Cell Reports, Volume *35*

Supplemental information

Targeted delivery of CRISPR-Cas9 and transgenes enables complex immune cell engineering

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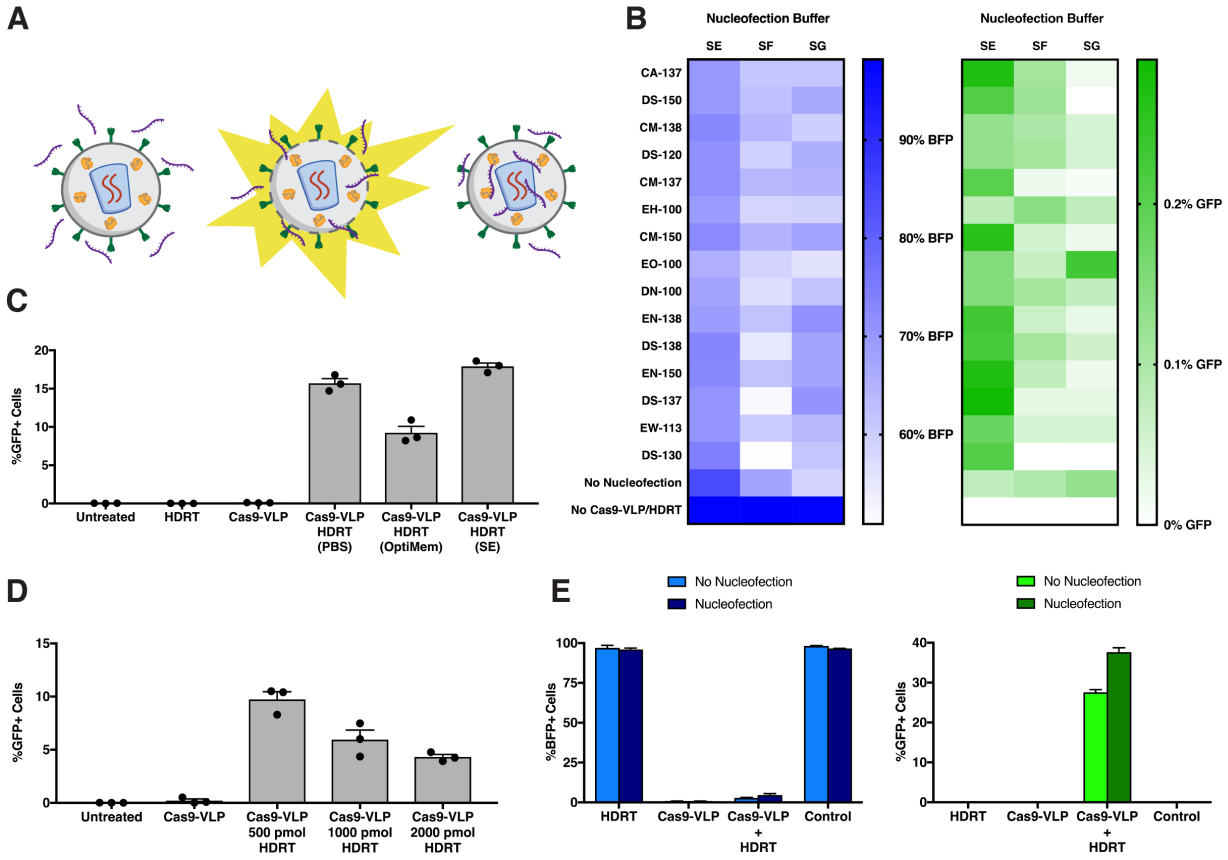

Figure S1. Cas9-VLPs mediate homology-directed repair (HDR), Related to Figure 2. (**A**) Schematic of nucleofection of Cas9-VLPs and single-stranded DNA homology-directed repair templates (HDRT, purple). (**B**) Assessment of different Lonza nucleofection buffers and pulse codes, 5 days post treatment. Cas9-VLPs packaging BFP-targeting RNPs were mixed with 80 pmol HDRT and nucleofected using the indicated nucleofection buffers and pulse codes. Nucleofected HDRT/Cas9-VLPs were subsequently used to treat a BFP-to-GFP HDR reporter HEK293 cell line (Richardson et al., 2016) where BFP knockout is indicative of non-homologous end joining and GFP expression is representative of HDR. (**C**) HDR-mediated GFP expression induced treatment with Cas9-VLPs nucleofected (Lonza, CM-150) with 500 pmol HDRT in different buffers, 7 days post treatment. (**D**) HDR-mediated GFP expression with varying concentrations of HDRT nucleofected (Lonza, CM-150) with Cas9-VLPs in SE buffer (Lonza), 7 days post treatment. (**E**) Pre-nucleofection of Cas9-VLPs and HDRT enhances HDR activity. Cas9-VLPs ($2.59x10^6$ pg CA) and 500pmol HDRT were mixed in SE buffer and either directly added to BFP-to-GFP reporter cells or subjected to nucleofection (Lonza, CM-150) prior to cell treatment. BFP-positive and GFP-positive cells were quantitated by flow cytometry at 7 days post treatment. All error bars represent standard error of the mean.

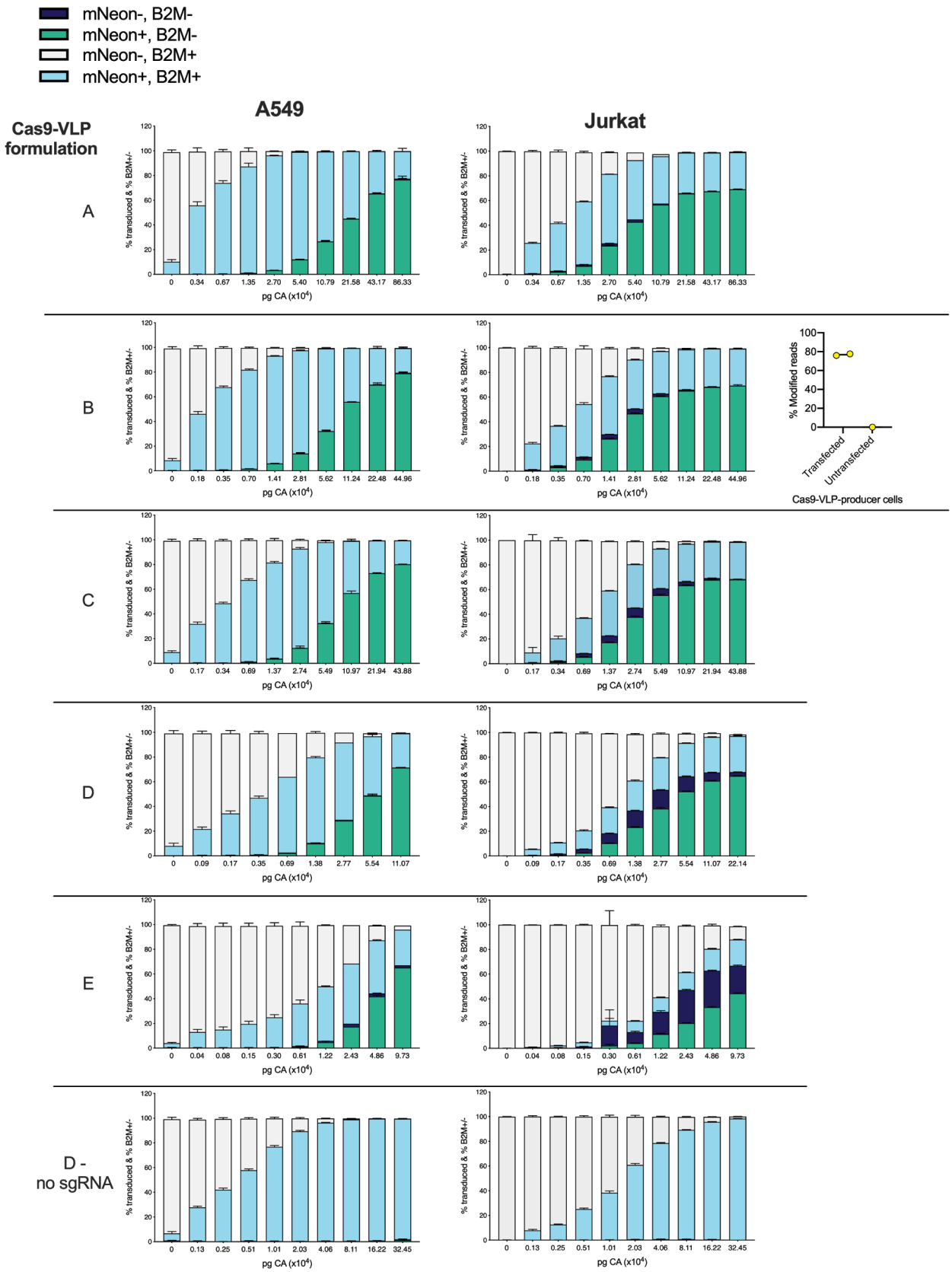

Figure S2. All Cas9-VLP formulations mediate genome editing, Related to Figure 2. Jurkat or A549 cells were

treated with B2M-Cas9-VLP formulations A-E and transduction (mNeonGreen+) and B2M expression were assessed by flow cytometry 6 days post treatment. Of note, cells transfected to produce *B2M*-targeted Cas9-VLPs themselves undergo genome editing (DNA isolated 3 days post transfection). n = 3 technical replicates were performed at each Cas9-VLP treatment dose and error bars represent standard error of the mean.

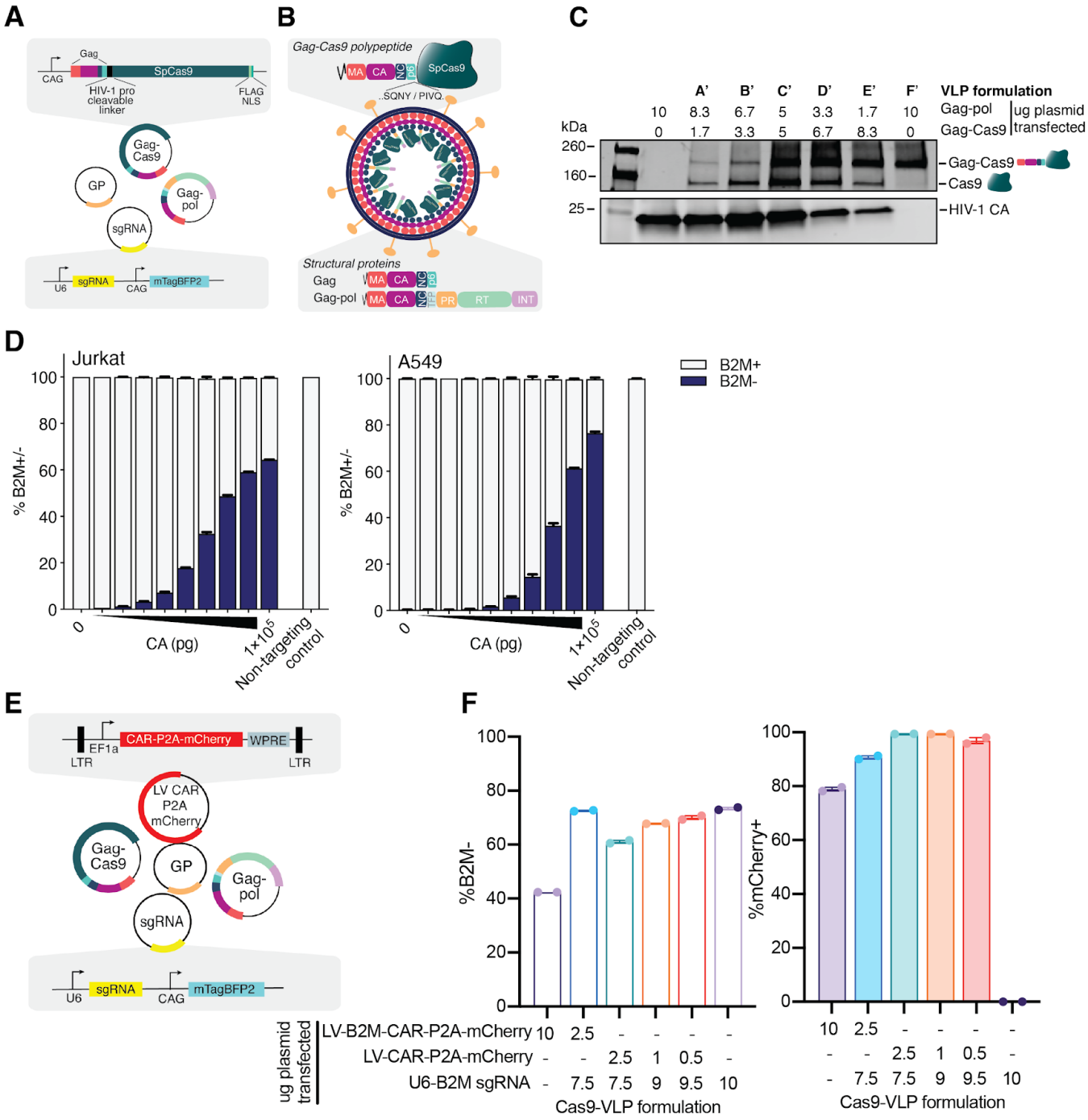

Figure S3. Traceless Cas9-VLPs mediate genome editing without viral transgene insertion and hybrid Cas9-VLPs do not require a lentiviral-encoded guide RNA expression cassette, Related to Figure 2. (**A**) Schematic of plasmids used for the production of traceless Cas9-VLPs. GP = glycoprotein. (**B**) Schematic of an immature, pre-proteolytically processed Cas9-VLP, produced through transient transfection and lacking a lentiviral genome. An HIV-1 protease cleavable linker containing SQNY/PIVQ was inserted between the c-termini of Gag and the n-termini of Cas9 to promote the separation during proteolytic virion maturation. (**C**) Western blot of Cas9-VLP content when various ratios of Gag-pol to Gag-Cas9 plasmids are used for production. An anti-Flag antibody was

used for Cas9 detection and an anti-HIV-1 capsid (CA) antibody was used to detect Cas9-VLP production. A' is used to indicate VLP formulation "A" lacking a packaged lentiviral genome. (**D**) Flow cytometry quantification of B2M expression in A549 and Jurkats 6 days post treatment with traceless Cas9-VLPs. Non-targeting control = Cas9-VLPs packaging the tdTom298 sgRNA. n = 3 technical replicates were performed at each Cas9-VLP treatment dose and error bars indicate standard error of the mean. (**E**) Schematic of plasmids used for the production of Cas9-VLPs that co-package Cas9 RNPs and a lentiviral genome that lacks a guide RNA expression cassette ("hybrid Cas9-VLPs"). (**F**) Optimization of hybrid Cas9-VLPs. Cas9-VLPs were produced as indicated and used to treat Jurkat cells. Targeted protein disruption (% of cells negative for B2M expression) and transduction (% of cells mCherry positive) was quantified at day 7. LV-B2M-CAR-P2A-mCherry = lentiviral transfer plasmid that encodes the U6-promoter driven expression of a *B2M*-targeting guide RNA and the EF1a-promoter driven expression of a CAR-P2A-mCherry transgene. LV-CAR-P2A-mCherry = lentiviral transfer plasmid that encodes the CAR-P2A-mCherry expression cassette alone. U6-B2M = a transient guide RNA expression plasmid.

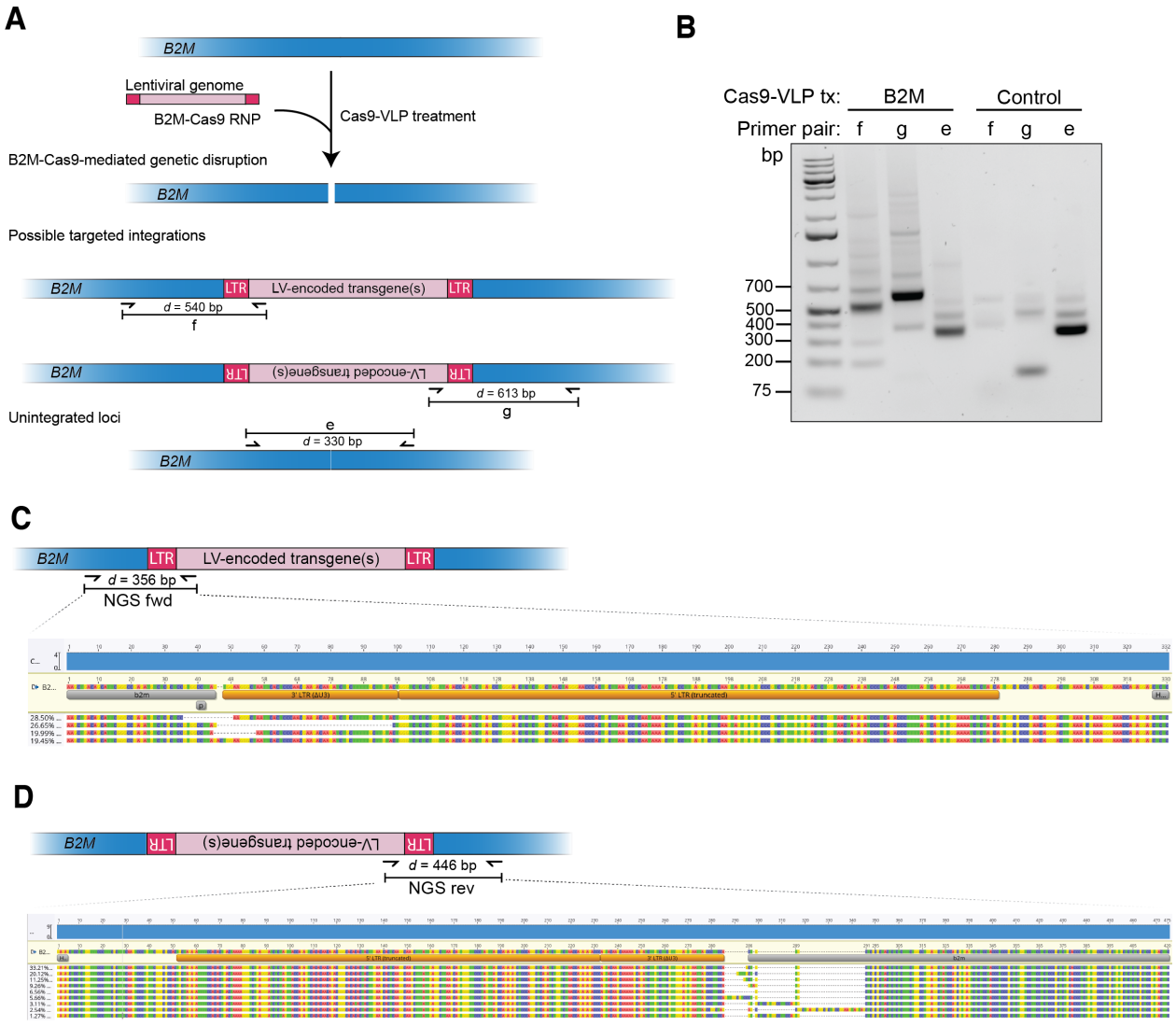

Figure S4. Targeted integration of the lentiviral genome into the Cas9 RNP target site, Related to Figure 2. **(A)** Schematic of hypothetical lentiviral insertion at the Cas9 RNP-induced double-stranded DNA break. **(B)** PCR to assess targeted lentiviral integration. DNA was isolated from 293T cells 3 days post treatment with B2M-targeting or non-targeting Cas9-VLPs and the indicated primer pairs were used for analysis. **(C)** MiSeq analysis of the targeted "forward" lentiviral integration in cells treated with B2M Cas9-VLPs. Reads mapped to the hypothetical B2M-lentiviral junction are shown. **(D)** MiSeq analysis of the targeted "reverse" lentiviral integration in cells treated with B2M Cas9-VLPs. Reads mapped to the hypothetical B2M-lentiviral junction are shown. Amplicon sizes include Illumina adaptor sequences, see Table S2.

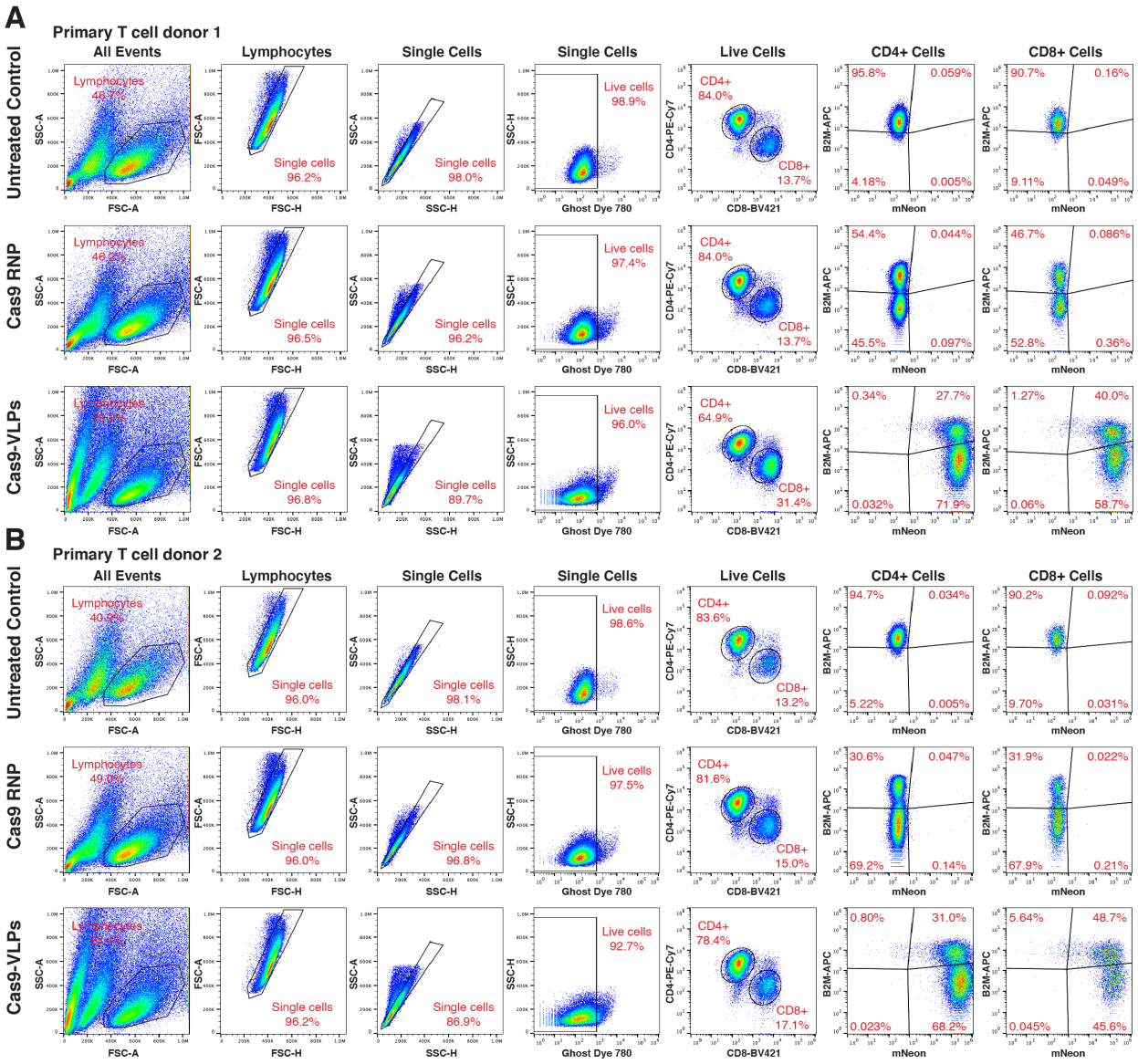

Figure S5. Representative flow cytometry gating strategy for quantifying genome editing in primary human T cells, Related to Figure 3. (A) Flow cytometry gating strategy to assess surface-expressed B2M in primary human T cells after no treatment, nucleofection of Cas9 RNPs, and treatment with Cas9-VLPs from donor 1. **(B)** Flow cytometry gating strategy to assess surface-expressed B2M in primary human T cells after no treatment, nucleofection of Cas9 RNPs, and treatment with Cas9-VLPs from donor 2.

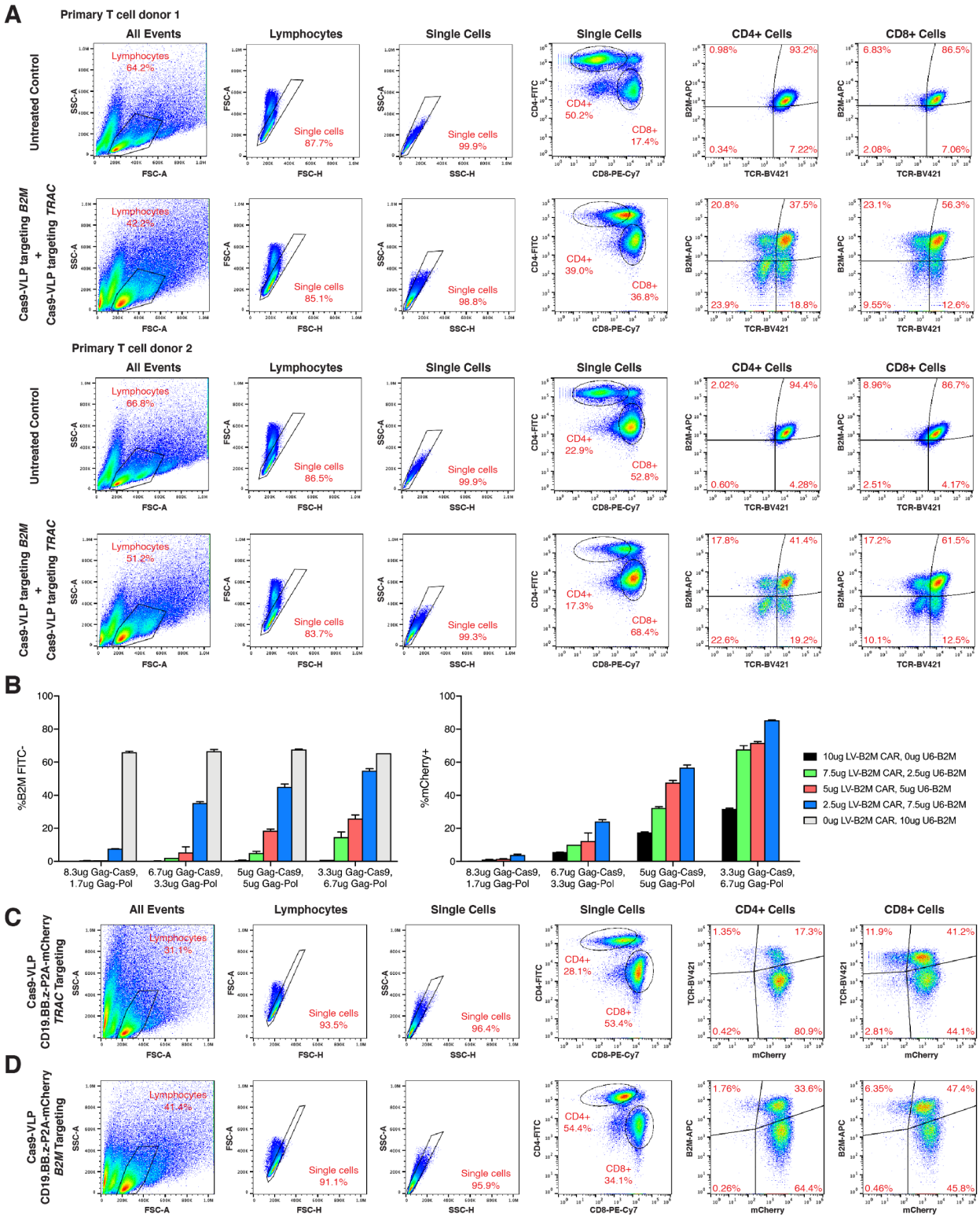

Figure S6. Optimization of CAR-Cas9-VLP production & representative flow cytometry gating strategy for Cas9-VLP-mediated multiplexed genome engineering of primary human CAR-T cells, Related to Figure 3.
(A) Flow cytometry gating strategy to assess the dual knockout of surface-expressed TCR and B2M by simultaneous treatment with Cas9-VLPs targeting *TRAC* and Cas9-VLPs targeting *B2M* in two independent T cell donors.

Cas9-VLPs optimized for simultaneous CAR transgene insertion and B2M knockout were used **(Fig. S5B). (B)** Optimization of Cas9-VLP production to maximize simultaneous CAR transgene integration and genome editing. Cas9-VLPs were produced with various ratios of plasmids encoding the Gag-Cas9 and Gag-pol structural proteins, and with various ratios of plasmids encoding a lentiviral transfer plasmid (encoding expression cassettes for U6-B2M CAR-P2A-mCherry) and a U6-B2M guide RNA expression plasmid. Jurkats were treated, passed at day 4 post treatment to maintain subconfluent culture conditions and flow cytometry was performed at 6 days post treatment to quantify B2M expression (**B,** left) and CAR-P2A-mCherry expression (**B,** right). Cas9-VLPs produced through transient transfection with the following plasmids were most efficient at mediating simultaneous knockout of B2M and CAR-P2A-mCherry transgene expression: 1μg VSV-G, 3.3μg Gag-Cas9, 6.7μg Gag-pol plasmid, 2.5μg LV-B2M, and 7.5μg U6-B2M. n = 2 replicates per treatment, error bars represent standard error of the mean. **(C)** Flow cytometry gating strategy to assess the knockout of surface-expressed TCR and expression of CAR-P2A-mCherry in primary human T cells by treatment with Cas9-VLPs. **(D)** Flow cytometry gating strategy to assess the knockout of surface-expressed B2M and expression of CAR-P2A-mCherry in primary human T cells by treatment with Cas9-VLPs.

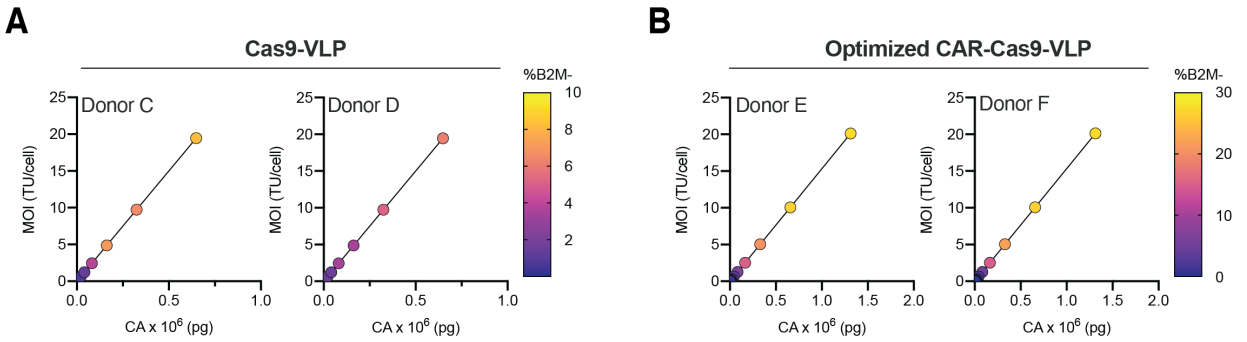

Figure S7. Cas9-VLP genome editing as a function of MOI and quantity of CA, Related to Figure 3. (A) Cas9-VLPs co-packaging *B2M*-targeting Cas9 RNPs and a lentiviral genome encoding mNeonGreen were generated (as used in Figure 3) and **(B)** Cas9-VLPs optimized to co-package *B2M*-targeting Cas9 RNPs and a lentiviral genome encoding CAR-P2A-mCherry were produced. The transducing units/mL (TU/mL) titer and capsid (CA) content were quantified for each Cas9-VLP preparation. Primary T cells from two human donors were treated with indicated multiplicity of infection (MOI) and picogram (pg) CA and cells negative for B2M protein were quantified by flow cytometry at day 7.

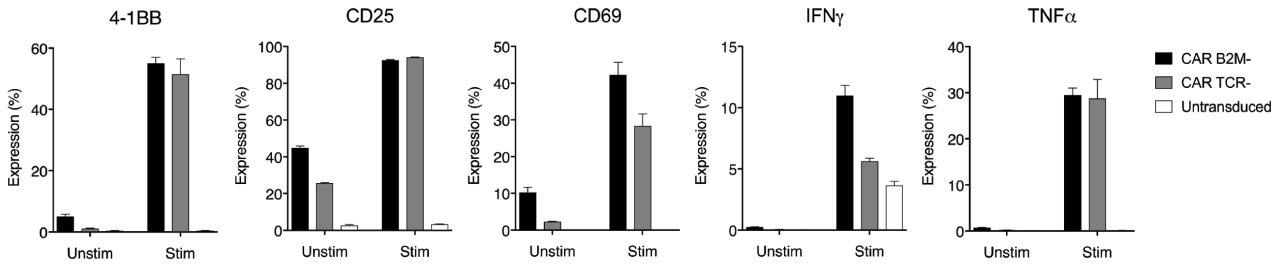

Figure S8. Functional cytokine production and surface receptor expression in Cas9-VLP generated CAR-T cells, Related to Figure 3. Cytokine and surface receptor expression were quantified in stimulated and unstimulated CAR-T cells generated from Cas9-VLPs at 24 h. For all, n = 2 biological replicates from independent donors were used and error bars indicate standard error of the mean.

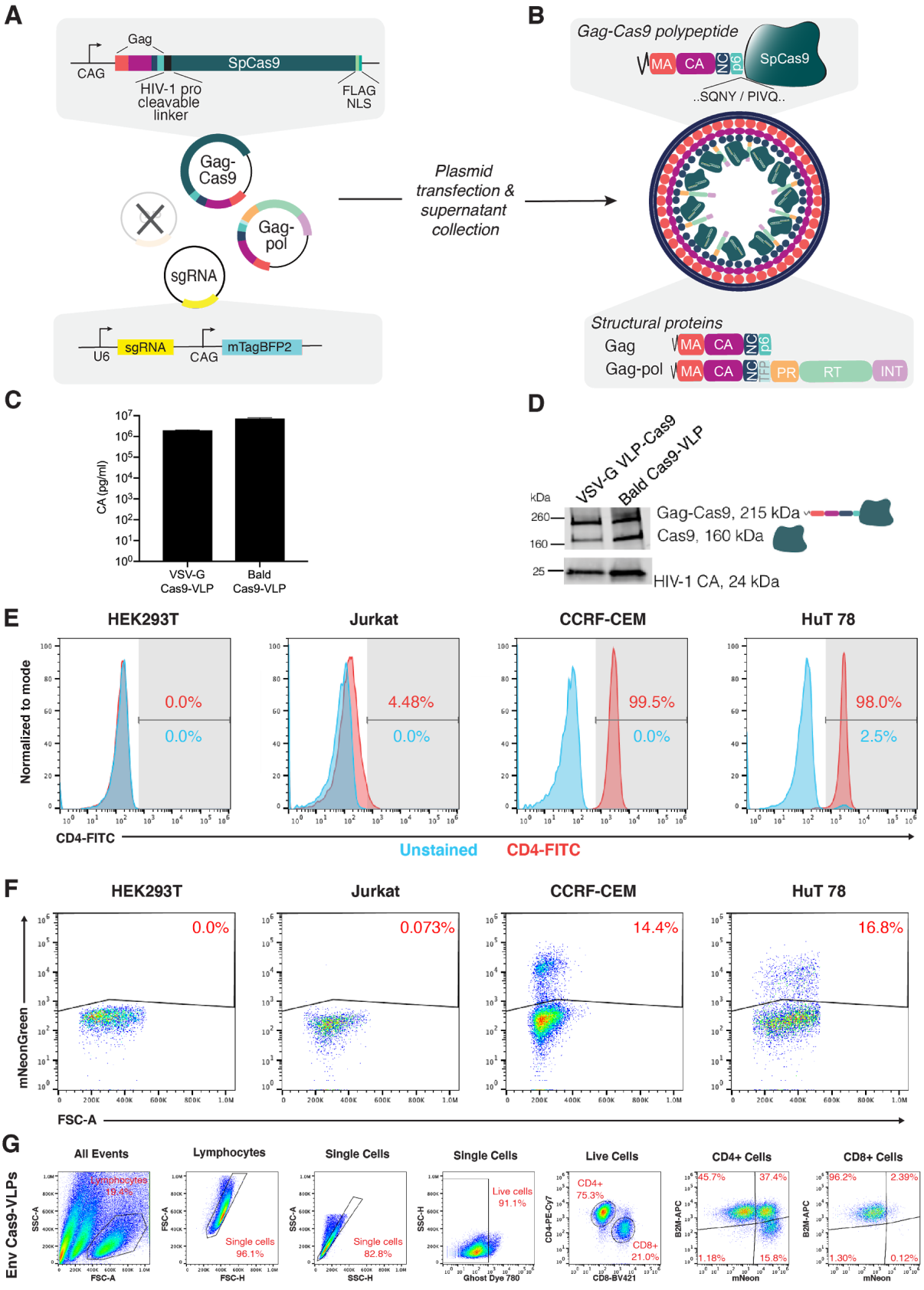
A
Gag
CAG
SpCas9
HIV-1 pro cleavable linker
FLAG NLS
Gag-Cas9
Gag-pol
sgRNA
U6
sgRNA
CAG
mTagBFP2
Plasmid transfection & supernatant collection
B
Gag-Cas9 polypeptide
MA
CA
NC
p6
SpCas9
..SQNY / PIVQ..
Structural proteins
Gag
Gag-pol
PR
RT
INT
C
CA (pg/ml)
VSV-G Cas9-VLP
Bald Cas9-VLP
D
kDa
260
160
25
VSV-G VLP-Cas9
Bald Cas9-VLP
Gag-Cas9, 215 kDa
Cas9, 160 kDa
HIV-1 CA, 24 kDa
E
HEK293T
Jurkat
CCRF-CEM
HuT 78
Normalized to mode
0.0%
0.0%
4.48%
0.0%
99.5%
0.0%
98.0%
2.5%
CD4-FITC
Unstained
CD4-FITC
F
HEK293T
Jurkat
CCRF-CEM
HuT 78
mNeonGreen
0.0%
0.073%
14.4%
16.8%
FSC-A
G
Env Cas9-VLPs
All Events
Lymphocytes
Single Cells
Single Cells
Live Cells
CD4+ Cells
CD8+ Cells
Lymphocytes 19.4%
Single cells 96.1%
Single cells 82.8%
Live cells 91.1%
CD4+ 75.3%
CD8+ 21.0%
45.7%
37.4%
1.18%
15.8%
96.2%
2.39%
1.30%
0.12%
FSC-A
FSC-H
SSC-A
SSC-H
SSC-H
Ghost Dye 780
CD4-PE-Cy7
CD8-BV421
B2M-APC
mNeon

Figure S9. Characterization of bald and HIV-1 Env pseudotyped Cas9-VLPs, Related to Figure 4. (A) Production of "bald" Cas-VLPs. Schematic of plasmids used for the production of bald Cas9-VLPs that lack a glycoprotein. **(B)** Schematic of an immature, pre-proteolytically processed Cas9-VLP produced through transient transfection. (**C)** Quantification of Cas9-VLP production by CA ELISA. Amount of CA produced per transfected p100 dish is shown. **(D)** Western blot of Cas9-VLP content. An anti-Flag antibody was used for Cas9 detection and an anti-HIV-1 capsid (CA) antibody was used to detect Cas9-VLP production. **(E)** Env-Cas9-VLPs are specific for CD4+ cells. Cell surface expression of CD4 in HEK293T, Jurkat, CCRF-CEM, and HuT 78 cell lines. **(F)** Transduction of Cas9-VLPs pseudotyped with the HIV-1 envelope correlates with cellular CD4 expression. **(G)** Representative flow cytometry gating strategy to assess the cell-type specificity of B2M knockout by Env-Cas9-VLPs within a mixed population of primary human T cells.

Table S1. Protospacer sequences for mammalian genome editing, Related to STAR Methods.

Target	Spacer sequence	PAM
B2M	5’-GAGTAGCGCGAGCACAGCTA	AGG
TRAC	5’-AGAGTCTCTCAGCTGGTACA	CGG
BFP	5’-GCTGAAGCACTGCACGCCAT	GGG
Control (tdTom298)	5’-AAGTAAAACCTCTACAAATG	TGG
Control (non-targeting guide used for integration site analysis)	5’-GTATTACTGATATTGGTGGG	

Table S2. Genomic amplification and sequencing primers, Related to STAR Methods.

Target	Sequence
B2M_Sanger_F	5'-TCACCCAGTCTAGTGCATGC
B2M_Sanger_R	5'-GACGCTTATCGACGCCCTAA
TRAC_Sanger_F	5'-CATCACTGGCATCTGGACTCCA
TRAC_Sanger_R	5'-TGCTCTTGAAGTCCATAGACCTCA
B2M_NGS1_F	5'-**GCTCTTCCGATCT**TGCGGGCCTTGTCCTGATTG
B2M_NGS1_R	5'-**GCTCTTCCGATCT**AGATCCAGCCCTGGACTAGC
B2M_NGS2_F	5'-**GCTCTTCCGATCT**AAGCTGACAGCATTCGGGC
B2M_NGS2_R	5'-**GCTCTTCCGATCT**GAAGTCACGGAGCGAGAGAG
Integration_a_F	5'-**GCTCTTCCGATCT**TGCGGGCCTTGTCCTGATTG
Integration_a_R	5'-GTTCGGGCGCCACTGCTAGA
Integration_b_F	5'-TTAAGCCTCAATAAAGCTTGCC
Integration_b_R	5'-**GCTCTTCCGATCT**AGATCCAGCCCTGGACTAGC
Integration_c_F	5'-**GCTCTTCCGATCT**TGCGGGCCTTGTCCTGATTG
Integration_c_R	5'-TTAAGCCTCAATAAAGCTTGCC
Integration_d_F	5'-GTTCGGGCGCCACTGCTAGA
Integration_d_R	5'-**GCTCTTCCGATCT**AGATCCAGCCCTGGACTAGC
Integration_e_F	5'-**GCTCTTCCGATCT**TGCGGGCCTTGTCCTGATTG
Integration_e_R	5'-**GCTCTTCCGATCT**AGATCCAGCCCTGGACTAGC
Integration_f_F	5'-**GCTCTTCCGATCT**TGCGGGCCTTGTCCTGATTG
Integration_f_R	5'-TACTGACGCTCTCGCACCCAT
Integration_g_F	5'-TACTGACGCTCTCGCACCCAT
Integration_g_R	5'-**GCTCTTCCGATCT**AGATCCAGCCCTGGACTAGC
Integration_NGS fwd_F	5'-**GCTCTTCCGATCT**AAGCTGACAGCATTCGGGC
Integration_NGS fwd_R	5'-**GCTCTTCCGATCT**GAGAGCTCCTCTGGTTTCCC
Integration_NGS rev_F	5'-**GCTCTTCCGATCT**GAGAGCTCCTCTGGTTTCCC

Integration_NGS rev_R	5'-**GCTCTTCCGATCT**GAAGTCACGGAGCGAGAGAG
Integration_Nested_1_F	5'-TCACCCAGTCTAGTGCATGC
Integration_Nested_1_R	5'-GACGCTTATCGACGCCCTAA
Integration_Nested_2_F	5'-**GCTCTTCCGATCT**AGGTCCGAGCAGTTAACTGG
Integration_Nested_2_R	5'-**GCTCTTCCGATCT**ACTTAGCGGGCGCCTAGA

Illumina adapter sequences used for library prep are in **bold**.

Table S3. HDR template, Related to Fig. S1, STAR Methods.

Target	Sequence
BFP_GFP_HDRT	5'-GCCACCTACGGCAAGCTGACCCTGAAGTTCATCTGCACCA CCGGCAAGCTGCCCGTGCCCTGGCCCACCCTCGTGACCACC CTGACGTACGGCGTGCAGTGCTTCAGCCGCTACCCCGACCA CATGA

Table S4. Primary human T cell donors, Related to STAR Methods.

Treatment	Figures	Number of Donors
Nucleofection	Fig. 4, Supp. Fig. 5	2 (Donors A-B)
VSV-G Cas9-VLP	Fig. 4, Supp. Fig. 5, Supp. Fig. 7	4 (Donors A-D)
Env Cas9-VLP	Fig. 4, Supp. Fig. 9	2 (Donors A-B)
B2M Cas9-VLP + TRAC Cas9-VLP	Fig. 4, Supp. Fig. 6	2 (Donors E-F)
B2M CAR-P2A-mCherry Cas9-VLP	Fig. 4, Supp. Fig. 6, Supp. Fig. 7	4 (Donors C-D, E-F)
TRAC CAR-P2A-mCherry Cas9-VLP	Fig. 4, Supp. Fig. 6	2 (Donors E-F)