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CRISPR-Cas9 Can Inhibit HIV-1 Replication but NHEJ Repair Facilitates Virus Escape

Gang Wang[1], Na Zhao[1], Ben Berkhout[1] and Atze T Das[1]

[1]*Laboratory of Experimental Virology, Department of Medical Microbiology, Center for Infection and Immunity Amsterdam (CINIMA), Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands*

Several recent studies demonstrated that the clustered regularly interspaced short palindromic repeats (CRISPR)-associated endonuclease Cas9 can be used for guide RNA (gRNA)-directed, sequence-specific cleavage of HIV proviral DNA in infected cells. We here demonstrate profound inhibition of HIV-1 replication by harnessing T cells with Cas9 and antiviral gRNAs. However, the virus rapidly and consistently escaped from this inhibition. Sequencing of the HIV-1 escape variants revealed nucleotide insertions, deletions, and substitutions around the Cas9/gRNA cleavage site that are typical for DNA repair by the nonhomologous end-joining pathway. We thus demonstrate the potency of CRISPR-Cas9 as an antiviral approach, but any therapeutic strategy should consider the viral escape implications.

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INTRODUCTION

The clustered regularly interspaced short palindromic repeats-Cas9 system represents a versatile tool for genome engineering by enabling the induction of double-stranded breaks at specific sites in DNA.[1] Sequence specificity is due to the gRNA that directs Cas9 to the complementary sequence present immediately upstream of a 3-nt protospacer adjacent motif in the target DNA. In mammalian cells, the double-stranded breaks can be repaired by the nonhomologous end-joining (NHEJ) pathway, which results in the frequent introduction of insertions, deletions, and nucleotide substitutions at the cleavage site, or by homology-directed repair, which depends on the presence of homologous DNA sequences.[1,2]

Several studies demonstrated that the Cas9/gRNA system can be used for inhibition of human pathogenic DNA viruses, including hepatitis B virus,[3–8] Epstein–Barr virus,[9] and human papilloma virus.[10] Replication of retroviruses, like HIV-1, can also be inhibited with the Cas9/gRNA system by targeting the reverse-transcribed HIV-1 DNA replication intermediate or the proviral DNA upon integration into the cellular genome.[2,11–13] Gene therapy approaches for the treatment of HIV-1 infected individuals have been proposed in which the Cas9 and antiviral gRNAs are directed to HIV-1 infected cells to inactivate or delete the integrated provirus, or in which blood stem cells are harnessed against new infections. However, Cas9/gRNA-mediated inhibition of virus production and/or replication has been shown only in short-term experiments, while we know that HIV-1 can escape from most if not all types of inhibitors, including small molecule antiviral drugs and sequence-specific attack by RNA interference. We therefore set out to identify viral escape strategies from Cas9/gRNA-mediated inhibition.

RESULTS AND DISCUSSION

Design of gRNAs that effectively target the HIV-1 DNA genome

In silico algorithms were used to select 19 gRNAs that should target HIV-1 DNA with high efficiency and exhibit no off-target effects on cellular DNA (see **Supplementary Table S1**). Seven gRNAs were selected that target the long terminal repeat (LTR) region present at the 5′ and 3′ ends of the proviral genome (**Figure 1a**). Five of these (gLTR1–5) also target the accessory nef gene that overlaps the 3′ LTR, but that is not essential for *in vitro* virus replication. Twelve gRNAs target sequences that encode other viral proteins, including well-conserved domains in the essential gag, pol and env genes and sequences of overlapping reading frames, like the tat and rev genes (**Figure 1a**). Nine selected gRNAs target sequences that are highly conserved among different HIV-1 isolates (Shannon entropy <0.2; gLTR7, gGag1, gGagPol, gPol1–4, gTatRev, and gEnv2), while the other gRNAs target less conserved HIV-1 domains (Shannon entropy ≥0.20; gLTR1–6, gGag2, gVpr, gEnv1, and gNef).

We first tested the antiviral activity in transient transfections of 293T cells with plasmids expressing HIV-1, Cas9 and one of the anti-HIV gRNAs or control gRNAs targeting non-HIV sequences (luciferase, GFP). To quantify HIV-1 gene expression, we measured viral capsid protein (CA-p24) produced at 2 days after transfection (**Figure 1b**). A similar high CA-p24 level was observed when different control gRNAs were tested, but this level was significantly reduced for all anti-HIV gRNAs, which is likely due to Cas9/gRNA induced cleavage of the HIV-1 plasmid. Accordingly, the inhibitory effect was not observed in control experiments with only Cas9 or gRNA (data not shown). There may be some small differences in antiviral activity among the gRNAs, but we decided to move all inhibitors forward to antiviral tests in stably transduced T cells.

Inhibition of HIV-1 replication by the Cas9/gRNA system

SupT1 T cells were first transduced with a Cas9-expressing lentiviral vector. Stably transduced cells were selected and subsequently transduced with a lentiviral vector expressing one of

Correspondence: Atze T Das and Ben Berkhout, Laboratory of Experimental Virology, Academic Medical Center, Meibergdreef 15, 1105 AZ Amsterdam, The Netherlands. E-mail: a.t.das@amc.uva.nl; b.berkhout@amc.uva.nl

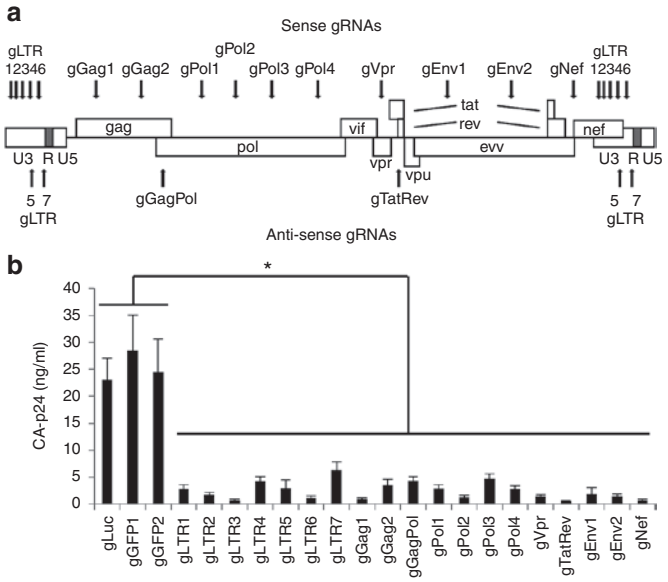

Figure 1 Cas9/gRNA targeting of the HIV-1 genome. (**a**) The HIV-1 proviral DNA with the position of gRNAs tested in this study. (**b**) The efficiency of gRNAs to silence HIV-1 DNA was tested in 293T cells transfected with plasmids expressing Cas9, gRNA, and HIV-1 LAI. To quantify viral gene expression, the viral capsid protein (CA-p24) was measured in the culture supernatant at 2 days after transfection. Average values (±SD) of four experiments are shown. Statistical analysis (independent samples' *t*-test analysis) demonstrated that CA-p24 expression in the presence of antiviral gRNAs differed significantly from values measured with control gRNAs against luciferase and GFP (*$P < 0.05$).

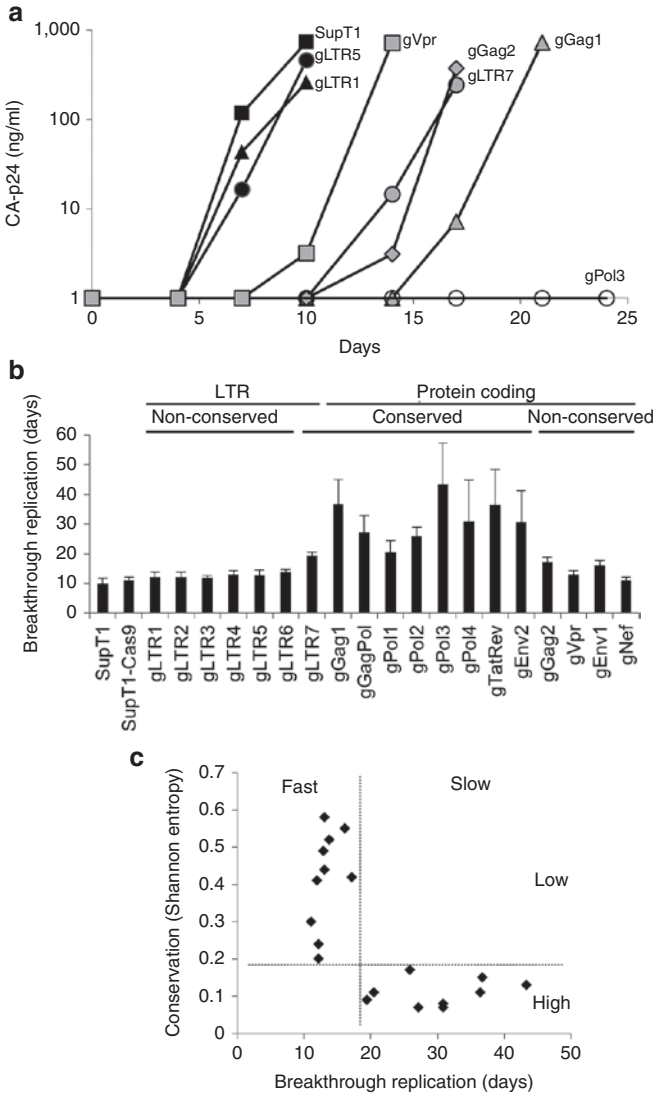

Figure 2 HIV-1 replication in Cas9 and gRNA expressing cells. (**a**,**b**) SupT1 cells stably transduced with Cas9 and gRNA expressing lentiviral vectors were infected with HIV-1 LAI. Virus replication was monitored by measuring the CA-p24 level in the culture supernatant (**a**) and by scoring the formation of virus-induced syncytia (**b**). The day at which massive syncytia were observed, which reflects breakthrough virus replication, is indicated. Average values of four experiments (±SD) are shown. SupT1, control nontransduced cells. SupT1-Cas9, cells transduced only with the Cas9 expressing vector. (**c**) Correlation between the level of inhibition (day of breakthrough replication; as shown in **b** and the conservation of target sequence amongst different HIV-1 isolates (Shannon entropy as shown in **Supplementary Table S1**). The Pearson's correlation coefficient was calculated: $r = -0.58$.

the antiviral gRNAs. Of note, none of the selected gRNAs target the lentiviral vectors. Upon infection of transduced cells with the HIV-1 LAI isolate, virus replication was monitored by measuring the CA-p24 level in the culture supernatant. Efficient virus replication was apparent in control nontransduced SupT1 cells and in Cas9-only transduced cells, as reflected by a rapid increase in the CA-p24 level (**Figure 2a**) and the appearance of large virus-induced syncytia and cell death around day 10 after infection (**Figure 2b**; average time of HIV-1 breakthrough replication of four experiments are shown). HIV-1 replication in cells transduced with Cas9 and gRNAs targeting poorly conserved LTR sequences (gLTR1–6) was only marginally delayed (**Figure 2a** and data not shown) and breakthrough replication resulting in large syncytia was observed at 12–14 days (**Figure 2b**). Replication in cells transduced with Cas9 and gLTR7, which targets the highly conserved and essential TATA-box region of the LTR promoter, was more delayed and resulted in breakthrough replication at 19 days. A similar split was observed when targeting protein-coding regions. Targeting highly conserved HIV-1 sequences (gGag1, gGagPol, gPol1–4, gTatRev, and gEnv2) exhibits a more sustained antiviral effect (breakthrough replication in 20–43 days; **Figure 2b**) than targeting less conserved domains (gGag2, gVpr, gEnv1, and gNef; breakthrough replication in 11–17 days; **Figure 2b**). Surprisingly, despite their potency to suppress virus production (**Figure 1b**), some of the gRNAs inhibited virus replication only briefly and none prevented breakthrough virus replication. Moreover, the time required for breakthrough replication did not correlate with the potency of inhibiting HIV-1 production in 293T cells (see **Supplementary Figure S1**).

The breakthrough viruses could represent viral escape variants that are no longer suppressed by the Cas9/gRNA system. Interestingly, the time required for breakthrough virus replication was longer for target sequences that are more conserved (**Figure 2c**: inverse correlation between the day of breakthrough replication and the Shannon entropy). Along these lines, the early escape observed for the gRNAs targeting nonconserved domains could be explained by many escape options

that are available to the virus, whereas the relatively late escape observed for gRNAs targeting conserved domains could be due to the fewer escape options because important sequences are targeted. Nevertheless, the poor inhibition and very swift viral escape observed for some of the gRNAs is remarkable, as the evolutionary process underlying viral escape, *i.e.*, the generation of sequence variation and subsequent outgrowth of variants with improved fitness, usually takes several weeks or even months, *e.g.*, for RNA interference inhibitors tested in the same experimental system.[14]

NHEJ-induced mutations around the Cas9 cleavage site cause rapid HIV-1 escape

We first tested whether the breakthrough viruses were indeed resistant to the specific Cas9/gRNA set by passage onto fresh matching Cas9/gRNA SupT1 cells and control nontransduced cells. The breakthrough viruses replicated with similar efficiency on both cell lines (see **Supplementary Figure S2**), which confirmed the escape phenotype. Both cell lines were also infected with wild-type HIV-1 LAI, showing the selective replication block in restricted Cas9/gRNA cells.

We next sequenced the gRNA-target region of breakthrough viruses in multiple independent cultures. Strikingly, we observed mutations in the target for all escape viruses (**Figure 3** and **Supplementary Figure S3**). The viruses that escaped rapidly from gRNAs targeting nonconserved LTR domains (gLTR1–6) frequently acquired deletions (1–31 nt in size; in 20 cultures) and insertions (1–3 nt; in six cultures), and a single culture acquired a point mutation in the target. In contrast, the gLTR7-resistant viruses that evolved more slowly had acquired substitutions (1 or 2 nt; in three cultures, once in combination with a 1-nt deletion) and 1-nt insertions (three cultures). The gLTR7 target includes the TATA box, which could explain why large deletions are not tolerated. The occurrence of single-nucleotide substitutions at critical target positions confirms the exquisite sequence specificity of Cas9/gRNA action.

This trend of a differential mutational spectrum between conserved and less conserved targets was confirmed for the gRNAs that target protein-coding regions. We predominantly observed nucleotide substitutions (1 or 2 nt) in conserved essential genes. Insertions were restricted to the size of 3 nt, such that a codon is added but the open reading frame is not disrupted. A fair percentage of the acquired substitutions represents silent codon changes, again suggesting pressure on the virus to maintain the coding potential. In contrast, nucleotide deletions and insertions that shift the open reading frames were

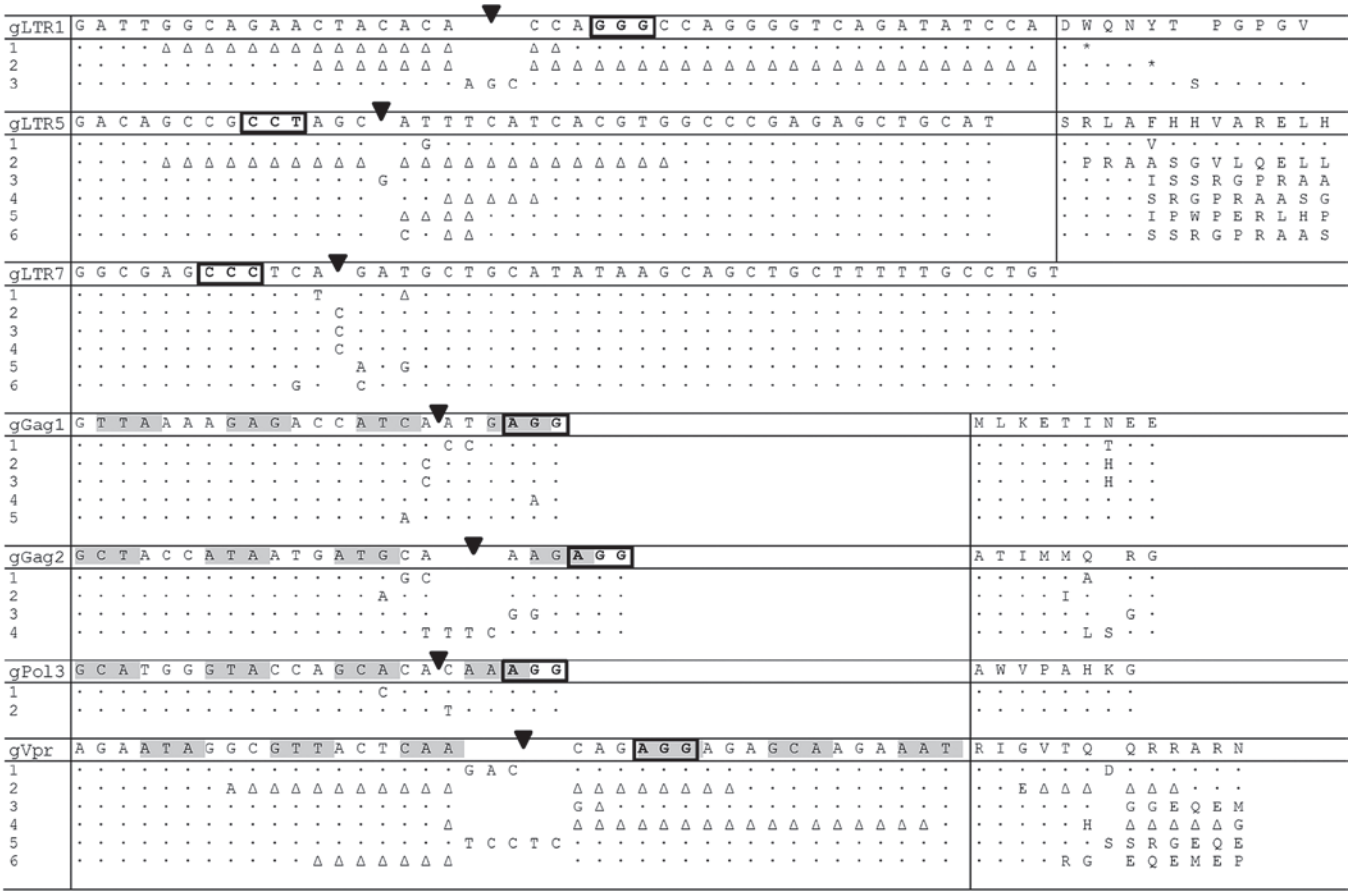

Figure 3 HIV-1 escapes from Cas9/gRNA inhibition through mutations in the target region. The gRNA target region in breakthrough viruses obtained in two to six independent HIV-1 cultures on the different SupT1-Cas9-gRNA cells was sequenced. For every gRNA, the wild-type HIV-1 nucleotide sequence is shown on top. Codons are boxed in grey if applicable, with the translated amino acid sequence on the right hand side. The protospacer adjacent motif (PAM) sequence is boxed and the arrowhead indicates the Cas9 cleavage site at position −3. Nucleotide and amino acid substitutions, insertions and deletions (Δ) are indicated. Data for all tested gRNAs are shown in **Supplementary Figure S3**.

frequently observed when targeting the less conserved vpr and nef genes that are not required for HIV-1 replication on SupT1 cells.

The position of all observed mutations was plotted relative to the gRNA target (position −1 to −20; **Figure 4**) and 3-nt protospacer adjacent motif (position 1–3) and we indicated the position of the expected double-stranded breaks. Except for a single point mutation in the protospacer adjacent motif region, all mutations cluster around the Cas9 cleavage site at position −3,[15–17] suggesting that the escape mutations were generated in the process of HIV-1 inhibition. More specifically, we propose that Cas9/gRNA inhibits by DNA cleavage, but subsequent repair by the NHEJ pathway will generate the mutations that provide viral resistance. The coupled Cas9 cleavage and NHEJ repair explains the immediate HIV-1 escape when noncritical sequences are targeted.

Insertions and deletions form the hallmark of NHEJ action, but such genome changes are not acceptable in critical HIV-1 sequences, which explains the frequent observation of nucleotide substitutions in conserved targets. Alternatively, these mutations could have been generated during the error-prone reverse transcription process during viral replication or by cellular APOBEC activity, as documented in more standard virus evolution scenarios.[18] Regular HIV-1 evolution is dominated by transitions with G-to-A as the predominant mutation. For example, in a similar virus evolution study with RNA interference antivirals,[19] 80% of the acquired mutations were transitions (91 of 113 substitutions; see **Supplementary Table S2**) and 46 G-to-A changes were scored. A completely different pattern was observed in this Cas9/gRNA study: only 44% transitions (27 of 62 mutations) and the A-to-C transversion was the most frequent mutation. Together with the clustering of the mutations around the Cas9 cleavage site and their rapid appearance, these findings strongly suggest the involvement of the Cas9-NHEJ pathway in the generation of most and perhaps all escape mutations. On the other hand, we cannot formally exclude that regular HIV-1 evolution contributed to virus escape, *e.g.* by creating the G-to-A mutation in the protospacer adjacent motif.

Implications for antiviral strategies

Taken together, we demonstrate that HIV-1 can be targeted effectively by the Cas9/gRNA system, but that the coupled NHEJ repair process creates viral escape variants. This results in immediate escape when nonessential viral sequences are targeted. When conserved protein-coding HIV-1 sequences are targeted, viral escape can be significantly delayed and the level of inhibition is comparable to that observed for some antiviral shRNAs targeting conserved HIV-1 domains. Combinations of such potent shRNAs provide durable inhibition of virus replication.[20–22] The clustered regularly interspaced short palindromic repeats/Cas9 antiviral strategy may similarly provide a sustained therapeutic effect when gRNAs targeting highly conserved HIV-1 sequences are applied in a combinatorial mode. Since Cas9 cleaves the DNA at position −3 and most escape mutations cluster around this position, this subdomain of the target sequence should be particularly conserved to reduce the viral escape options. The coupled Cas9-NHEJ cleavage-repair action may also suggest an alternative anti-viral strategy where mutations are introduced to weaken the fitness of the persisting virus. We observed rapid mutation-mediated escape when nonessential sequences were attacked, but even these mutations will likely reduce the viral replicative fitness *in vivo*, suggesting the potency of such an attenuation strategy. Alternatively, cellular genes that are essential for HIV-1 replication, like the genes encoding the CCR5 coreceptor, could be targeted by Cas/gRNAs. Moreover, these clustered regularly interspaced short palindromic repeats/Cas9 strategies could be combined with other antiviral approaches, either regular antiviral drugs or gene therapy strategies.

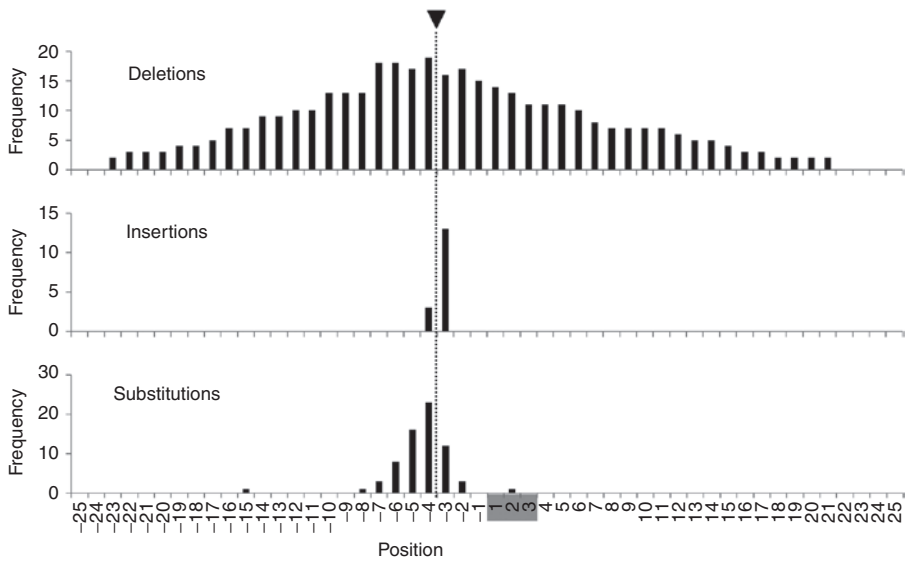

Figure 4 HIV-1 escape mutations cluster around the Cas9 cleavage site. The position of all observed nucleotide deletions, insertions, and substitutions (as shown in **Figure 3** and **Supplementary Figure S3**) was plotted relative to the gRNA target sequence (position −1 to −20) and PAM (position 1–3). The arrowhead indicates the Cas9 cleavage site at position −3.

MATERIALS AND METHODS

Plasmids. The lentiviral vector LentiCas9-Blast (Addgene plasmid # 52962) containing the human codon-optimized *Streptococcus pyogenes* Cas9-expression cassette and LentiGuide-Puro (Addgene plasmid # 52963) with gRNA expression cassette were gifts from Feng Zhang.[23] Oligonucleotides encoding the gRNAs were ligated into the BsmB1 site of the LentiGuide-Puro vector. Control gRNAs targeting the firefly luciferase and EGFP gene[13] were included (see **Supplementary Table S3**). The plasmid pLAI encodes the HIV-1 subtype B isolate LAI.[24]

Cell culture and transfection. Human embryonic kidney 293T cells and SupT1 T cells were cultured as described previously.[25] 293T cells was transfected with 200 ng pLAI, 500 ng LentiCas9-Blast plasmid, and 500 ng LentiGuide-Puro plasmid by calcium phosphate precipitation.

Lentiviral vector production and transduction. The lentiviral vector was produced and titrated as previously described.[20] Briefly, the vector was produced by transfection of 293T cells with the lentiviral vector plasmid and packaging plasmids pSYNGP, pRSV-rev, and pVSV-g with Lipofectamine 2000 (Invitrogen, Life Technologies, Bleiswijk, The Netherlands). After transfection, the medium was replaced with OptiMEM (Invitrogen, Life Technologies, Bleiswijk, The Netherlands) and the cells were cultured for 48 hours. The lentiviral vector containing supernatant was filtered (0.45 μm), aliquoted and stored at −80 °C. SupT1 cells (2×10^5 cells in 1-ml culture medium) were transduced with an equal amount of LentiCas9-Blast (Cas9) virus particles (based on CA-p24) and cultured with 1 ng/ml blasticidin for 1 week to select transduced cells. The cells were subsequently transduced with an equal amount of the different LentiGuide-Puro (gRNA) virus particles and cultured with 1 ng/ml puromycin to select for dually transduced cells.

HIV-1 infection and evolution. The HIV-1 LAI stock was produced by transfection of 293T cells with the pLAI molecular clone. Virus production was measured by CA-p24 enzyme-linked immunosorbent assay.[26] SupT1 T cells (2×10^5 cells in 1 ml culture medium) were infected with an equal amount of HIV-1 LAI virus corresponding to 1 ng CA-p24. Cells were passaged twice a week. Virus spread was monitored by measuring the CA-p24 production in the culture supernatant and scoring the formation of syncytia every 3 or 4 days. At the peak of infection, when massive syncytia were observed, cell-free virus was passaged to fresh, matching-transduced cells. When syncytia were apparent in the newly infected cells, cellular DNA containing the integrated provirus was isolated for sequencing analysis of the gRNA target region (PCR and sequencing primers listed in **Supplementary Table S4**), as previously described.[27]

SUPPLEMENTARY MATERIAL

Figure S1. The capacity of Cas9/gRNA to inhibit HIV-1 replication does not correlate with the efficiency of silencing viral gene expression.
Figure S2. Efficient replication of HIV-1 escape variants on SupT1-Cas9/gRNA expressing cells.
Figure S3. Sequence of the gRNA target region in breakthrough viruses obtained in 2 to 6 independent cultures of HIV-1 on different SupT1-Cas9-gRNA cells.
Table S1. Selected gRNAs targeting HIV-1.
Table S2. Nucleotide substitution patterns observed in HIV-1 escape variants.
Table S3. Target sequence of control gRNAs.
Table S4. Primers used for sequencing of gRNA target regions in HIV-1.
Supplementary References

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