Supporting Information

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SI Materials and Methods

Human T-Cell Isolation and Culture. Human primary T cells were isolated from either fresh whole blood or buffy coats (Stanford Blood Center). Whole blood was collected from human donors in sodium heparinized vacutainer tubes (Becton Dickinson) with approval by the UCSF Committee on Human Research and processed within 12 h. PBMCs were isolated by Ficoll gradient centrifugation. Fresh blood was mixed in a 1:1 ratio with Ca^{2+} and Mg^{2+} free HBSS, Buffy coats were diluted in a 1:10 ratio with HBSS. Thirty milliliters of the respective HBSS/blood solution were transferred to 50-mL Falcon tubes and underlaid with 12 mL Ficoll-Paque PLUS (Amersham/GE healthcare). After density gradient centrifugation (1,000 × *g*, 20 min, no brakes) the PBMC layer was carefully removed and the cells were washed twice with Ca^{2+} and Mg^{2+} free HBSS. $CD4^+$ T cells were pre-enriched with a Easysep Human $CD4^+$ T-cell enrichment kit (Stemcell Technologies) according to the manufacturer's protocol. Pre-enriched $CD4^+$ T cells were stained with the following antibodies: αCD4-PerCp (SK3; Becton Dickinson), αCD25-APC (BC96; TONBO Biosciences), αCD127-PE (R34-34; TONBO Biosciences), αCD45RA-violetFluor450 (HI100; TONBO Biosciences), and αCD45RO-FITC (UCHL1; TONBO Biosciences). $CD4^+CD25^{lo}CD127^{hi}$ Teffs were isolated using a FACS Aria Illu (Becton Dickinson). Teff purity was >97%.

For Cas9 RNP transfections, the effector $CD4^+$ T cells isolated from whole blood were preactivated on αCD3 (UCHT1; BD Pharmingen) and αCD28 (CD28.2; BD Pharmingen) coated plates for 48 h. Plates were coated with 10 μg/mL αCD3 and αCD28 in PBS for at least 2 h at 37 °C. Buffy coat-derived T cells were activated on plates coated with 10 μg/mL αCD3 (in PBS for at least 2 h at 37 °C) with 5 μg/mL αCD28 added directly to the RPMI complete medium.

The T cells were activated in RPMI complete, RPMI-1640 [UCSF Cell Culture Facility (CCF)] supplemented with 5 mmol/L 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes) (UCSF CCF), 2 mmol/L Glutamax (Gibco), 50 μg/mL penicillin/streptomycin (Corning), 50 μmol/L 2-mercaptoethanol (Sigma-Aldrich), 5 mmol/L nonessential amino acids (Corning), 5 mmol/L sodium pyruvate (UCSF CCF), and 10% (vol/vol) FBS (Atlanta Biologicals). After electroporation the medium was supplemented with 40 IU/mL IL-2.

Expression and Purification of Cas9. The recombinant *S. pyogenes* Cas9 used in this study carries at the C terminus an HA tag and two nuclear localization signal peptides that facilitate transport across the nuclear membrane. The protein was expressed with a N-terminal hexahistidine tag and maltose binding protein in *Escherichia coli* Rosetta 2 cells (EMD Millipore) from plasmid pMJ915. The His tag and maltose-binding protein were cleaved by TEV protease, and Cas9 was purified by the protocols described in Jinek et al. (1). Cas9 was stored in 20 mM Hepes at pH 7.5, 150 mM KCl, 10% (vol/vol) glycerol, 1 mM TCEP at −80 °C.

In Vitro T7 Transcription of sgRNA with PAGE Purification. The DNA template encoding for a T7 promoter, a 20-nt target sequence, and the chimeric sgRNA scaffold was assembled from synthetic oligonucleotides by overlapping PCR. Briefly, for the CXCR4 sgRNA template, the PCR contains 20 nM premix of SLKS3 (5′-TAA TAC GAC TCA CTA TAG GAA GCG TGA TGA CAA AGA GGG TTT TAG AGC TAT GCT GGA AAC AGC ATA GCA AGT TAA AAT AAG G -3′) and SLKS1 (5′-GCA CCG ACT CGG TGC CAC TTT TTC AAG TTG ATA ACG GAC TAG CCT TAT TTT AAC TTG CTA TGC TGT TTC CAG C-3′), 1 μM premix of T25 (5′-TAA TAC GAC TCA CTA TAG-3′) and SLKS1 (5′-GCA CCG ACT CGG TGC CAC TTT TTC AAG-3′), and 200 μM dNTP and Phusion Polymerase (NEB) according to the manufacturer's protocol. The thermocycler setting consisted of 30 cycles of 95 °C for 10 s, 57 °C for 10 s, and 72 °C for 10 s. The PCR product was extracted once with phenol:chloroform:isoamylalcohol and then once with chloroform before isopropanol precipitation overnight at −20 °C. The DNA pellet was washed three times with 70% (vol/vol) ethanol, dried by vacuum, and dissolved in diethylpyrocarbonate-treated water. The PD-1 sgRNA template was assembled from T25, SLKS1, SLKS2, and SLKS11 (5′-TAA TAC GAC TCA CTA TAG CGA CTG GCC AGG GCG CCT GTG TTT TAG AGC TAT GCT GGA AAC AGC ATA GCA AGT TAA AAT AAG G-3′) by the same procedure.

A 100-μL T7 in vitro transcription reaction consisted of 30 mM Tris·HCl (pH 8), 20 mM $MgCl_2$, 0.01% Triton X-100, 2 mM spermidine, 10 mM fresh DTT, 5 mM of each ribonucleotide triphosphate, 100 μg/mL T7 Pol, and 0.1 μM DNA template. The reaction was incubated at 37 °C for 4 h, and 5 units of RNase-free DNaseI (Promega) was added to digest the DNA template 37 °C for 1 h. The reaction was quenched with 2× STOP solution [95% (vol/vol) deionized formamide, 0.05% bromophenol blue, and 20 mM EDTA] at 60 °C for 5 min. The RNA was purified by electrophoresis in 10% (vol/vol) polyacrylamide gel containing 6 M urea. The RNA band was excised from the gel, ground up in a 50-mL tube, and eluted overnight in 25 mL of 300 mM sodium acetate (pH 5) overnight at 4 °C with gentle rocking. The solution was then centrifuged at 4,000 × *g* for 10 min, and the RNA supernatant was passed through a 0.45-μm filter. One equivalent of isopropanol was added to the filtered supernatant to precipitate the RNA overnight at −20 °C. The RNA pellet was collected by centrifugation, washed three times with 70% (vol/vol) ethanol, and dried by vacuum. To refold the sgRNA, the RNA pellet was first dissolved in 20 mM Hepes (pH 7.5), 150 mM KCl, 10% (vol/vol) glycerol, and 1 mM TCEP. The sgRNA was heated to 70 °C for 5 min and cooled to room temperature. $MgCl_2$ was added to a final concentration of 1 mM. The sgRNA was again heated to 50 °C for 5 min, cooled to room temperature, and kept on ice. The sgRNA concentration was determined by OD_{260nm} using Nanodrop and adjusted to 100 μM using 20 mM Hepes (pH 7.5), 150 mM KCl, 10% (vol/vol) glycerol, 1 mM TCEP, and 1 mM $MgCl_2$. The sgRNA was stored at −80 °C.

In Vitro T7 Transcription of sgRNA with Phenol/Chloroform Extraction. DNA templates for in vitro T7 transcription were generated by annealing complementing single-stranded ultramers (Ultramer sequences: CXCR4_1: 5′-TAA TAC GAC TCA CTA TAG GAA GCG TGA TGA CAA AGA GGG TTT TAG AGC TAT GCT GGA AAC AGC ATA GCA AGT TAA AAT AA GGC TAG TCC GTT ATC AAC TTG AAA AAG TGG CAC CGA GTC GGT G-3′; CXCR4_2: 5′-CAC CGA CTC GGT GCC ACT TTT TCA AGT TGA TAA CGG ACT AGC CTT ATT TTA ACT TGC TAT GCT GTT TCC AGC ATA GCT CTA AAA CCC TCT TTG TCA TCA CGC TTC CTA TAG TGA GTC GTA TTA-3′; PD-1_1: 5′-TAA TAC GAC TCA CTA TAG CGA CTG GCC AGG GCG CCT GTG TTT TAG AGC TAT GCT GGA AAC AGC ATA GCA AGT TAA AAT AAG GCT AGT CCG TTA TCA ACT TGA AAA AGT GGC ACC GAG TCG GTG C-3′; PD-1_2: 5′-GCA CCG ACT CGG TGC CAC TTT TTC AAG TTG ATA ACG GAC TAG CCT TAT TTT AAC TTG CTA TGC TGT TTC CAG CAT AGC TCT AAA ACA CAG GCG CCC TGG CCA GTC GCT ATA GTG

AGT CGT ATT A-3′). Ultramers were mixed in a 1:1 ratio in nuclease-free duplex buffer (IDT) and heated up to 95 °C for 2 min followed by a 30-min incubation at room temperature.

A 100-μL T7 in vitro transcription reaction contained 1× Transcription Optimized buffer (Promega), 10 mM fresh DTT, 2 mM of each ribonucleotide triphosphate, 400 U T7 Pol (Promega), 0.5 U pyrophosphatase (Life Technologies), and 2 μg DNA template. The reaction was incubated for 4 h at 37 °C. Five units of RNase-free DNaseI (Promega) were added to digest the DNA template at 37 °C for 30 min. The reaction was stopped with 5 μL 0.5 M EDTA.

Given concern for the possibility of nucleic acid exchange between wells during PAGE purification, we tested phenol/chloroform-purified sgRNAs side by side with PAGE-purified sgRNAs as indicated in Fig. 4 and Fig. S1*A*. Phenol/chloroform extraction was performed after addition of 190 μL RNA-free H_2O. sgRNA was precipitated with 80 μL 3 M sodium acetate and 420 μL isoproponal and incubation at −20 °C for 4 h. The RNA pellet was washed twice with 70% (vol/vol) EtOH and once with 100% (vol/vol) EtOH. The vacuum-dried pellet was reconstituted, and the sgRNAs refolded as described in *In Vitro T7 Transcription of sgRNA with PAGE Purification.*

Cas9 RNP Assembly and Electroporation. Cas9 RNPs were prepared immediately before experiments by incubating 20 μM Cas9 with 20 μM sgRNA at a 1:1 ratio in 20 μM Hepes (pH 7.5), 150 mM KCl, 1 mM $MgCl_2$, 10% (vol/vol) glycerol, and 1 mM TCEP at 37 °C for 10 min to a final concentration of 10 μM.

T cells were electroporated with a Neon transfection kit and device (Invitrogen). A total of 2.5×10^5 T cells was washed three times with PBS before resuspension in 8 μL of buffer T (Neon kit, Invitrogen). Cas9 RNP (2 μL of 10 μM Cas9 CTRL without sgRNA or 1–2 μL Cas9:sgRNA RNP; final concentration: 0.9–1.8 μM) and HDR template (0–200 pmol as indicated) were added to the cell suspension to a final volume of 11 μL (adjusted with Cas9 storage buffer) and mixed. Ten microliters of the suspension was electroporated with a Neon electroporation device (Invitrogen; 1,600 V, 10 ms, three pulses). The HDR templates for CXCR4 and PD-1 are single-stranded oligonucleotides complementary (antisense strand) to the target sequence and contain a HindIII restriction sequence along with 90-nt homology arms. Upon successful HDR, the respective PAM sites are deleted, which should prevent recutting of the edited site by the Cas9 RNPs. The PD-1 HDR template additionally causes a frameshift and nonsense mutation as early as amino acid position 25 by replacing 12 nt with 11 nt (CXCR4 HDR template: 5′-GGG CAA TGG ATT GGT CAT CCT GGT CAT GGG TTA CCA GAA GAA ACT GAG AAG CAT GAC GGA CAA GTA CAG GCT GCA CCT GTC AGT GGC CGA AAG CTT GGA TCC CAT CAC GCT TCC CTT CTG GGC AGT TGA TGC CGT GGC AAA CTG GTA CTT TGG GAA CTT CCT ATG CAA GGC AGT CCA TGT CAT CTA CAC AGT-3′; PD-1 HDR template: 5′-AAC CTG ACC TGG GAC AGT TTC CCT TCC GCT CAC CTC CGC CTG AGC AGT GGA GAA GGC GGC ACT CTG GTG GGG CTG CTC CAG GCA TGC AGA TAA TGA AAG CTT CTG GCC AGT CGT CTG GGC GGT GCT ACA ACT GGG CTG GCG GCC AGG ATG GTT CTT AGG TAG GTG GGG TCG GCG GTC AGG TGT CCC AGA GC-3′). The CXCR4 HDR control donor is a sequence scrambled version on the original CXCR4 HDR template containing a HindIII restriction site (CXCR4 control HDR template: 5′-TTC AAA ACT AGC GTC AGG GGC TCG ATT TAC TCG GGA CTT GCT ACA ACA TCG CAG TCA CGC GCA CGA TCC TTC CAG GAT TGG AGG TGG ACT TAG ATA AAG CTT CCG TGT GCA CCG TAT AGA TTC GTT GAT GCA GGC TAT TCC CGT GAT CCC ACG CGG AGG TGA TGG AGC GTC AAG CAT AGC TAG CAC AGA TGA-3′).

Electroporated T cells were transferred to 500 μL of their respective culture medium in a αCD3/CD28-coated 48-well plate. Plates were coated with 10 μg/mL αCD3 (UCHT1; BD Pharmingen) and αCD28 (CD28.2; BD Pharmingen) in PBS for at least 2 h at 37 °C. Twenty-four hours after electroporation cells were resuspended and transferred to a noncoated well plate. Three to four days after electroporation, T cells were analyzed by FACS and T7 endonuclease I assay.

FACS Analysis of Edited T Cells. Cell-surface staining was performed with αCXCR4-APC (12G5; BD Pharmingen) and αPD-1-PE (EH12.2H7; Biolegend) for 15 min on ice. Cells were kept at 4 °C throughout the staining procedure until cell sorting to minimize antibody-mediated internalization and degradation of the antibody. Cells were sorted using a FACS Aria Illu (Becton Dickinson).

PCR Amplification of Target Region. A total of 5×10^4 to 2×10^5 cells were resuspended in 100 μL of Quick Extraction solution (Epicenter) was added to lyse the cells and extract the genomic DNA. The cell lysate was incubated at 65 °C for 20 min and then at 95 °C for 20 min and stored at −20 °C. The concentration of genomic DNA was determined by NanoDrop (Thermo Scientific).

Genomic regions, containing the *CXCR4* or *PD-1* target sites, were PCR-amplified using the following primer sets: for CXCR4—forward 5′-AGA GGA GTT AGC CAA GAT GTG ACT TTG AAA CC-3′ and reverse 5′-GGA CAG GAT GAC AAT ACC AGG CAG GAT AAG GCC-3′ (938 bp); and for PD-1—forward 5′-GGG GCT CAT CCC ATC CTT AG-3′ and reverse 5′-GCC ACA GCA GTG AGC AGA GA-3′ (905 bp). Both primer sets were designed to avoid amplifying the HDR templates by annealing outside of the homology arms. The PCR contained 200 ng of genomic DNA and Kapa Hot start high-fidelity polymerase (Kapa Biosystems) in high GC buffer according to the manufacturer's protocol. The thermocycler setting consisted of one cycle of 95 °C for 5 min, 35 cycles of 98 °C for 20 s, 62 °C for *CXCR4* or 68 °C for *PD-1* for 15 s, and 72 °C for 1 min, and one cycle of 72 °C for 1 min. The PCR products were purified on 2% (wt/vol) agarose gel containing SYBR Safe (Life Technologies). The PCR products were eluted from the agarose gel using QIAquick gel extraction kit (Qiagen). The concentration of PCR DNA was quantitated with a NanoDrop device (Thermo Scientific). A total of 200 ng of PCR DNA was used for T7 endonuclease I and HindIII analyses. For Fig. 1*E*, PCR product was cloned with TOPO Zero Blunt PCR Cloning Kit (Invitrogen) and submitted for Sanger sequencing.

Analysis of Editing Efficiency by T7 Endonuclease I Assay. Editing efficiency was estimated by T7 endonuclease I assay. T7 endonuclease I recognizes and cleaves mismatched heteroduplex DNA that arises from hybridization of wild-type and mutant DNA strands. The hybridization reaction contained 200 ng of PCR DNA in KAPA high GC buffer and 50 mM KCl and was performed on a thermocycler with the following setting: 95 °C, 10 min, 95–85 °C at −2 °C/s, 85 °C for 1 min, 85–75 °C at −2 °C/s, 75 °C for 1 min, 75–65 °C at −2 °C/s, 65 °C for 1 min, 65–55 °C at −2 °C/s, 55 °C for 1 min, 55–45 °C at −2 °C/s, 45 °C for 1 min, 45–35 °C at −2 °C/s, 35 °C for 1 min, 35–25 °C at −2 °C/s, 25 °C for 1 min, and hold at 4 °C. Buffer 2 and 5 units of T7 endonuclease I (NEB) were added to digest the reannealed DNA. After 1 h of incubation at 37 °C, the reaction was quenched with 6× blue gel loading dye (Thermo Scientific) at 70 °C for 10 min. The product was resolved on 2% agarose gel containing SYBR gold (Life Technologies). The DNA band intensity was quantitated using Image Lab. The percentage of editing was calculated using the following equation $[1 - (1 - (b + c/a + b + c))^{1/2}] \times 100$, where a is the band intensity of DNA substrate and b and c are the cleavage products. For the quantification of the *PD-1* T7E1 assay (Fig. 4*D*), the intensity of the DNA substrate was calculated as the sum of the two large bands seen under all conditions. Calculation of the percentage of total edit based on T7E1 assays allows only an estimate of cleavage efficiency.

Analysis of HDR by HindIII Restriction Digestion. HDR templates were designed to introduce a HindIII restriction site into the targeted gene locus. To test for successful introduction of the HindIII site into the *CXCR4* locus, the 938-bp region was PCR-amplified using the primers 5′-AGA GGA GTT AGC CAA GAT GTG ACT TTG AAA CC-3′ and 5′-GGA CAG GAT GAC AAT ACC AGG CAG GAT AAG GCC-3′. For the *PD-1* locus, a 905-bp region was amplified using the primers 5′-GGG GCT CAT CCC ATC CTT AG-3′ and 5′-GCC ACA GCA GTG AGC AGA GA-3′. The reaction consisted of 200 ng of PCR DNA and 10 units of HindIII High Fidelity in CutSmart Buffer (NEB). After 2 h of incubation at 37 °C, the reaction was quenched with 1 vol of gel loading dye at 70 °C for 10 min. The product was resolved on 2% (wt/vol) agarose gel containing SYBR gold (Life Technologies). The band intensity was quantitated using Image Lab. The percentage of HDR was calculated using the following equation: $(b + c/a + b + c) \times 100$, where a is the band intensity of DNA substrate and b and c are the cleavage products.

Deep-Sequencing Analysis of On-Target and Off-Target Sites. The genomic regions flanking the Cas9 target site for the CXCR4 on-target and two off-target genes were amplified by the two-step PCR method using the following primers: CXCR4 on-target (5′-ACA CTC TTT CCC TAC ACG ACG CTC TTC CGA TCT NNN NNC TTC CTG CCC ACC ATC TAC TCC ATC ATC TTC TTA ACT G-3′ and 5′-GTG ACT GGA GTT CAG ACG TGT GCT CTT CCG ATC TNN NNN CAG GTA GCG GTC CAG ACT GAT GAA GGC CAG GAT GAG GAC-3′); off-target #1 [*POU domain*, *class 2*, *transcription factor 1 isoform 1* (*POU2F1*)] locus; 5′-ACA CTC TTT CCC TAC ACG ACG CTC TTC CGA TCT NNN NNG CTA TAA TAG TAC AAG TAT ATG TTA AAT AAG AGT CAT AGC ATG-3′ and 5′-GTG ACT GGA GTT CAG ACG TGT GCT CTT CCG ATC TNN NNN CTG GCT TTA TAT ATA TAC ATA GAT AGA CGA TAT AGA TAG C-3′); and off-target #2 [*glutamate receptor 1 isoform 1 precursor* (*GRIA1*)] locus; 5′-ACA CTC TTT CCC TAC ACG ACG CTC TTC CGA TCT NNN NNC CTG GTC CCA GCC CAG CCC CAG CTA TTC AGC ATC C-3′ and 5′-GTG ACT GGA GTT CAG ACG TGT GCT CTT CCG ATC TNN NNN ACT CTG CAC TGG TAT ATC AAT ACA CTT GTT TTT CTC ATC CC-3′). First, 100–150 ng of the genomic DNA from the edited and control samples was PCR-amplified using Kapa Hot start high-fidelity polymerase (Kapa Biosystems) according to the manufacturer's protocol. The thermocycler setting consisted of one cycle of 95 °C for 5 min and 15–20 cycles of 98 °C for 20 s, 63 °C for 15 s, 72 °C for 15 s, and one cycle of 72 °C for 1 min. The resulting amplicons were resolved on 2% (wt/vol) agarose gel, stained with SYBR Gold, and gel-extracted using Qiagen gel extraction kit.

Illumina TruSeq Universal adapter (5′-AAT GAT ACG GCG ACC ACC GAG ATC TAC ACT CTT TCC CTA CAC GAC GCT CTT CCG ATC T-3′) and modified Illumina RNA PCR barcode primer (5′-CAA GCA GAA GAC GGC ATA CGA GAT-Index- GTG ACT GGA GTT CAG ACG TGT GCT CTT CCG ATC T-3′) were attached to the amplicon in the second PCR step using Kapa Hot start high-fidelity polymerase (Kapa Biosystems). The thermocycler setting consisted of one cycle of 98 °C for 30 s, 8–10 cycles of 98 °C for 20 s, 65 °C for 15 s, 72 °C for 15 s, and one cycle of 72 °C for 5 min. The resulting amplicons were resolved on 2% (wt/vol) agarose gel, stained with SYBR Gold, and gel-extracted using a Qiagen gel extraction kit. Barcoded and purified DNA samples were quantified by Qubit 2.0 Fluorometer (Life Technologies), size-analyzed by BioAnalyzer (Agilent), quantified by qPCR, and pooled in an equimolar ratio. Sequencing libraries were sequenced with the Illumina HiSEq 2500.

Analysis of Deep-Sequencing Data. Sequencing reads that contained the unique 12 nt resulting from the HDR template were extracted and analyzed separately from those that did not contain HDR template-derived sequence. All reads that did not contain the replaced 12 nt were aligned to the reference hg19 genome, and all of the reads that contained the replaced 12 nt were aligned to a modified hg19 genome with the expected substitutions using Burrows-Wheeler Aligner. The samtools mpileup utility was then used to quantify the total number of reads that mapped to each position of the *CXCR4* gene, and a custom script examining the CIGAR string was used to estimate the number and locations of insertions and deletions for each read. Insertion efficiency was estimated for the experiment with CXCR4 RNP (without HDR template) as the following: (number of reads with insertions ±100 bp from cut site)/(total number of reads ± from cut site). For deletion efficiency the experiment with CXCR4 RNP (without HDR template) was estimated as the following: (number of reads with deletions ±100 bp from cut site)/(total number of reads ± from cut site). For experiments with CXCR4 RNP + HDR template, insertion and deletion efficiencies were calculated based only on reads that that did not contain the 12-nt replacement derived from HDR (these are the fractions shown in Fig. 3*B*). Total editing efficiency was estimated as (number of reads with indels ±100 bp from cut site)/(total number of reads ± from cut site). In Dataset S1, "%Indels in Total Reads" refers to total editing efficiency and includes reads with HDR template sequence incorporated except in rows where these reads have been removed. HDR efficiency was estimated as the following: (number of reads containing HindIII site ± 100 bp from cut site)/(total number of reads ± 100 bp from cut site). Distribution of insertion and deletion sizes were estimated for a region ± 20 bp from the cut site.

1. Jinek M, et al. (2012) A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science* 337(6096):816–821.

Fig. S1. Effects of "on-target" and control HDR templates on PD-1 and CXCR4 surface expression levels. (*A*) The effects on CXCR4 expression were tested for two different HDR templates with the same nucleotide composition. In cells that were treated with CXCR4 Cas9 RNP, CXCR4 HDR template (dark red) was compared with a control HDR template consisting of the same nucleotides as the original CXCR4 HDR in randomized order including a HindIII restriction site (light red) and with no HDR template treatment (orange). Further controls are Cas9 CTRL (Cas9 without sgRNA; dark gray) and scrambled guide Cas9 RNP (no predicted cut within the human genome) with 100 pmol CXCR4 HDR template (light gray). The histograms show the results of four experiments with two differently in vitro-transcribed CXCR4 sgRNAs (two different purification strategies; *Materials and Methods*) tested in two different blood donors. As in Fig. 4, for each blood donor, experiments done with phenol/chloroform-extracted sgRNAs are shown on *Top* and experiments with PAGE-purified sgRNAs are shown

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at the *Bottom*; scrambled guides were prepared for both experiments with phenol/chloroform extraction. (*B*) PD-1 (blue, *Left*) and CXCR4 (red, *Right*) surface expression levels after editing with the respective Cas9 RNPs and on- or off-target HDR templates. Targeted cells were compared with cells treated with Cas9 CTRL (dark gray) or scrambled guide Cas9 RNP (light gray).

CXCR4 Cas9 RNP

Deletions
Insertions

Percentage of Reads

Insertion or Deletion Length (nt)

CXCR4 Cas9 RNP + HDR Template
HindIII Reads Removed

Percentage of Reads

Insertion or Deletion Length (nt)

CXCR4 Cas9 RNP + HDR Template
HindIII Reads Only

Percentage of Reads

Insertion or Deletion Length (nt)

Fig. S2. Distribution of insertion and deletion lengths near expected *CXCR4* cut site. Histograms show the percentage of reads that contain varying sizes of deletions (gray bars) and insertions (black bars) within ±20 nt of the predicted cut site. (*Top*) Insertions and deletions for CXCR4 RNP-treated cells. (*Middle*) Insertions and deletions in reads without HDR template-derived sequence incorporated in the cells treated with CXCR4 RNP and CXCR4 HDR template. (*Bottom*) Insertions and deletions in reads that did incorporate in the HDR template-derived sequence in the cells treated with CXCR4 RNP and CXCR4 HDR template.

Dataset S1. Summary of editing frequencies based on deep sequencing

Dataset S1

Indicated are the numbers of reads (and percentages of total reads) with insertions, deletions, both insertions and deletions, or any indels in cells treated with Cas9, CXCR4 Cas9 RNP, and CXCR4 Cas9 RNP + HDR template (based on deep-sequencing results analyzed in Fig. 3). Here, "%Indels in Total Reads" refers to total editing efficiency and includes reads with HDR template sequence incorporated except in rows where these reads have been removed. Total number of reads with indels was calculated as the following: (no. of reads with insertions) + (no. of reads with deletions) – (no. of reads with insertions and deletions).