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Silencing Integrated SIV Proviral DNA with TAR-specific CRISPR Tools

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Abstract

Background—One approach for a functional HIV cure is to prevent transcription from integrated proviral DNA. A critical step in HIV transcription is the Tat protein interaction with the TAR element viral RNA. We tested the strategy of blocking this Tat-TAR interaction in the SIVmac model.

Methods—We designed five CRISPR short guiding RNAs (sgRNAs) targeting the SIVmac TAR element, along with inactive versions of Cas9 (dCas9). These sgRNA constructs were delivered as ribonucleoproteins or plasmid DNA, along with SIV DNA. The constructs were also tested in integrated viral DNA in a cell line chronically infected by SIV.

Results—The sgRNAs targeting the coding strand of the TAR element inhibited SIV RNA transcription in association with dCas9-KRAB, but not with dCas9.

Conclusions—Induction of epigenetic modifications may be more effective in inactivating provirus than transcriptional interference, and thus may be a better strategy to achieve a functional cure *in vivo*.

Keywords

Epigenetic Editing; Dead Cas9; KRAB

INTRODUCTION

Current combined antiretroviral therapy (cART) effectively reduces HIV loads in patients to undetectable levels and prevents disease progression. However, cART does not affect the reservoir of latently infected cells, which is not sensitive to antivirals and not detected by the

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ETHICS STATEMENT

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to. Ethical approval was not required because no animals were used for research in this study.

CONFLICT OF INTEREST STATEMENT

The authors have no conflicts of interest to declare.

immune system, leading in most cases to virus rebound from this reservoir when therapy is interrupted. One strategy to potentially achieve a functional cure is the “block and lock” approach, which aims at locking out proviruses in a deep latency that prevents viral reactivation by inhibiting viral transcription[1,2], thereby suppressing the residual viremia arising from reactivation of latently infected cells. However, HIV transcription is a complex process regulated at the levels of chromatin organization, transcription initiation, polymerase recruitment, and transcription elongation. The first nucleotides of the HIV-1 nascent RNA constitute the trans-activation response (TAR) element, which forms a single stem loop structure, whereas for HIV-2 and SIVmac the TAR element forms a complex, multi-stem loop structure[3]. The viral Trans-Activator of Transcription (Tat) enters the nucleus and binds to TAR RNA along with the positive transcription elongation factor b (P-TEFb), and this orchestrates the assembly of the Super Elongation Complex (SEC), consisting of additional transcriptional elongation factors and scaffold proteins[4], leading to inhibition of RNA polymerase II (RNAPII) regulatory subunits and hyperphosphorylation of RNAPII, thus allowing for enhanced viral transcription elongation by RNPII. In the absence of Tat, RNAPII only transcribes randomly terminated transcripts, making Tat an indispensable viral protein and a target for drug development.

Newly genome-editing strategies also offer the potential of curing individuals of HIV by removing or inactivating the integrated proviral DNA from infected cells. Of these, CRISPR/Cas system tools have been shown to be the most flexible and amenable to modifications[5]. Mutation of the two catalytic sites of Cas9 nuclease yielded an inactive Cas9 that retained the guiding (g)RNA-mediated specific DNA-binding affinity; this “dead” Cas9 (dCas9) variant has been used to repress the expression of targeted genes by interfering with transcriptional elongation, RNA polymerase binding or transcription factor binding[6,7], a phenomenon known as CRISPR interference (CRISPRi). Further improvement in transcriptional inhibition was achieved with the addition to dCas9 of repression domains such as the Krüppel-associated box (KRAB), with the resultant dCas9–KRAB fusion protein being the current gold standard for dCas9-based transcription repression studies[6,8,9]. The KRAB domain represses transcription via interaction with the KAP1 protein, which functions as a scaffold to recruit co-repressors including heterochromatin protein 1 (HP1), histone deacetylases, and the histone methyltransferase SETDB1[9]; DNA methylation of upstream promotor sequences silences gene transcription, and deacetylation and methylation of histones at promoter sites also inactivate genes. These modifications of DNA and histones that change gene expression without changing the gene sequence are known as epigenetic modifications.

Here we present an approach that combines a “block and lock” strategy with dCas9 variants that induce CRISPRi or epigenetic editing of integrated SIV provirus. In total, five short guiding RNAs (sgRNA) targeting the proviral TAR element of SIV were designed: TAR1, TAR4, and TAR5 sgRNAs target the sense strand of the TAR element, and TAR2 and TAR3 sgRNAs target the antisense strand. We investigated the ability of these TAR-targeting sgRNA to prevent transcription of the SIV provirus via CRISPRi and epigenetic editing utilizing dCas9 and dCas9-KRAB, respectively. We demonstrate that epigenetic editing results in sustained inhibition.

MATERIALS AND METHODS

Design of SIV TAR-specific short guiding RNAs

Conserved regions of the SIV proviral DNA were identified by aligning in Geneious R7 the following SIV_{mac251} lineage viruses[10]: SIVMM251 (accession M19499), SIVMM239 (accession M33262), SIV1A11AA (accession M76764), and SIVMM32H (accession D01065). The TAR element region (nt 500–800 of the SIVmac sequence) was screened to find potential truncated short guiding RNA (sgRNA) targets, 17 nucleotides in length to enhance specificity[11]. Once candidate protospacer targets were found in these highly conserved SIV regions, the sequences were searched in both the human and rhesus macaque genomes (BLAST, GenBank NCBI NIH) to find potential cleavage sites that might target the host genome. Blast parameters in GenBank NCBI NIH Nucleotide Blast "blastn suite" consisted of the full 17 nucleotide protospacer sequence plus the four potential protospacer adjacent motif (PAM) sites that would allow for the Cas9 nuclease of *Streptococcus pyogenes* cleavage (AGG, CGG, GGG, and TGG). Therefore, for each potential protospacer sequence, eight total BLAST searches were made: four potential PAM sequences for each protospacer sequence in both human and rhesus genomes. Searching for potential off-target cleavage events in the human genome, sequences were blasted against "Human genomic plus transcript (Human G+T)" in the database "GPIPE/9606/current/all_top_level (Homo sapiens GRCh38.p12 [GCF_000001405.38]) chromosomes plus unplaced and unlocalized scaffolds (reference assembly in Annotation Release 109)" and "GPIPE/9606/current/rna (Homo sapiens Annotation Release 109 RNAs)" which are the human genome and human transcriptome, respectively.[12] Because of the short 20 nucleotide sequence imputed, the search parameters were adjusted to search for a shorter input sequence automatically by "blastn suite." The BLAST conditions were the same for rhesus macaques as for humans, but using the "rhesus macaque (taxid:9544)" database. If any of the protospacer with PAM sequences hit a 100% match in either the human or rhesus genomes, that candidate protospacer target was eliminated from the potential SIV proviral target. Five sgRNAs were found to have a SIV-specific target to the SIV_{mac} TAR element (TAR1, TAR2, TAR3, TAR4, and TAR5). The oligonucleotide sequences we designed to generate these sgRNAs are listed in Table 1.

Generation of plasmids encoding Cas9 variants and sgRNA

Top and bottom protospacer oligonucleotides (Table 1) were resuspended to 100 μM in nuclease-free water, and pairs were phosphorylated with T4 polynucleotide kinase and annealed together. Double-stranded oligonucleotides were ligated into BbsI digested and Antarctic phosphatase-treated pSpCas9(BB)-2A-GFP (pX458, Addgene plasmid # 48138), gifts from Feng Zhang[13]. Briefly, this plasmid encodes the chimeric sgRNA driven by the RNA polymerase III human U6 promoter and the SpCas9 fused to enhanced green fluorescent protein (GFP) with a 2A self-cleaving peptide sequence, driven by the RNA polymerase II chicken beta-actin hybrid (CBh) promoter. Unmodified pX458 was used as a control expressing empty sgRNA and Cas9. Plasmids that contained protospacer sequences were verified by Sanger sequencing. In order to generate plasmids that encode the catalytically inactive "dead" SpCas9 (dCas9, with D10A and H840A inactivating mutations), the dCas DNA sequence from pX330A_dCas9–1×6 (Addgene plasmid #63600),

a gift from Feng Zhang[14] was cloned into pX458 using AgeI and PmlI restriction endonucleases for a plasmid we denote as dCas9-pX458.

In order to make lentiviral particles for the transduction of lymphoid cells to express dCas9-KRAB, pLV hU6-sgRNA hUbC-dCas9-KRAB-T2a-Puro (pLV) was used as the lentiviral vector, a gift from Charles Gersbach (Addgene plasmid # 71236)[15]. The phosphorylated, annealed protospacer oligonucleotides for TAR1, TAR2, TAR3, TAR4, and TAR5 were ligated into BsmBI digested pLV plasmid using T4 DNA ligase. To make lentiviral particles for the expression of dCas9 only, CSII-U6-gRNA-CBh-3XFLAG-PA-dCas9-P2A-Puro (CSII) was used as the lentiviral vector, a gift from Tohru Kimura (Addgene plasmid # 83306). The phosphorylated, annealed protospacer oligonucleotides for TAR1 was ligated into BbsI digested CSII plasmid. Plasmids were digested with BsmBI to look for protospacer insertion, as inserted protospacers have lost the BsmBI recognition sites and will not linearize. Unmodified pLV and CSII were used as controls as they expressed an empty gRNA.

Cell lines

Human embryonic kidney cell line 293T/17 (HEK293T, ATCC CRL-11268) was maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum (FBS), 2 mM GlutaMAX (Life Technologies), 25 mM HEPES, 1 mM Sodium Pyruvate, and 1X nonessential amino acids (NEAA) at 37°C with 5% CO_2 incubation.

CEM.NK^R-CCR5 cells were obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID (catalog number 4376) deposited by Dr. Alexandra Trkola[16–18]. CEM.NK^R-CCR5 cells were infected with SIVmac239 at a MOI of 0.1 using ViroMag R/L magnetofection (OZ Biosciences). Infected CEM.NK^R-CCR5 cells were isolated from uninfected cells by sorting CD4 negative (CD4$^-$) and CCR5 negative (CCR5$^-$) cells. The double negative CD4 and CCR5 cells were serially diluted to isolate single cell clones with a limiting dilution assay. One clone, 2C11, was found to produce high amounts of SIV Gag p27 and did not express CD4 or CCR5. 2C11 cells were maintained in Roswell Park Memorial Institute 1640 medium (RPMI) supplemented with 10% FBS, 2 mM GlutaMAX (Life Technologies), 100 U/mL penicillin, 100 μg/mL streptomycin, 25 mM HEPES, 1X NEAA (RPMI-10) at 37°C with 5% CO_2 incubation.

Ribonucleoprotein Transfections

Purified Cas9 fused with a nuclear localization signal (Cas9-NLS, Cat. No. Cas9-NLS-50) and dCas9 fused with a nuclear localization signal (Cas9-Dead-NLS, Cat. No. Cas9d-NLS-50) were purchased from Eupheria Biotech. Synthetic sgRNA with 2'-O-methyl 3' phosphorothioate modifications of the first and last three nucleotides were purchased from Synthego and resuspended to 3 μM per manufacture's recommended protocol (CRISPRevolution sgRNA EZ kit). To generate ribonucleoproteins, the Cas9 and dCas9 proteins were complexed with the synthetic sgRNA for 10 minutes at room temperature in Opti-MEM™ I Reduced serum medium (Gibco™). HEK293T cells were seeded onto 24-well plates (Corning) at a density of 1.5×10^5 cells/well, 16–24 hours prior to transfection. Cells were transfected using Transit-X2 (Mirus) at 70–80% confluency following

manufacture's recommended protocol. A total of 250 ng of pMA239 (a plasmid that contains the entire $SIV_{mac}239$ proviral genome), 290 ng of pmaxGFP (a plasmid that expresses GFP, Lonza), and freshly prepared RNP made of 6 nM sgRNA and 1 nM of Cas9 or dCas9 were transfected into each well. Supernatant was collected 24-hours post-transfection while cells were harvested for flow cytometry analysis.

Plasmid Transfections

HEK293T cells were seeded onto 6-well plates (Corning) at a density of 7×10^5 cells/well 16–24 hours prior to transfection. Cells were transfected with plasmids at 70–80% confluence using Transit-LT1 (Mirus) following manufacture's recommended protocol. A total of 500 ng of pMA239 and 2 μg of pX458 or dead-pX458 plasmid DNA were transfected per well. Supernatant was collected 24-hours post-transfection and cells were harvested for flow cytometry analysis or cell sorting based on eGFP expression.

The 2C11 cells were electroporated for plasmid transfection. Four to eight million 2C11 cells were resuspended at a concentration of one million cells per 50–100 uL 1SM buffer[19] (5 mM KCl; 15 mM $MgCl_2$; 120 mM NaH_2HPO_4/Na_2HPO_4 pH 7.2; 25 mM Mannitol, and 25 mM Sodium Succinate). Cells were mixed with 5 μg of plasmid DNA (encoding gRNA and Cas9, dCas9, or dCas9-KRAB) in an electroporation cuvette, and nucleofected in the Nucleofector 2b device (Lonza) using program X-001. Cells were sorted based on eGFP expression in a FACS Aria III instrument (BD Biosciences).

Transduction of 2C11 cells with lentiviral vectors encoding TAR sgRNA and dCas9 or dCas9-KRAB

The pLV and CSII plasmids were transfected into HEK293T cells to generate lentivirus particles. Cells were seeded onto 6-well plates (Corning) at a density of 7×10^5 cells/well 16–24 hours prior to transfection. Cells were transfected with either one of these plasmids, along with plasmids pREV, pGAG, and pVSV-G (System Biosciences, SBI, catalog number CD500 series). Supernatant was collected 48-hours post-transfection and centrifuged at 300 x g for five minutes to clarify supernatant, and then filtered through a 0.45 μm PVDF membrane and froze at −80°C. Cells were harvested for intracellular staining to detect HIV p24 Gag capsid protein using flow cytometry analysis to assess transfection efficiency. Lentivirus particles in supernatant were pelleted through a 10% sucrose cushion using an ultracentrifuge at 10,000 x g for 4 hours at 4°C, stopped with a low break. After centrifugation, the supernatant was carefully decanted and a total of 100 μL sterile 1X PBS was added to the viral pellet, tubes were wrapped in parafilm and placed overnight at 4°C to fully resuspend pellet. The resuspend pellet was transferred to a sterile cryovial and froze at −80°C.

2C11 cells were transduced by the pLV or CSII lentivirus particles (generated from HEK293T cells) using ViroMag R/L magnetofection (OZ Biosciences). Per lentivirus condition, 5×10^5 2C11 cells were pelleted via centrifugation at 300 x g for 5 min, liquid was removed by vacuum as to not resuspend the cell pellet, and the lentivirus preparation was gently pipetted on top of the cells. Tubes were placed on a rack with magnet underneath and were placed in a 37°C incubator for 2 hours. Cells were resuspended in up to 0.5 ml of

warmed RPMI-10 (complete with pen/strep) and transferred to a 24-well plate. The plate was incubated at 37°C for 48 hours, at which time the entire medium was replaced with RPMI-10 (complete with pen/strep) supplemented with puromycin for selection of transduced cells. The entire medium was replaced with fresh RPMI-10 with puromycin twice a week for 7–10 days. After 7–10 days of puromycin selection, cells were grown in RPMI-10. Transduced cells were surface and intracellularly stained to detect CD4 and SIV p27 Gag expression using flow cytometry analysis.

Flow cytometry

HEK293T cells were harvested 24-hours post-transfection via trypsinization followed by a wash with DMEM complete medium. Cells were fixed in 1X BD FACS Lysing solution (BD Biosciences), washed with 1X PBS + 1% BSA (washing buffer), and permeabilized with 1X BD FACS Permeabilizing Solution 2 (BD Biosciences) as per manufacture's recommended protocol. Cells were intracellularly stained by incubating with anti-SIV Gag p27 antibody (clone 55–2F12, NIH AIDS Reagent Program) conjugated with Dylight633 at room temperature for one hour. Cells were then washed twice with washing buffer and then fixed with 1X PBS containing 1.6% methanol-free formaldehyde (Polysciences) and analyzed on a three-laser CyAn ADP (Beckman-Coulter) and data was evaluated on FlowJo version 10 software. HEK293T cells transfected with lentiviral vectors were harvested for intracellular staining similarly described above, except an anti-HIV Gag 24 FITC-conjugated antibody was used (clone FH190–1-1, Beckman Coulter, catalog number KC57-FITC).

Electroporated 2C11 cells were harvested day 15 or 17 of culture post-sorting for surface and intracellular staining. Cells were washed with cold 1X PBS and resuspended in 100 μL cold RPMI-10. Cells were surface stained with anti-CD4 PerCP-Cy5.5 (clone L200, BD-Biosciences) antibody for 30–60 minutes at 4°C in the dark. Cells were then washed with washing buffer and intracellularly stained with anti-SIV Gag p27 Dylight633 (clone 55–2F12, NIH AIDS Reagent Program). Transduced 2C11 cells with pLV and CSII lentiviral particles were surface and intracellularly stained as described above.

Luminex assays

Virus production was determined by quantifying SIV Gag p27 from cell culture supernatants as described in Obregon-Perko *et al* 2019[20]. The levels of SIV p27 from the supernatants were calculated by generating a standard curve from a SIVmac239 viral stock of known concentration.

RESULTS

Characterization of SIV TAR-specific sgRNAs

The TAR element located in the 5' LTR R region of SIV is critical for initiating transcription of the entire viral RNA, which makes it a desirable target of CRISPR. Five sgRNAs targeting the TAR element were designed: TAR1, TAR4, and TAR5 sgRNAs target the sense strand of the TAR element, while TAR2 and TAR3 sgRNAs target the antisense strand (Figure 1A). TAR1 protospacer binds the TAR element DNA sequence that corresponds to

the first stem loop structure of the transcribed RNA, TAR4 protospacer binds the second stem loop, and TAR5 binds the second and third stem loop (Figure 1B).

We set out to test in HEK293T cells the efficiency of viral inhibition between two different transient transfection assays, ribonucleoprotein (RNP) and plasmid transfections, co-transfecting the plasmid pMA239, which contains the complete SIVmac239 proviral DNA and serves as the target for the sgRNAs and Cas variants; another benefit of this setting was that HEK cells lack expression of CD4 and CCR5, thus preventing the newly produced SIV from initiating new rounds of viral infection and replication. Virus inhibition was assessed by quantification of SIV p27 protein released into the supernatant. HEK293T cells transfected with plasmids encoding Cas9 nuclease and TAR1, TAR2, TAR3, TAR4, and TAR5 sgRNAs produced significant less SIV than cells transfected with empty guided Cas9 nuclease, compared to cells transfected with a plasmid co-expressing Cas9 and no gRNA (Figure 2A). This degree of SIV inhibition was more dramatically seen when presented as % inhibition over the control with Cas9 and no gRNA, where it can be seen that Cas9 in association with TAR1, TAR3, TAR4, and TAR5 gRNAs inhibited more than 98% of virus production (Figure 2B). Similarly, HEK293T cells transfected with plasmids encoding dCas9 and TAR1, TAR3, TAR4, and TAR5 suppressed SIV production, with TAR 1 and TAR5 having the most dramatic inhibition (Figure 2). Remarkably, when using plasmid transfections, TAR1 and TAR5 suppressed virus production when complexed with dCas9 as much as when complexed with Cas9 nuclease (Figure 2A), which was not the case for TAR2, TAR3, and TAR4; the latter showed a 2-log higher concentration of SIV when associated with dCas9 (Figure 2A).

RNP transfections has the advantage of being a rapid methodology to assess the effects of CRISPR constructs without the need for tedious molecular cloning. Purified Cas9 and dCas9 were purchased (Eupheria Biotech) and complexed with stable, synthesized sgRNA and co-transfected into HEK293T cells, along with plasmids pMA239 and pmaxGFP; the plasmid pmaxGFP, which expresses GFP, was used in this assay to assess transfection efficiency. Production of SIV antigens was similar when pMA239 and pMaxGFP were transfected by themselves (Figure 3A), with the addition of purified Cas9 nuclease (Figure 3B), or with the addition of purified dCas9 (Figure 3C); the same was observed for transfections of pMA239 and pmaxGFP along with TAR1 sgRNA, but in the absence of any Cas9 protein (Figure 3F). Finally, transfection of HEK293T cells with RNP consisting of TAR1 sgRNA and purified Cas9 (Figure 3D) or dCas9 (Figure 3E) resulted in complete inhibition of SIV expression. Similarly, HEK293T cells transfected with TAR1, TAR4 or TAR5 sgRNA complexed with Cas9 nuclease resulted in less SIV p27 protein detected in culture supernatants (Figure 4A); however, when part of an RNP with dCas9, only TAR1 gRNA was able to suppress SIV p27 production (Figure 4A, 4B); TAR4 and TAR5 dCas9 RNP, although targeting the TAR element on the same strand, had much lower levels of inhibition.

In summary, the five TAR-specific sgRNAs that we designed were effective when combined with the Cas9 nuclease, with the three sgRNAs that targeted the coding strand (TAR1, TAR4, and TAR5) showing higher inhibitory effect than the two sgRNAs that targeted the opposite strand (TAR2, and TAR3). Additionally, RNP and plasmid transfections in our HEK293T co-transfection assays had different outcomes on virus production when

comparing Cas9 and dCas9. TAR1 and TAR5 sgRNAs had similar transient inhibitory effects on SIV production, either in combination with Cas9 or dCas9, only when co-expressed from a single plasmid; TAR4 was consistently less effective when delivered as part of an RNP, with either Cas9 or dCas9. The ineffectiveness of dCas9 RNP to inhibit SIV, while plasmid encoded dCas9 could, may be due to the fact that plasmid encoded sgRNA and Cas9 or dCas9 are constitutively expressed in the cells during the 24-hour transfection as opposed to a fixed bolus of RNP that is transfected into the cells and is not replenished.

Testing of TAR gRNAs in 2C11 cells to silence proviral DNA

Since we demonstrated in HEK293T cells that plasmids co-expressing SIV TAR-specific sgRNAs in association with dCas9 were able to inhibit transiently SIV RNA transcription and expression from plasmid DNA (pMA239), we investigated the ability of these same TAR-specific sgRNAs and dCas9 plasmids to suppress virus production from an integrated SIV DNA in a chronically infected cell line. We also expanded the scope of our study by exploring the potential viral inhibitory capacity of targeted epigenetic changes induced by plasmids co-expressing the same TAR-specific sgRNAs along with dCas9-KRAB fusion protein. For all these purposes we used 2C11 cells, a cell line derived from CEM.NK^{R}-CCR5^{+} cells infected with SIVmac239 that is predominantly CD4 negative and produces abundant SIV particles.

In a first approach, 2C11 cells were electroporated with plasmid encoding TAR1 sgRNA with either Cas9 or dCas9; electroporated cells were sorted for GFP expression 24 hours after transfection and then cultured for 15–17 days. 2C11 cells electroporated with empty sgRNA and Cas9 and then sorted and cultured showed an increase in SIV Gag p27 concentration in supernatant cultures for 15 days post-nucleofection (Figure 5). In contrast, 2C11 cells electroporated with TAR1 Cas9 or TAR1 dCas9-KRAB maintained relatively stable SIV Gag p27 concentrations in supernatant cultures over 15–17 days. Absence of detectable GFP expression after 3 days post-sorting suggested that electroporated plasmids were no longer found in 2C11 cells, most likely due to dilution of the plasmid DNA through replication cycles or intracellular degradation (data not shown). Therefore, the effects of TAR1 sgRNA with Cas9 and dCas9-KRAB occurred early in the 24-hour period post electroporation and first few days post-sorting, and these effects were maintained through cycles of cell replication for the remainder of the culture timepoints. On the contrary, 2C11 cells electroporated with the TAR1 dCas9 plasmid had no inhibition of SIV Gag p27 in supernatant cultures and instead had a dramatic accumulation of SIV Gag p27 in supernatant cultures (Figure 5).These data indicated a more effective virus suppression by epigenetic modifications mediated by dCas9-KRAB than CRISPRi mediate by dCas9.

Lentiviral Transductions of 2C11 Cells for Epigenetic Editing of Provirus

In order to assure that both TAR-specific sgRNAs and dCas9-KRAB were expressed in a more durable manner through several rounds of cell replication, we transduced 2C11 cells with lentivirus vectors co-expressing dCas9-KRAB with each of the five TAR sgRNAs. 2C11 cells transduced with the lentiviral vectors were selected in puromycin, and SIV transcription inhibition was examined by measuring upregulation of CD4 expression by surface staining, and measuring capsid production via p27 intracellular staining. Compared

to 2C11 cells transduced with lentiviral vectors expressing dCas9-KRAB and no sgRNA (Figure 6A), vectors co-expressing TAR1, TAR4, and TAR5 showed a significant reduction in the percentage of cells expressing SIV p27 and a concomitant recovery in the percentage of CD4 expressing cells (Figure 6B, E, and F); 2C11 cells transduced with dCas9-KRAB and TAR2 or TAR3 had much lower percentages of $CD4^+$ $SIVp27^-$ cells, but still had some inhibitory effect on SIV, particularly TAR3 (Figure 6C and D). Since the 2C11 cells transduced with dCas9-KRAB and TAR1, TAR4, and TAR5 sgRNA had a higher percentage of CD4 expression that inversely correlated with p27 expression, $CD4^+$ cells were sorted and cultured to determine viral production and compare virus production to their matched unsorted cells (Figure 6G). Cells were passaged four times after the initial antibody staining; with these additional passages, these cells had an even higher percentage of cells expressing CD4 from 93.0% to 96.0% for TAR1, 90.2% to 94.5% for TAR4, and 88.7% to 89.8% for TAR5. These data suggested that the dCas9-KRAB constructs continued to inhibit transcription from SIV proviral DNA throughout continuous culturing.

We also made a lentiviral vector co-expressing dCas9 and TAR1 sgRNA, which was used to transduce 2C11 cells and sort stably transduced cells. These cells were kept in culture along with the cell lines derived from the lentiviral vectors expressing dCas9-KRAB and TAR-specific sgRNAs. Flow cytometry analysis of these cell lines showed that 2C11 cells transduced with TAR1 dCas9 had levels of $CD4^+$ $SIVp27^-$ cells similar to 2C11s transduced with empty sgRNA and dCas9 controls (Figure 7) suggesting that even continuous expression of TAR1 sgRNA and dCas9 did not result in sustained inhibition of SIV transcription.

DISCUSSION

This work is part of a larger project aimed at developing a translatable, proof-of-concept cure strategy for HIV that utilizes highly specific CRISPR/dead Cas9 reagents to inactivate virus replication in both latently and actively infected cells using a “block and lock” approach, whereby viral transcription is “blocked”, “locking” proviruses in deep latency[1,2]. Here we target a critical step in HIV transcription, i.e. the interaction of the Tat protein with the TAR element in the nascent viral RNA. We examined the use of TAR specific dCas9 and dCas9-KRAB to inhibit SIV transcription of the nascent TAR element viral RNA via CRISPRi or epigenetic editing, respectively, in order to block a critical step for full-length transcription of proviral DNA.

Initially we tested RNP and plasmid delivery of sgRNAs that targeted different areas of the SIV proviral genome that coded for the TAR element (Figure 1) along with Cas9 nuclease or dCas9 in HEK293T cells to measure transient inhibition of virus production. When transfecting plasmid DNA, the sgRNA targeting the proviral DNA coding strand (TAR1, 4, and 5) showed higher degrees of viral inhibition compared to the sgRNAs that targeted the non-coding strand (TAR2 and 3; Figure 2). In general, for almost all the TAR-specific sgRNAs, the Cas9 nuclease was more effective than dCas9, except for TAR5 that had similar activities with either version of Cas9. However, when using RNPs for TAR1, 4, and 5, only TAR1 sgRNA with dCas9 could suppress virus production in HEK293T cells (Figures 3 and 4). These data suggest TAR1 sgRNA, which binds to the TAR element region coding for

loop 1, targets an important component of the TAR element that can be disrupted just by dCas9 binding the DNA via CRISPRi. The inhibitory effect of dCas9 with TAR4 and TAR5, observed with plasmid DNA delivery was not reproduced by the RNPs, which suggests that there is an advantage to plasmid transfections over RNP transfections in HEK293Ts, and that this advantage may have implications for *in vivo* delivery of CRIPSR/dCas9 reagents.

In order for CRISPRi or epigenetic editing to be successful in inhibiting SIV replication *in vivo*, these molecules first must be able to target provirus integrated into host cell chromosomes of lymphoid cells. Therefore, we used a lymphoblastoid cell line chronically infected with SIVmac239 (2C11) developed in the laboratory that has a single integrated provirus (Smith, L.M. et al, manuscript in preparation), and electroporated it with plasmid DNAs coding for TAR1 sgRNA in combination with Cas9, dCas9, or dCas9-KRAB (Figure 5). For these cells, evidence for the presence of the introduced DNA was lost after a few days in culture, and only TAR1 sgRNA with Cas9 and dCas9-KRAB inhibited viral transcription for more than 2 weeks in culture. This suggests that Cas9, which introduces indels in the proviral DNA[21], and dCas9-KRAB, which represses transcription by inducing epigenetic modification in the DNA[22], were able to act early after transfection and induce changes that remained in place through continued cell division. On the other hand, inhibition of transcription induced by dCas9 and TAR1 sgRNA was lost after cell division, along with dilution or degradation of the plasmid DNA coding for these molecules.

Since plasmid-derived expression of TAR-specific sgRNAs and Cas9 variants is of a transient nature in dividing cells such as 2C11, we developed lentiviral vectors to study the long-term SIV inhibitory effect of these molecules. 2C11 cells transduced with lentiviral vectors expressing TAR1, TAR4, or TAR5 sgRNAs and dCas9-KRAB were able to inhibit SIV transcription, which was documented by reduction in SIV p27 and upregulation of CD4 (Figure 6). However, when we transduced 2C11 cells with TAR1 dCas9, there were no detectable changes in the percentage of cells expressing p27 or CD4. Thus, a more prolonged expression of dCas9 binding the TAR1 target site did not inhibited integrated provirus via CRISPRi. Altogether, these data suggest that the expected epigenetic modifications that are the result of KRAB-mediated histone modification[22] are more effective at inactivating integrated proviral DNA than CRISPRi, providing evidence that epigenetic editing may be a more effective approach over CRISPRi to inactive provirus *in vivo*.

In our experiments for inducing epigenetic modifications we utilized the KRAB domain, which when fused to dCas9[6,15] as well as to other DNA binding proteins such as zinc-finger nucleases (ZFNs)[23,24] and transcription activator-like effectors (TALEs)[25], is a transcriptional repressor known for robust, albeit transient, epigenetic modifications; however, other repressor domains could also be employed. For example, transcription repression by the basic region-helix-loop-helix-zipper (bHLHZip) protein Mad1 requires DNA binding as a ternary complex with Max and mSin3A or mSin3B, the mammalian orthologs of the Saccharomyces cerevisiae transcriptional corepressor SIN3; the transcriptional repressor domain of Mad1, mSin interaction domain (SID), recruits histone deacetylases 1 and 2 (HDAC 1/2) that remove activating histone acetylation[26] thereby modifying the epigenome through histone deacetylation. It has been shown that fusing both

SID and KRAB to dCas9 can induce highly specific gene silencing using a novel Enhancer-interference (Enhancer-i) technique[27]. Similarly, there are reports about the use of a combined dCas9 with a bipartite repressor made of KRAB and the repressor MeCP2 for improved gene silencing[8], which could also be tested. Therefore, combining two different mechanisms of epigenetic modifications for the purpose of transcription suppression may be an improved strategy in the implementation of the "block and lock" approach to provirus inactivation.

In future studies, it will be important to determine the type of histone modifications that are induced by each of the more effective sgRNAs in order to verify epigenetic editing. In addition, a further improvement in our strategy will be to utilize simultaneous expression of multiple sgRNAs against TAR to prevent escape mutants that may render a single sgRNA from binding to its target sequence. Viruses isolated *in vivo* are not clonal as in our model system of SIV_{mac239} infected T-lymphoblast cells, 2C11s, but are instead a diverse population that is changing over the course of infection[28]. Because a heterogeneous population of viruses poses a challenge for a single target CRISPR therapy, multiplexing sgRNAs may overcome this problem. Therefore, the strategy of using multiplex plasmids that target three conserved regions of the SIV provirus may reduce viral escape while enhancing virus inactivation.

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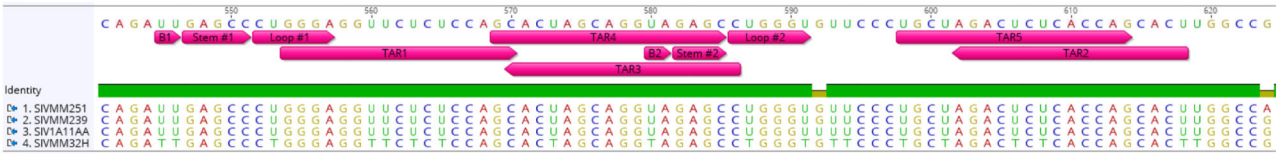
(A)



(B)

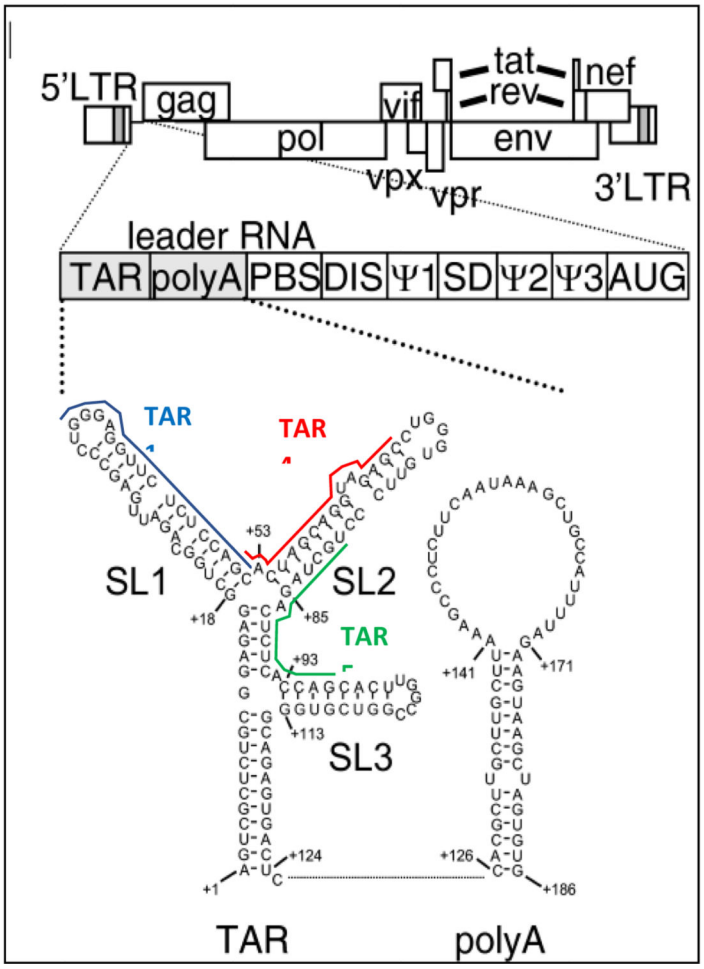


FIGURE 1.

Schematic representation of TAR sgRNA target sites in SIV_{mac} proviral genome. The TAR element in the R region of the LTR was targeted with TAR1, TAR2, TAR3, TAR4, and TAR5 sgRNA. A) Alignment of gRNA sequences to SIV_{mac} lineage sequences. TAR1, TAR4, and TAR5 sgRNAs target the sense strand of the TAR element, and TAR2 and TAR3 sgRNAs target the antisense strand. B1 and B2 annotate Bulge 1 and Bulge 2, respectively. B) TAR1, TAR4, and TAR5 protospacer binding regions of the SIV_{mac239} TAR Element represented on the transcribed RNA: blue line represents where TAR1 binds, red line is where TAR4 binds, and green line is where TAR5 binds. Adapted from Centlivre, M., Klaver, B., Berkhout, B., and Das, A. Functional analysis of the complex trans-activating

response element RNA structure in simian immunodeficiency virus. *Journal of Virology* **78**, 13522–13533 (2004).

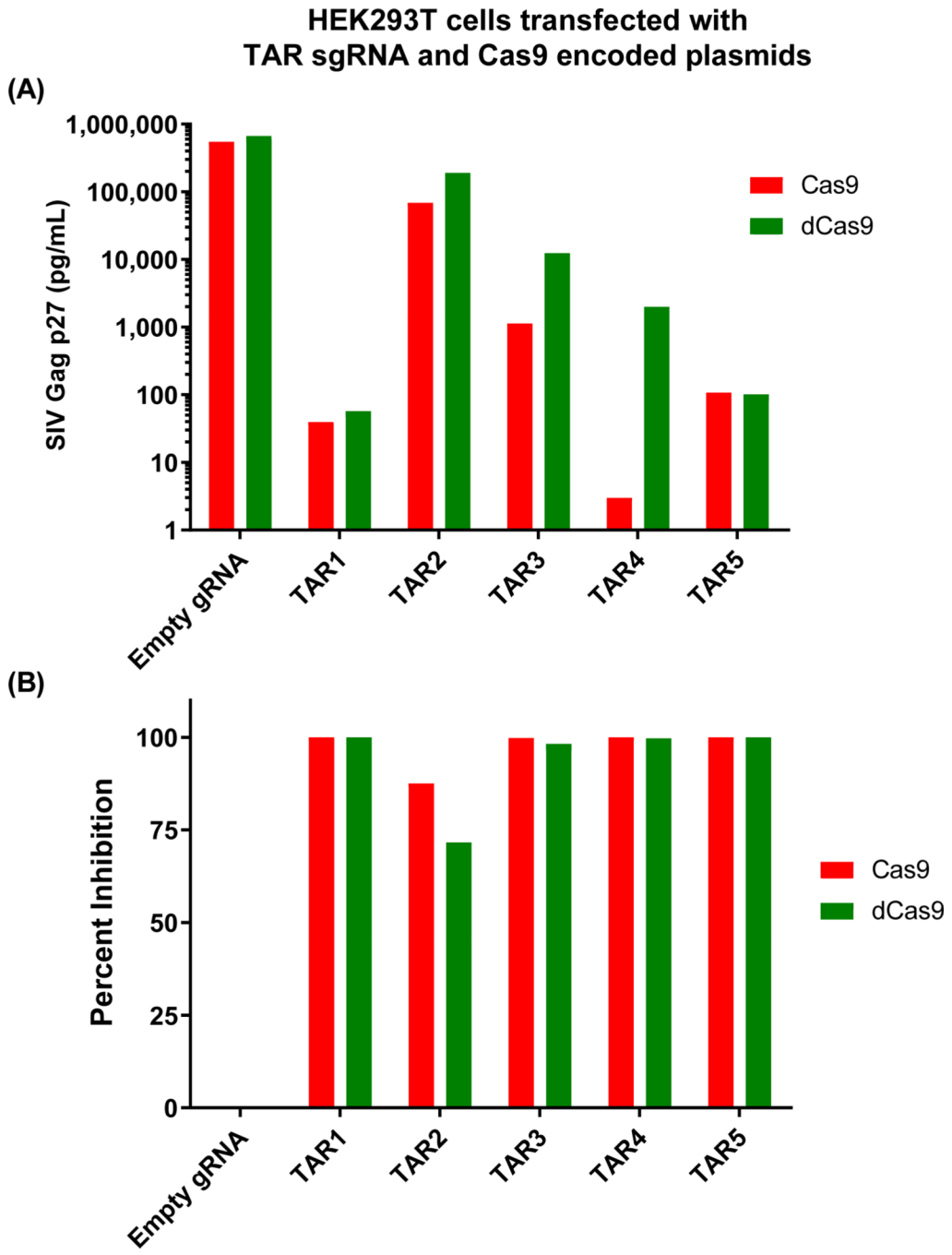


FIGURE 2. Transient Inhibition of SIV Production by Plasmids that Encode TAR-targeting sgRNAs and Cas9 variants.

HEK293T cells were co-transfected with pMA293 and plasmids encoding TAR sgRNA and either Cas9 nuclease or inactive dead Cas9. (A) Release of SIV virions after 24-hours post-transfection. Supernatant was harvested for Luminex assay to detect SIV Gag p27 in the supernatant. (B) Inhibition of SIV expressed as percentage of reduction in SIV p27 compared to plasmids that did not expressed any sgRNA.

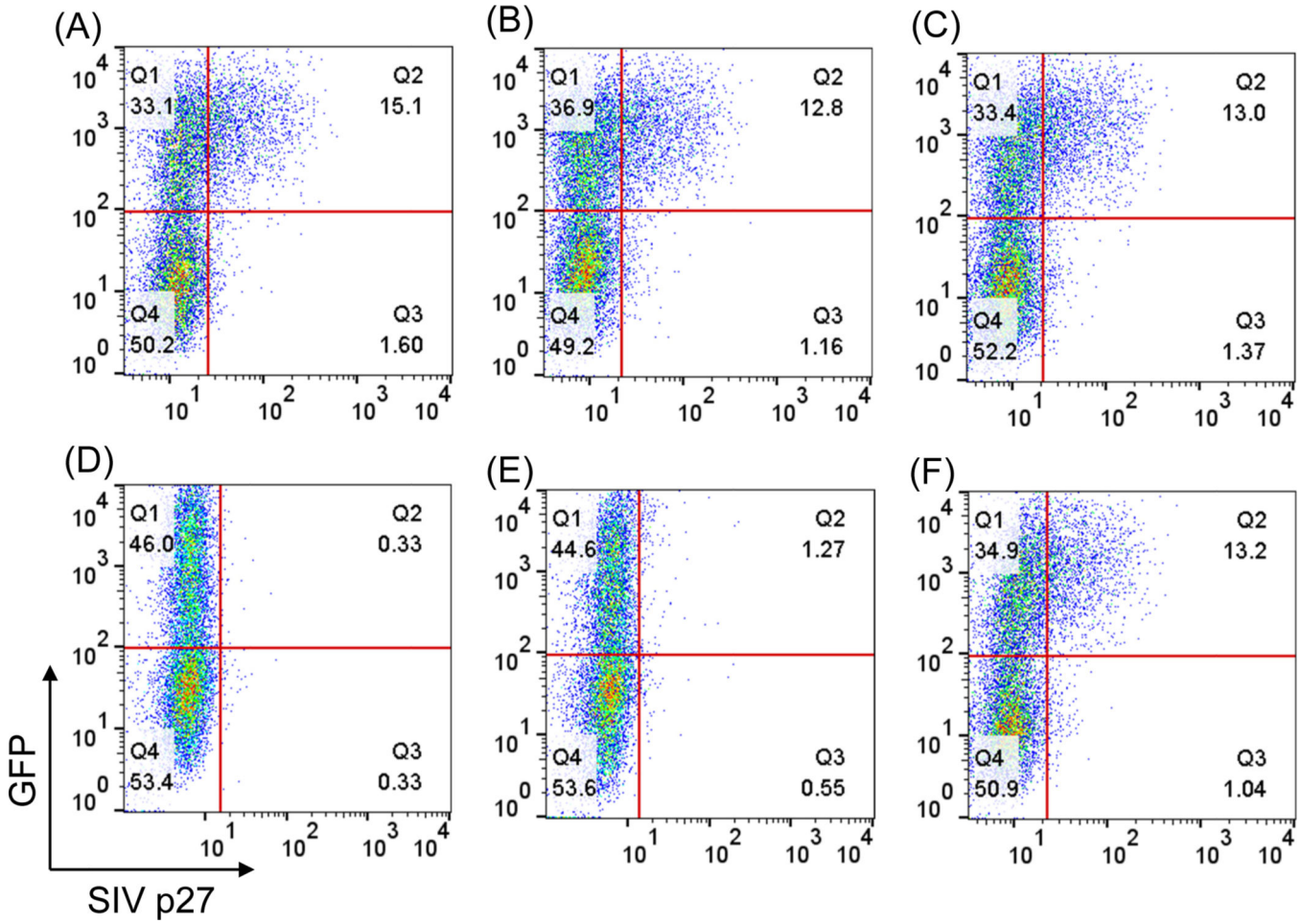


FIGURE 3. Transient Inhibition of SIV production by TAR-specific RNP.

.HEK293T cells were co-transfected with: (A) plasmids pMA293 and pmaxGFP alone; (B) pMA239 and pMax along with purified Cas9; (C) pMA239 and pMax along with purified dCas9; (D) pMA239 and pMax, along with RNP consisting of TAR1 sgRNA and Cas9; (E) pMA239 and pMax, along with RNP consisting of TAR1 sgRNA and dCas9; (F) pMA239 and pMax, along with TAR1 sgRNA alone. After 24-hours post-transfection, cells were harvested for detection of GFP and intracellular detection of SIV Gag p27.

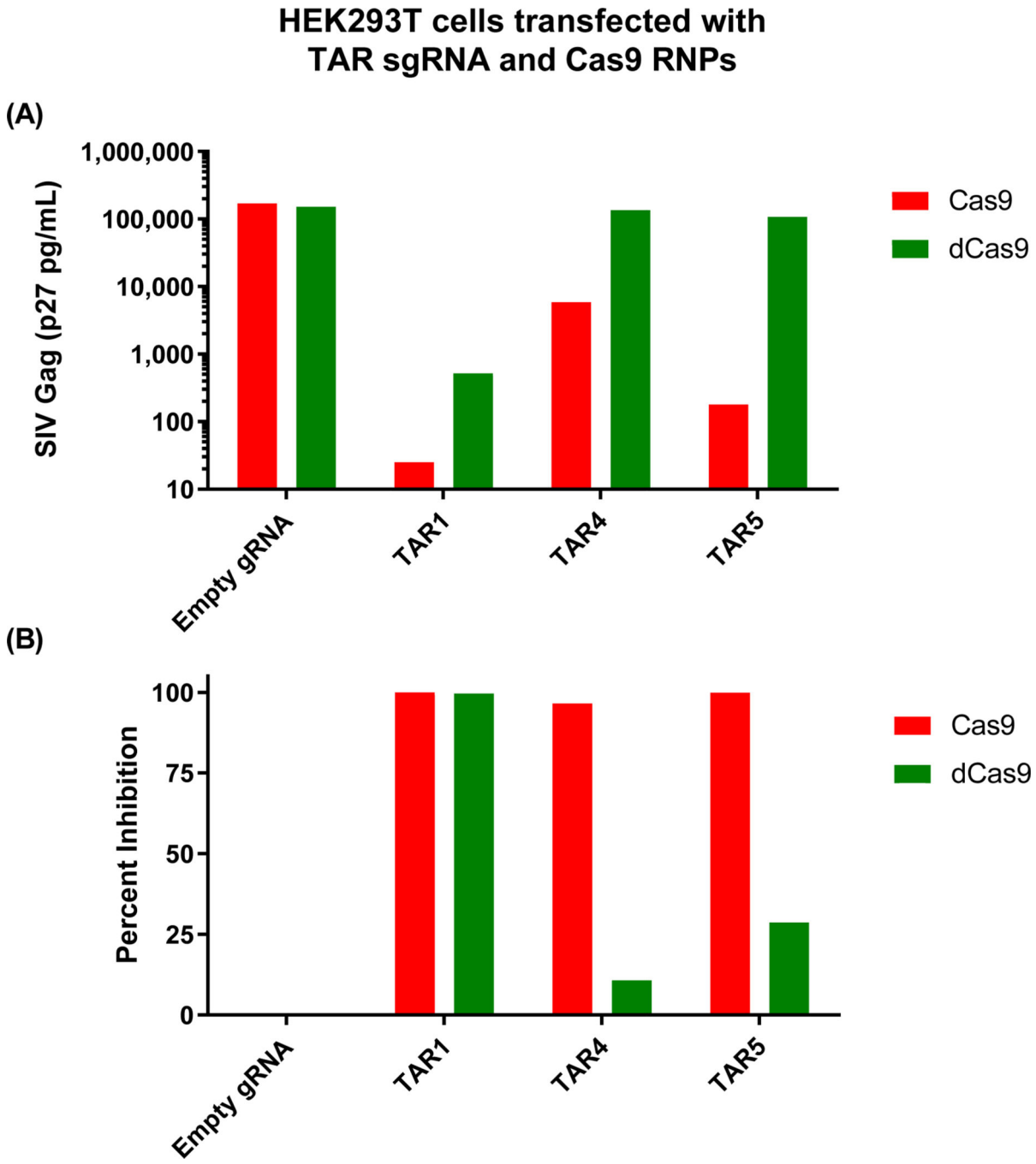


FIGURE 4. Transient Inhibition of SIV production by TAR-specific RNP.

HEK293T cells were co-transfected with plasmids pMA293, pmaxGFP, and reconstituted RNPs. (A) After 24-hours post-transfection, supernatants were harvested for a Luminex assay to detect SIV Gag p27. (B) Percent viral inhibition by each TAR-specific gRNA compared to SIV p27 content in the absence of gRNAs.

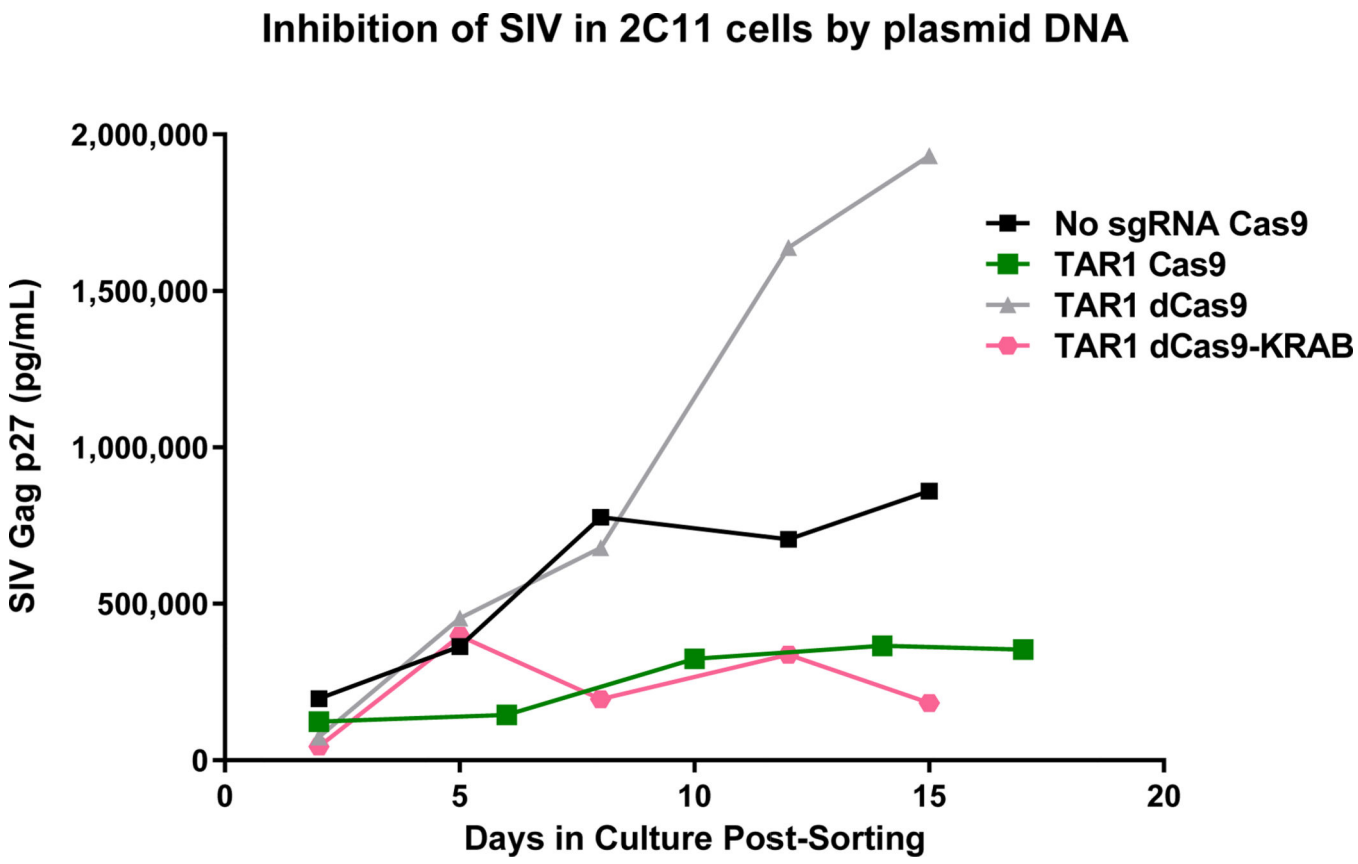


FIGURE 5. Inhibition of integrated SIV transcription with Plasmids that Encode TAR1 sgRNA and either Cas9, dCas9, or dCas9-KRAB.

2C11 cells were electroporated with plasmids and sorted 24-hours post-electroporation. Cells were cultured for 15 days adjusting twice a week the cell concentration at 1×10^6 cells/mL with fresh culture medium. SIV p27 was quantified by a Luminex assay.

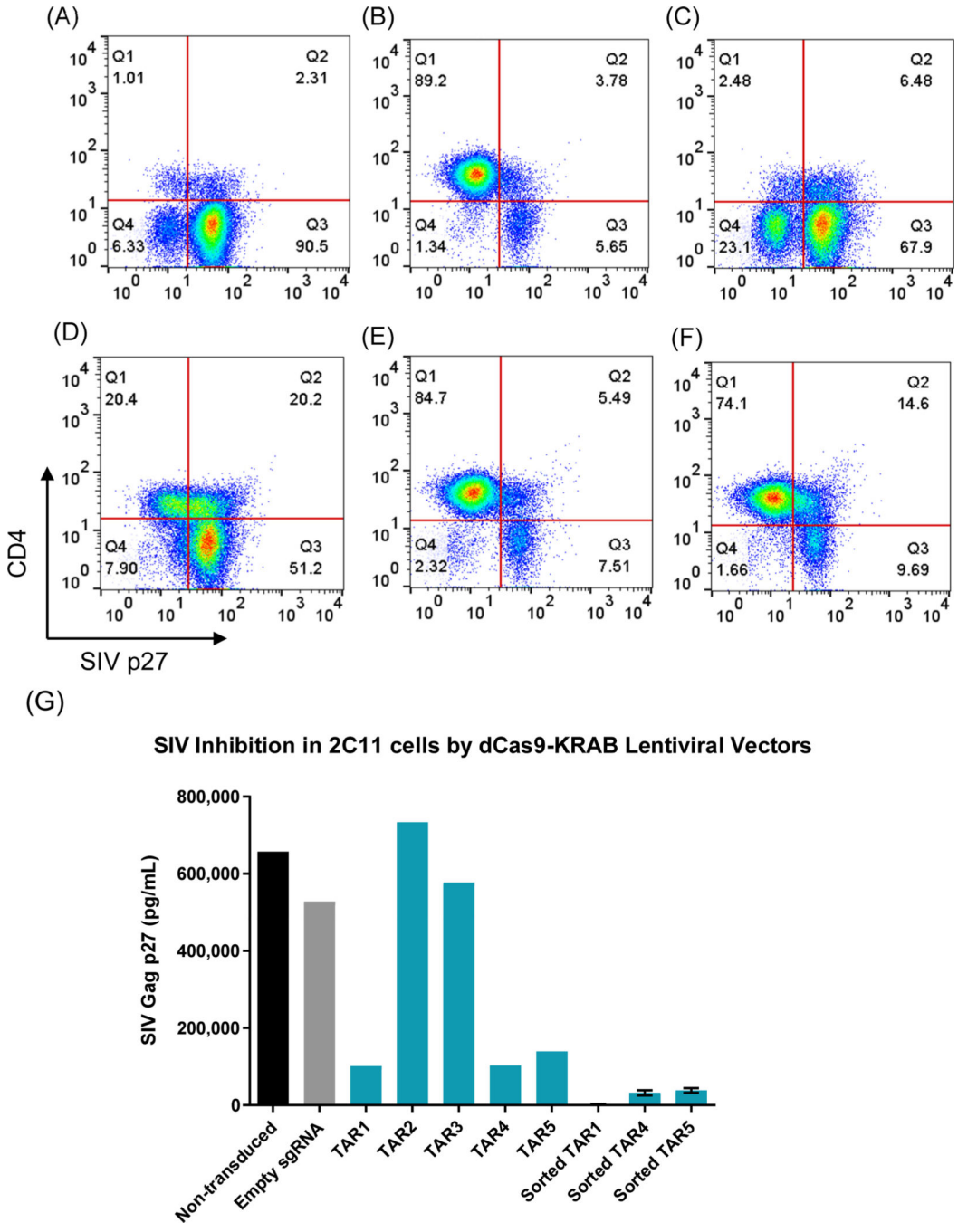


FIGURE 6. Inhibition of SIV in 2C11 Cells Transduced with lentiviral vectors co-expressing TAR-specific sgRNAs and dCas9-KRAB.

2C11 cells were transduced with lentiviral vectors co-expressing dCas9-KRAB and (A) no sgRNA; (B) TAR1 sgRNA; (C) TAR2 sgRNA; (D) TAR3 sgRNA; (E) TAR4 sgRNA; and (F) TAR5 sgRNA. Cells were stained for flow cytometry to assess surface CD4 and intracellular SIV p27 expression. (G) Release of SIV p27 in the supernatant of 2C11 cells transduced with lentiviral vectors.

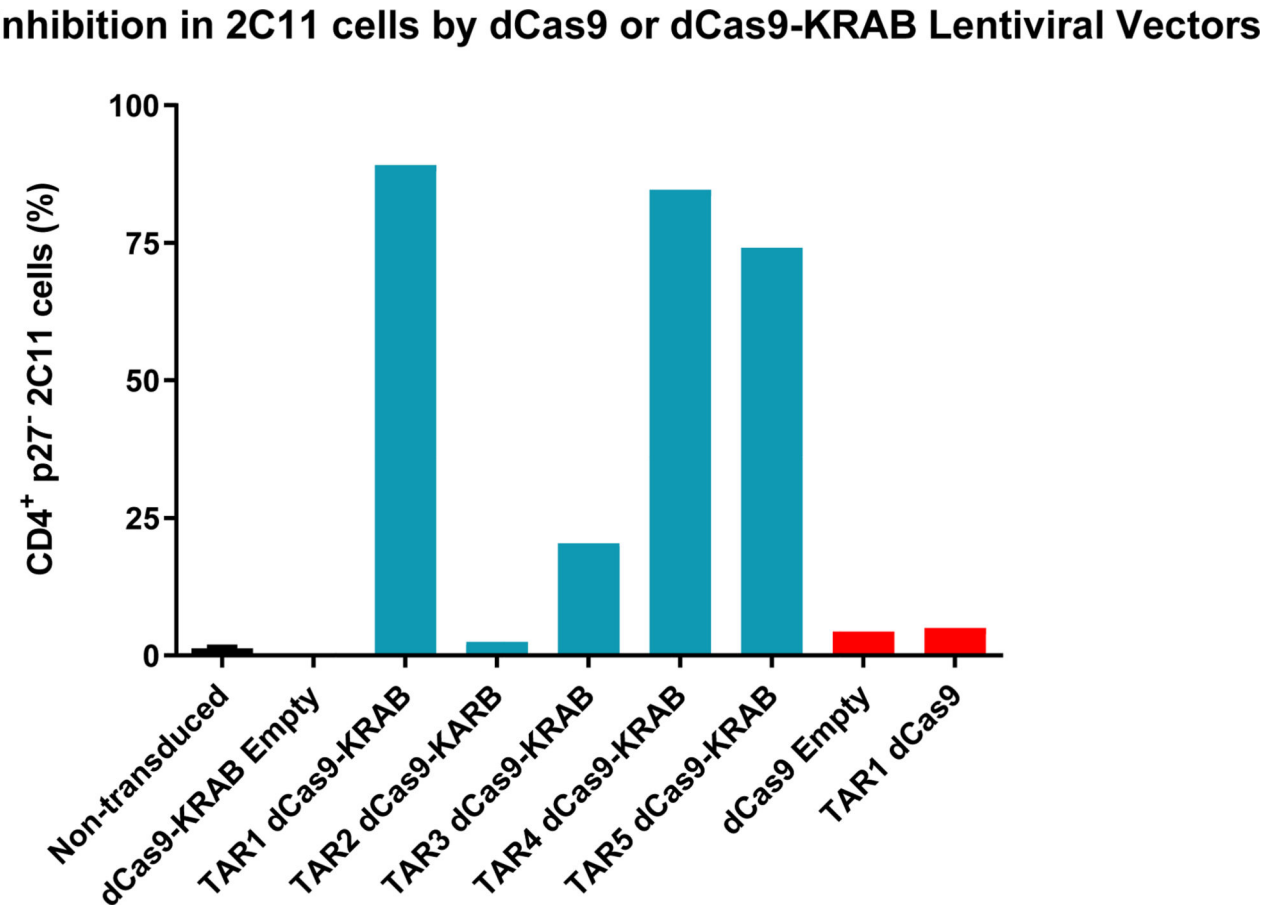


FIGURE 7. Inhibition of SIV in 2C11 Cells Transduced with lentiviral vectors co-expressing TAR-specific sgRNAs and dCas9-KRAB or dCas9.

2C11 cells were transduced with lentiviral vectors co-expressing dCas9-KRAB and (A) no sgRNA; (B) TAR1 sgRNA; (C) TAR2 sgRNA; (D) TAR3 sgRNA; (E) TAR4 sgRNA; and (F) TAR5 sgRNA. Cells were stained for flow cytometry to assess surface CD4 and intracellular SIV p27 expression.

TABLE 1.

List of Oligonucleotides (5'→3') used to Generate SIV_{mac} TAR-Specific sgRNAs

Top TAR1	CACCGGCTGGAGAGAACCTCCC
Bottom TAR1	AAACGGGAGGTTCTCTCCAGCC
Top TAR2	CACCGAGACTCTCACCAGCACT
Bottom TAR2	AAACAGTGCTGGTGAGAGTCTC
Top TAR3	CACCGCACTAGCAGGTAGAGCC
Bottom TAR3	AAACGGCTCTACCTGCTAGTGC
Top TAR4	CACCGGCTCTACCTGCTAGTGC
Bottom TAR4	AAACGCACTAGCAGGTAGAGCC
Top TAR5	CACCGCTGGTGAGAGTCTAGCA
Bottom TAR5	AAACTGCTAGACTCTCACCAGC