed strong inhibition of virus replication for all cell lines relative to the control JS1 up to 7 days postinfection (Fig. 5B). At 13 days postinfection, all six infected Gag-5 cell cultures showed signs of virus replication as an increase in CA-p24 production (Fig. 5B). At 13 days postinfection, three of the six Pol-47 cultures were positive, and at 22 days the three remaining cultures also became positive. We observed a more durable inhibition when the two shRNAs were coexpressed, with no signs of virus replication up to 17 days postinfection. Nevertheless, four cultures became positive at 22 days postinfection. We sequenced several escape viruses and observed point mutations within the target sequences of Gag-5 and Pol-47 (Fig. 6A). When we infected control cells with the wild-type virus and the Pol-47 escape variant E1, both viruses showed a similar replication curve (Fig. 6B). In contrast, only the E1 variant replicated in Pol-47 cells.
FIG. 5. A more durable inhibition of HIV-1 with two shRNAs. (A) Lentiviral vector expressing two different shRNAs from individual promoters, JS1-shRNA2. (B) Six separate SupT1 cell cultures of each cell line were infected with HIV-1 LAI (5 ng CA-p24) and virus spread was monitored for 27 days by measuring CA-p24 with an ELISA. A single replication curve of the control JS1-transduced SupT1 cells is also shown.

FIG. 6. Single nucleotide substitutions result in viral escape. (A) Escape virus from Gag-5 and Pol-47 SupT1 cells was sequenced. The integrated provirus was PCR amplified with primers that amplify approximately 0.5 kb containing the Gag-5 or Pol-47 target sequence. Sequence analysis showed point mutations within the respective targets. (B) One nanogram of CA-p24 of the escape virus E1 (gray symbols) and the wild-type LAI virus (black symbols) was used to infect control cells and Pol-47 cells. The E1 mutant is resistant to Pol-47 (right). (C) Five nanograms of CA-p24 was used to infect control cells and the cell line expressing two shRNAs. A higher viral dose was used to differentiate among the inhibition. The E1 mutant is inhibited less potently than the wild-type virus (right).
showing that the point mutation in the Pol-47 target sequence causes viral resistance. Finally, we also infected the cells that express two shRNAs, this time with a higher viral input to differentiate inhibition (Fig. 6C). Both viruses were inhibited, but inhibition of the wild-type virus was more potent. We obtained similar results for other escape viruses (data not shown). These results confirm that the observed inhibition is highly sequence-specific. In addition, it shows that virus inhibition is more durable when two shRNAs are coexpressed.

**DISCUSSION**

For the effective treatment of HIV-1 infection with an RNAi-based gene therapy, the application of multiple shRNAs simultaneously, preferably targeting highly conserved HIV-1 regions, has been proposed [21–24]. We have identified multiple effective shRNAs against eight highly conserved targets within the HIV-1 RNA genome. The targets of most previously published siRNAs and shRNAs that are effective against HIV-1 are in fact not highly conserved [22]. Only a few studies focused on the need to target conserved HIV-1 sequences [10,25–27]. The largest study so far describes a set of anti-HIV shRNAs against conserved HIV-1 sequences that was selected based on the Tuschl rules for siRNA design, which require a target sequence to start with two adenosines [25,28]. Only 11 shRNAs obeyed these criteria, and of these several were shown to inhibit HIV-1 efficiently. In contrast, we ignored all design rules and constructed 86 shRNAs based solely on conservation of the target sequence among viral isolates. Of these, 21 are potent HIV-1 inhibitors that are ideal candidates for a multiple shRNA gene therapy.

In fact, many of these 21 shRNAs do not comply with known siRNA criteria, thus confirming the limited value of these criteria for shRNA design against HIV-1. We analyzed the expected siRNAs that would result from shRNA dicing [15]. Neither the rational design method of Dharmacon [29], the observed strand bias method [30], nor the BIOPREDSI algorithm [31] would have predicted these 21 shRNAs. We previously used a target site accessibility criterion, based on the in vitro binding of HIV-1 transcripts to DNA oligo microarrays, for target selection [4]. With the current shRNA set, a trend could be recognized; targets with low accessibility scores were poor targets (results not shown). However, as a selection criterion, this would not have been very useful, since most HIV-1 sequences were fairly accessible.

shRNA libraries generated at random from cDNA result, on average, in about one in four shRNAs being active [32,33], which is in agreement with our results. An attempt was made to identify sequence preferences in these active shRNAs, but none were detected [33]. We looked for apparent properties of the effective shRNAs from our dataset. The only trends we observed were related to polymerase III transcription. Most effective shRNAs start with G or A, which is the preferred transcription start nucleotide, and a stretch of four U’s was never observed in an effective shRNA, which is likely due to the fact that this motif may trigger termination of transcription [34]. It would be of interest to study the effectiveness of those shRNAs when expressed from a polymerase II promoter.

Some of the conserved targets that we have selected have previously been reported as effective siRNA targets. For instance, Surabhi and Gaynor [3] used an siRNA against the R/T-4 target and Dave et al. [26] targeted the Nef-9 sequence. In our screen, R/T-4 inhibited HIV-1 production efficiently, but the shRNA against Nef-9 did not. This particular shRNA target has a stretch of four adenosines, and consequently four U’s in the guide strand of the hairpin, which can act as a transcription termination signal, thereby explaining its inactivity.

In an eventual clinical application of this RNAi-based gene therapy, patients who received conventional antiretroviral drugs and failed the therapy will likely be treated. Therefore, it is important to consider the presence of possible drug-resistant HIV-1 variants with genome mutations that may affect shRNA targets. Only Pol-1 targets a site that is involved in protease-inhibitor resistance. The D30N mutation provides resistance to Nelfinavir [35]. In addition, the V32I mutation is often observed when protease inhibitors are used [36]. These mutations result in a G-to-A and G-to-T mutation at positions 13 and 19 in the Pol-1 target site, respectively. The mutation at position 19 is not likely to impact Pol-1 shRNA inhibition [37]. However, the mutation at position 13 may provide resistance to the Pol-1 shRNA. Therefore, patients on Nelfinavir therapy should be excluded from a gene therapy that includes Pol-1, but a different shRNA from the active set could be included in the multiple shRNA therapy. Alternatively, one could anticipate such genome variation and design a compensatory shRNA variant [9,22,38].

As with conventional drugs, siRNAs or shRNAs can induce side effects. We previously postulated that the m.o.i. in a gene therapy setting should be kept low to avoid high expression levels of shRNAs, thereby minimizing the risk of undesired side effects [22]. Recently, it was shown that expression of shRNAs led to fatality in mice due to oversaturation of the mi/siRNA pathways [39]. However, it is important to note that an exceptionally high copy number was used in these experiments, resulting in extremely high levels of shRNA expression. In contrast, we always use an m.o.i. of 0.15 or less, which results in only a single vector genome per transduced cell. Thus, we never reached the high levels required to saturate the si/miRNA pathway in our experiments, not even with our triple shRNA construct.

Another potential side effect is induction of the interferon pathway. Although initially the paradigm
was that only dsRNA larger than 30 bp induces this pathway, recently small dsRNA was also shown to evoke this reaction [40–44]. This effect is dose dependent, and some sequence motifs were implicated in the activation of this response [42,44]. Our lentiviral vector construct with a single or triple shRNA cassette did not induce interferon-β or phosphorylation of PKR in a transient transfection experiment. Considering the relatively low shRNA expression levels in the stable cell lines, the interferon pathway should not be induced.

Concerns remain involving off-target effects in which siRNAs silence partially complementary transcripts through an miRNA-like mechanism. Such an off-target effect requires at least complementarity between the siRNA seed region and the 3′UTR of a target gene [45,46]. Such a weak restraint results, for any effective siRNA, in numerous potential off-target genes. When multiple shRNAs are combined, the number of potential off-target genes will increase, increasing the chance of a potential negative effect on treated cells. However, we observed no obvious changes in our cell culture experiments, growth rates were comparable across all cell lines. Furthermore, the observed inhibition of luciferase reporters or HIV-1 remained similar in stable cell lines when these were cultured for up to 100 days (data not shown). This indicates that silencing off-target genes may not be significant due to the low multiplicity of infection or that silenced off-target genes did not affect cell viability. Nevertheless, off-targeting is a genuine concern for the development of a multiple shRNA approach against HIV-1 and the potential risks should be properly assessed before an eventual clinical application.

We have shown that multiple effective shRNAs inhibit HIV-1 production much more strongly compared to a single shRNA. Also, when we infected cells expressing a single shRNA or two shRNAs, escape mutants quickly emerged in the single-shRNA cells. In contrast, virus escape was delayed in the double-shRNA cell line. These data indicate that a combinatorial approach against HIV-1 based on RNAi results in an increase in the magnitude of inhibition combined with a reduced chance to escape from this inhibition.

We are currently evaluating safety and efficacy of individual shRNAs. The logical step forward is to include more shRNAs in the lentiviral vector. In silico analysis suggests that perhaps no more than four shRNAs will be required to prevent viral escape [22,47]. We have recently tested a four-shRNA construct and have not seen viral escape up to 60 days in multiple cultures of stably transduced cells. This result underscores the idea that durable viral suppression is within reach. The design of this multiple shRNA vector will be described elsewhere, as it involves the usage of four different polymerase III promoters to avoid recombination during the transduction step (ter Brake and Berkhout, manuscript in preparation). Currently, a gene therapy strategy with a lentiviral vector that transcribes three different anti-HIV RNA molecules, including a single shRNA [48], is undergoing preclinical evaluation and phase I clinical trials may be initiated this year. Our results indicate that RNAi has great antiviral potential in a gene therapy approach expressing multiple shRNAs from a single viral vector and support our effort in the development of the multiple shRNA approach for the treatment of HIV-1-infected individuals.

**MATERIALS AND METHODS**

**Plasmid construction.** The shRNA expression plasmids pSUPER-shRNA were constructed as previously described [15]. Lentiviral vector plasmids were derived from the construct pRLCpttgkppreSIN [19], which we renamed JS1. Expression cassettes for shRNAs were obtained by digestion of pSUPER constructs with Xhol and PstI and the fragment was inserted into the corresponding sites of JS1, resulting in JS1-shRNA. The double- and triple-shRNA-expressing lentiviral vectors were constructed as follows. First, the shPol-47 cassette was obtained by digestion of pSUPER-sh Pol-47 with Smal and XhoI and inserted in between the HindIII and XhoI sites of the pSUPER shGag-5 plasmid, resulting in pSUPER-shRNA2. The third shPol-1 cassette was inserted by repeating this procedure to yield pSUPER-shRNA3. The double- and triple-expression cassettes were digested with Smal and XhoI from pSUPER-shRNA2 and shRNA3, respectively, and inserted into the XhoI and EcoRV sites of JS1, resulting in JS1-shRNA2 and -shRNA3. Firefly reporter plasmids were constructed by insertion of a 50- to 70-nucleotide HIV-1 sequence, with the 19-nucleotide target region in the center, in the EcoRI and PstI sites of pGL3-Nef, resulting in pGL3-reporter [12].

**Cell culture.** Human embryonic kidney 293T adherent cells were grown in DMEM (Gibco BRL) and SupF1 suspension cells were grown in RPMI (Gibco BRL), both supplemented with 10% FCS, penicillin (100 U/ml), and streptomycin (100 μg/ml) in a humidified chamber at 37°C and 5% CO2. PBMCs were grown in RPMI supplemented with 10% FCS, penicillin (100 U/ml), and streptomycin (100 μg/ml) and stimulated with IL-2 (100 U/ml) and PHA (4 μg/ml) for 2 days after which CD8 cells were depleted; cells were subsequently cultured without PHA.

**Transfection experiments.** Cotransfections of pLAI and the shRNA vector were performed in a 96-well format. Per well, 2 × 104 293T cells were seeded in 200 μl DMEM with 10% FCS without antibiotics. The next day, 100 ng of pLAI, 20 ng of shRNA vector, and 0.6 ng of pRL (Renilla luciferase) were transfected with 0.5 μl Lipofectamine 2000 in a reaction volume of 50 μl according to the manufacturer’s instructions (Invitrogen). Eight hours posttransfection the medium was replaced with 200 μl medium containing antibiotics; 48 h after transfection samples of medium were taken for CA-p24 ELISA and cells were lysed for Renilla luciferase activity measurements with the Renilla Luciferase Assay System (Promega). CA-p24 is a measure of virus production. To correct for transfection variation, relative CA-p24 values were determined by dividing the CA-p24 measurements by the Renilla values obtained. We set the condition that for a valid experiment the ratio between the highest and the lowest Renilla values should not exceed 2 and also that the positive shRNA control, against HIV-1 Nef, should show strong inhibition. Stable cell lines were transfected with either 100 ng pLAI or 100 ng pGL3-reporter with 2 ng of pRL, with 1 μl Lipofectamine 2000, in a reaction volume of 100 μl according to the manufacturer’s instructions, in a 24-well format. Two days posttransfection with pLAI, supernatant was collected for CA-p24 ELISA and cells were lysed for measurement of Renilla luciferase activity. Following transfection of the pGL3-reporter, cells were lysed to measure firefly and Renilla luciferase activities with the Dual-Luciferase Reporter Assay System (Promega).

**Lentiviral vector production.** Lentiviral vector was produced as follows. 2.2 × 108 293T cells were seeded in a T25 flask the day prior to...
transfection. The next day, medium was replaced with 2.2 ml medium without antibiotics. Subsequently, lentiviral vector plasmid (2.4 µg) was cotransfected with packaging plasmids pSYNG (1.5 µg) [49], RVSV-rev (0.6 µg), and pVSVG (0.8 µg) [17,18] with 16 µl Lipofectamine 2000 and 1.5 µl OptiMEM (Gibco BRL). The second day, medium was replaced with fresh medium. On the third and fourth days, medium containing lentiviral vector was harvested in the morning. Cellular debris was removed by low-speed centrifugation and supernatant was stored at 4°C. On the fourth day, supernatants were pooled and filtered and aliquots of 0.8 ml were stored at −80°C.

**Lentiviral vector transduction.** Lentiviral vector stocks were titrated on 293T cells and SupT1 cells to determine vector titer. SupT1 cells and 293T cells were transduced after CD8 depletion. Three days posttransduction, cells were transduced at a multiplicity of infection of 0.15. PBMCs were used to determine vector titer. SupT1 cells and 293T cells were transduced cell clones, transduced single cells were obtained by live FACS. RNAi research in the Berkhout lab is performed with the lentiviral vector system and technical assistance. We thank Esther de Jong (AMC Cell Biology) for live cell sorting. RNAi research in the Berkhout lab is performed with the lentiviral vector system and technical assistance. We thank Esther de Jong (AMC Cell Biology) for live cell sorting.

**HIV-1 infection.** HIV-1 LAI was produced from transfected 293T cells. HIV-1 LAI was selected and pooled to avoid clone-to-clone variation. Transduced cell clones, transduced single cells were selected. For selection of transduced cell clones, transduced single cells were obtained by live FACS. The presence of the correct number of expression cassettes was tested by PCR. For the SupT1 cells expressing two HIV-1 variants (0.6 µg), multiple clones were selected and pooled to avoid clone-to-clone variation.

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