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Supplemental Information

Gesicle-Mediated Delivery of CRISPR/Cas9 Ribonucleoprotein Complex for Inactivating the HIV Provirus

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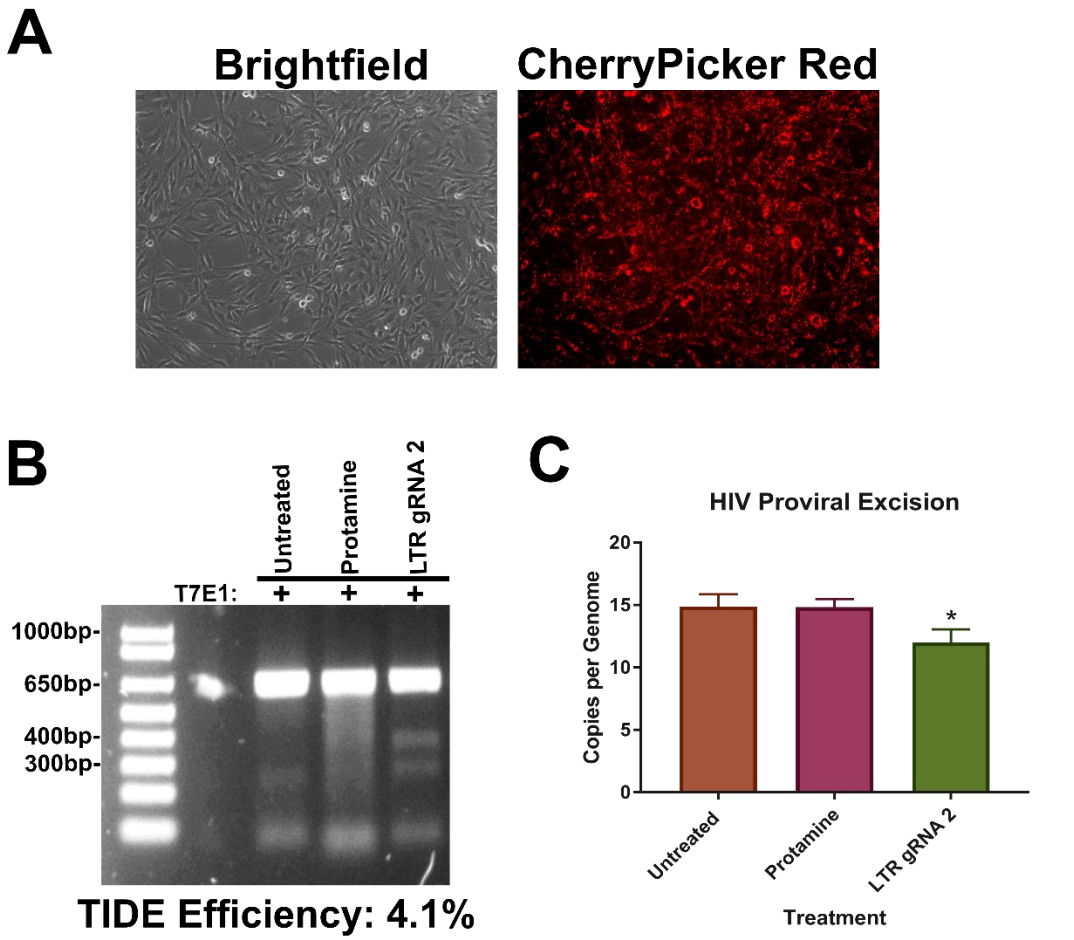
Supplemental Figures



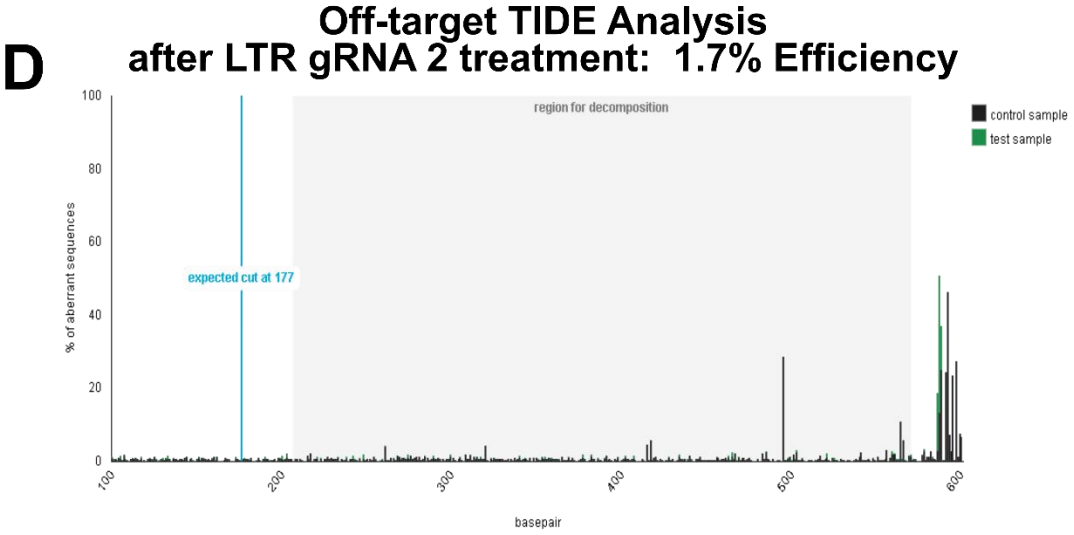
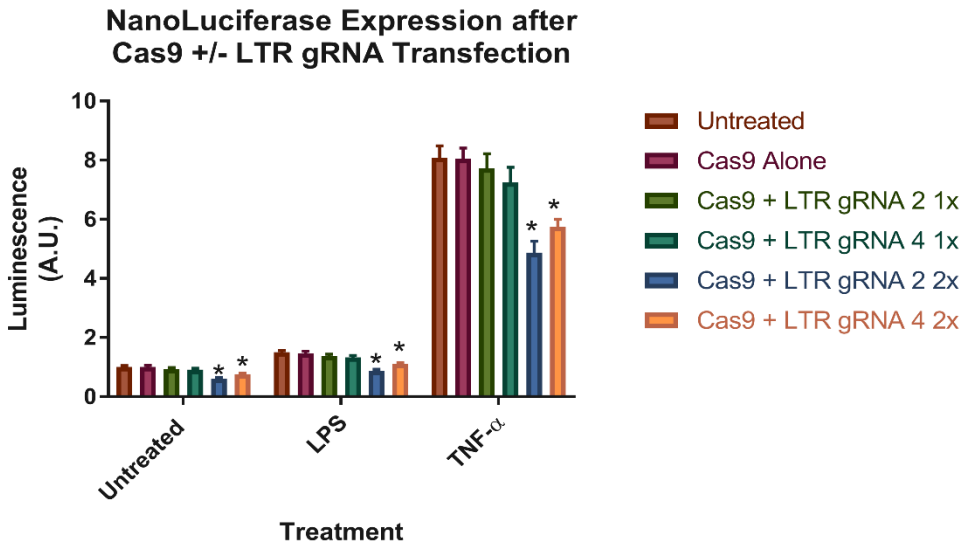
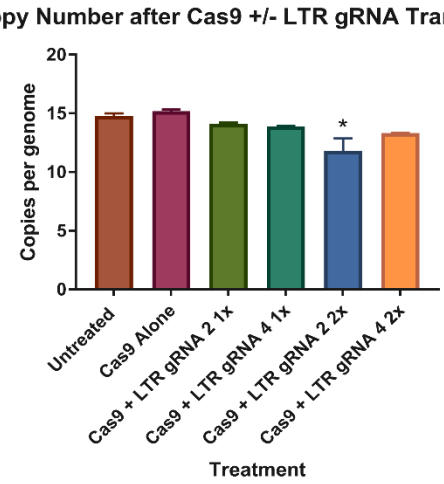


Figure S1. Molecular characterization of LTR gRNA 2. HIV-NanoLuc CHME-5 microglia were treated with gesicles containing LTR gRNA 2. (**A**) Live cell images of gesicle treatment showed CherryPicker Red expression. (**B**) T7E1 assay showed positive products for mutation by LTR gRNA 2, with a TIDE efficiency of 4.1%. (**C**) A significant loss of proviral copy number is observed by DDPCR. (**D**) Examination of the top off-target of LTR gRNA 2 resulted in a 1.7% mutation efficiency. Data are the mean $\pm$ SEM of three experiments, *$p<0.05$ vs untreated cells.

A


NanoLuciferase Expression after
Cas9 +/- LTR gRNA Transfection
Luminescence (A.U.)
10
8
6
4
2
0
Untreated
LPS
TNF-α
Treatment
Untreated
Cas9 Alone
Cas9 + LTR gRNA 2 1x
Cas9 + LTR gRNA 4 1x
Cas9 + LTR gRNA 2 2x
Cas9 + LTR gRNA 4 2x

B


Copy Number after Cas9 +/- LTR gRNA Transfection
Copies per genome
20
15
10
5
0
Untreated
Cas9 Alone
Cas9 + LTR gRNA 2 1x
Cas9 + LTR gRNA 4 1x
Cas9 + LTR gRNA 2 2x
Cas9 + LTR gRNA 4 2x
Treatment

C

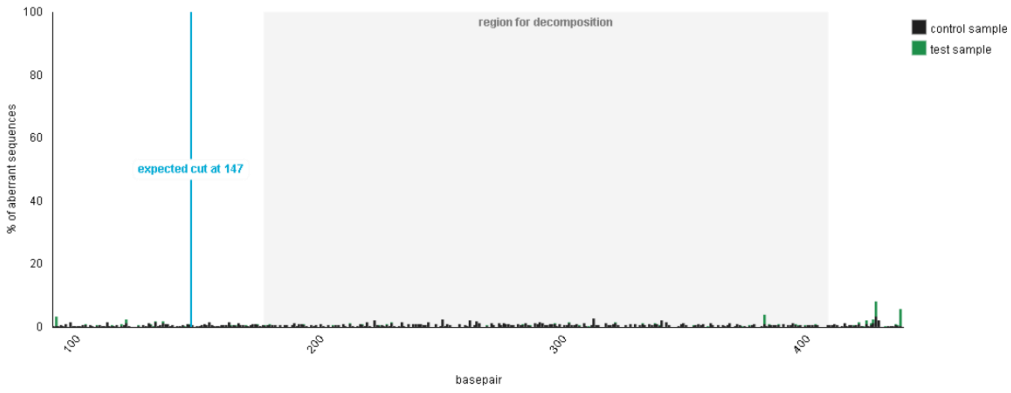

Off-target TIDE Analysis
after LTR gRNA 4 treatment: 2% Efficiency
region for decomposition
control sample
test sample
% of aberrant sequences
100
80
60
40
20
0
expected cut at 147
100
200
300
400
basepair

Figure S2. Plasmid transfection of HIV LTR gRNA 2 and 4. HIV-NanoLuc CHME-5 cells were transfected with separate plasmids containing Cas9 and the specified LTR gRNA. Significant changes from the control were only observed after 2 rounds of transfection. (**A**) Cells transfected 1x or 2x were assayed for HIV proviral activity by NanoLuciferase. LTR gRNA 2 and 4 showed significant reduction in activity after 2 rounds of transfection only. Raw luminescence values are shown. (**B**) Proviral copy number loss was assayed using ddPCR, with significant reduction observed with LTR gRNA 2 after 2 rounds of transfections. (**C**) Off-target analysis by TIDE showed a 2% efficiency using LTR gRNA 4 2x. Data are the mean $\pm$ SEM of two stable cultures, with three experiments each *$p<0.05$ vs untreated cells within each stimulation group.

Supplemental Materials and Methods (also found in main document)

NanoSight analysis: Analysis of size and concentration was performed on different gesicle preparations using the NanoSight NS500 (NanoSight/Malvern, Salisbury, UK). Light scatter mode was used to detect all particle in the solution. Gesicle preparations were diluted at a 200 µg/ml concentration and infused into the instrument. Six screen captures of 30 seconds each were used for particle analysis. Fluorescent mode using a 565 nm laser was utilized to detect CherryPicker Red positive particles only. Camera level was used at 16 (NTA 3.0 levels) and detection threshold was set at 3. For Supplemental Video 1.

EVOS Live Cell Imaging: For live cell time-course imaging of gesicle application, HIV-NanoLuc CHME-5 microglia were plated on a 24 well plate $0.5x10^5$ cell/well in 1 ml of media the day before experimentation. The day of experimentation, media was changed to 600 µl of Fluorbrite Imaging Media (Gibco) supplemented with 5% FBS (Hyclone), 1% penicillin/streptomycin (Gibco), and 8 µg/ml protamine sulfate. Gesicles were applied by centrifugation as described, and the plate was placed in an EVOS FL Auto2 incubator/microscope (EVOS/Thermo-Fisher). Brightfield and red fluorescent images were taken every 15 minutes for 16 hours. For Supplemental Video 2.

Determining off-target mutation by CRISPR/Cas9: Potential off target regions utilizing HIV LTR gRNAs were determined using crispor.tefor.net. The HIV LTR gRNA sequences were scanned against the rat genome due to CHME-5 cells originating from the rat. The top two predicted off-target sites for LTR gRNA 2 and 4 were chosen and primers were developed to amplify this region to be used for the resolvase assay. LTR gRNA 2 off-target 1 is located on chromosome #1 and off-target 2 is located on chromosome #17. LTR gRNA 4 off-target 1 is located on chromosome #14 and off target 2 is located on chromosome #17. After comparing the off-target sites with the gRNA sequence we confirmed that no gRNA sequence had 100% homology to the paired off-target. Furthermore, no off-target areas are located within exon regions. Primers used for analysis are as follows. LTR gRNA 4 Off-target 1: Fwd: 5'-GCGTGAGGGCTTTGTAGAGCTG -3', Rev: 5'- GCTAGCAAACATCACCACAG -3', TIDE primer 5'- GACTTTCATCAGCCAGGGCAC -3'; LTR gRNA 4 Off-target 2: Fwd: 5'-GACCAAGCCATCTTCTGACAC -3', Rev: 5'- GTTTGGGTTGCAGCCTTTCTCC -3', TIDE primer 5'-GGGTTGGTGTGGTTGGTAGAG-3'; LTR gRNA 2 Off-target 1: Fwd: 5'-GGAGCAACTGGTGTGATTCTG -3', Rev: 5'- GGTTCCATTCTGCGAAGATGAG -3', TIDE primer 5'- CATAGGGACAGGCATTATGG-3'; LTR gRNA 2 Off-target 2: Fwd: 5'-GGCTTCAGAAGCCTCAGAATG -3', Rev: 5'-GCTCTCCTTCCTGCCAGTGTAG -3', TIDE primer 5'- GCTTTCACTTTCCCAGTGCC-3'. For Supplemental Figure 1, Supplemental Figure 2.

PCR: The genomic DNA was isolated using the NucleoSpin® Tissue Column (Macherey-Nagel Bethlehem, PA) according to the manufacturer's instructions. DNA concentrations were approximated by A260 reading, using a Nanodrop 2000 (Thermo-Fisher). PCR reactions were prepared using Phusion High Fidelity GC Buffer (New England Biolabs, Ipswich, Ma) with 5 µM forward and reverse primers and 1 µl of DNA sample. PCR was carried out in a Bio-Rad C1000™ Thermal Cycler (Bio-Rad, Hercules, CA) using the following cycle. For amplification of HIV-LTR: 98ºC for 30 sec., 40x cycle of 98ºC → 72ºC, 72ºC 5min, 12ºC hold. PCR products were run on a 1% agarose gel made with 1xTAE (VWR, Radnor, PA) with SYBR Safe DNA gel stain (Thermo-Fisher) for 45 min. LTR amplification primers used were Fwd: 5'-CTCTGCTGCCTCCTGTCTTCTG-3', Rev: 5'-GTTTCAGAATCTCGGGGTGTCCG-3', TIDE primer 5'- CCTTCTAGCCTCCGCTAGTC- 3'. For Supplemental Figure 1.

T7 Endonuclease 1: The T7 Endonuclease 1 enzyme- "T7E1" (New England Biolabs) was used to detect mutations in the HIV-LTR PCR amplified region. Amplified products were denatured and slowly annealed in NEBuffer 2 (New England Biolabs) using the following cycle: 95ºC 5 min., 85ºC 30 sec. → ramp 2ºC per 5 sec., 25ºC 30 sec. → ramp 0.1ºC per sec., 25ºC hold. T7E1 was then added to the reannealed products and incubated at 37ºC for 1 hour. DNA was run on a 1% agarose gel and presence of T7E1 digestion was used to verify CRISPR/Cas9 mediated mutation. For Supplemental Figure 1.

Droplet Digital PCR: The relative copy number per genome of the modified HIV provirus was determined using Droplet Digital PCR (Bio-Rad-QX200) to quantify the number of provirus templates per microliter for the *NanoLuc* transgene relative to the autosomal reference gene *GGT1*. Primers and probes used are as follows: *NanoLuc* DDPCR- Fwd: 5'-ATTGTCCTGAGCGGTGAAA-3', Rev: 5'-CACAGGGTACACCACCTTAAA-3', Probe:

FAM-TGGGCTGAAGATCGACATCCATGT-Iowa Black; *GGT1* DDPCR- Fwd: 5'-CCACCCCTTCCCTACTCCTAC-3', Rev: 5'-GGCCACAGAGCTGGTTGTC-3', Probe: HEX-CCGAGAAGCAGCCACAGCCATACCT-Iowa Black. *Nef* EVAGreen assay: *Nef* Fwd 5'-GGCTGGATGGCCTACTGTAAGG-3', *Nef* Rev 5'-GTCTTTCCAGGTCTCGAGATACTGC-3'. Reaction conditions consisted of a master mix containing: 1x ddPCR™ Supermix for Probes no dUTP (Bio-Rad), 450 nM forward and reverse primers, 50 nM probe, 0.1U MseI restriction enzyme with 50 ng genomic DNA. Reactions were run analyzing *NanoLuc* and *GGT1* simultaneously as a duplex reaction. Reactions for *Nef* were performed using EVAGreen Dye (BioRad). Equation for calculating copy number is ((copies/µL *NanoLuc*)/(copies/µL *GGT1*))*(2) = copy number provirus per genome. For Supplemental Figure 1, Supplemental Figure 2.

Transient transfection of CRISPR/Cas9: HIV-NanoLuc CHME-5 cells were plated on a 24 well plate at $0.5x10^5$ cells per well in 1 ml of media. The plasmids utilized were FLAG-tagged NLS-Cas9-T2A-PuroR, (Addgene #48139) and the LTR gRNAs were ligated into the pGuide-it-sgRNA vector (previously described). Lipofectamine 2000 (Thermo-Fisher) was used as the transfection reagent and the concentration for each treatment are as follows: Cas9: 1.6 µg/ml + pBluescript II SK1.6 (Addgene #212205), Cas9: 1.6 µg/ml + LTR gRNA 2: 1.6 µg/ml, Cas9: 1.6 µg/ml + LTR gRNA 4: 1.6 µg/ml. Cells were treated with either one round of transfection or two rounds of transfection after the first cellular expansion and plating procedure. For Supplemental Figure 2.

Luciferase assay: NanoLuciferase was assay as previously described. Cells that were prepared for luciferase assay were lysed directly in an opaque 96 well using RIPA lysis buffer/1% NP 40 with protease inhibitor. Luminescence was measured in the opaque plate using the substrate coelenterazine (Regis Technologies Morton Grove, IL, USA) in a Bio-Tek Synergy 2 plate reader (Winooski, VT, USA). For Supplemental Figure 2.

Statistical Analysis: All analyses were evaluated by Prism Graphpad, GraphPad Software (Inc., La Jolla, CA,USA). Data were analyzed with one-way ANOVA with Bonferonni 's multiple comparison post-test, or with two-way ANOVA with Tukey's multiple comparison post-test. Data are expressed as the mean $\pm$ SEM. Statistically significant differences were considered as $p<0.05$.