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Generation of knock-in primary human T cells using Cas9 ribonucleoproteins

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T-cell genome engineering holds great promise for cell-based therapies for cancer, HIV, primary immune deficiencies, and autoimmune diseases, but genetic manipulation of human T cells has been challenging. Improved tools are needed to efficiently “knock out” genes and “knock in” targeted genome modifications to modulate T-cell function and correct disease-associated mutations. CRISPR/Cas9 technology is facilitating genome engineering in many cell types, but in human T cells its efficiency has been limited and it has not yet proven useful for targeted nucleotide replacements. Here we report efficient genome engineering in human CD4+ T cells using Cas9 single-guide RNA ribonucleoproteins (Cas9 RNPs). Cas9 RNPs allowed ablation of CXCR4, a co-receptor for HIV entry. Cas9 RNP electroporation caused up to ~40% of cells to lose high-level cell-surface expression of CXCR4, and edited cells could be enriched by sorting based on low CXCR4 expression. Importantly, Cas9 RNPs paired with homology-directed repair template oligonucleotides generated a high frequency of targeted genome modifications in primary T cells. Targeted nucleotide replacement was achieved in CXCR4 and PD-1 (PDCD1), a regulator of T-cell exhaustion that is a validated target for tumor immunotherapy. Deep sequencing of a target site confirmed that Cas9 RNPs generated knock-in genome modifications with up to ~20% efficiency, which accounted for up to approximately one-third of total editing events. These results establish Cas9 RNP technology for diverse experimental and therapeutic genome engineering applications in primary human T cells.

CRISPR/Cas9 genome engineering | Cas9 ribonucleoprotein | RNP | primary human T cells

The CRISPR/Cas9 system has been used increasingly to edit mammalian germline sequence and cell lines (1, 2). Considerable efforts are underway to use this powerful system directly in primary human tissues, but efficiency has been limited, especially in human CD4+ T cells. Plasmid delivery of cas9 and single-guide RNAs (sgRNAs) was efficient in other cell types, but ablated only 1–5% of target protein expression in CD4+ T cells (3). Improved ability to ablate key targets and correct pathogenic genome sequence in human T cells would have direct therapeutic applications, especially allowing T cells to be edited ex vivo and then reintroduced into patients.

Multiple scientific and clinical trials are underway to manipulate T-cell genomes with available technologies, including gene deletions with transcription activator-like effector nucleases and zinc finger nucleases and exogenous gene introduction by viral transduction (4, 5). Genetic manipulations have been attempted to “knock out” HIV coreceptors CXCR4 and CCR5 in T cells to gain resistance to HIV infection (6–8). There also has been marked success in engineering T cells to recognize and kill hematological malignancies, but additional genetic modifications appear necessary for solid organ tumor immunotherapy (9–11). Deletion of genes that encode key immune checkpoints such as PD-1 could prove useful for these efforts (12, 13). Further therapeutic opportunities would be possible if targeted T-cell genomic loci could be corrected with specific replacement sequence, rather than deleted (14). Efficient technology to promote homologous recombination in T cells could eventually allow therapeutic correction of mutations that affect specialized T-cell functions.

Recent reports in mammalian cell lines demonstrate that Cas9 ribonucleoproteins (RNPs; recombinant Cas9 protein complexed with an in vitro-transcribed single-guide RNA) can accomplish efficient and specific genome editing (15–17). Here we show that electroporation of Cas9 RNPs leads to efficient genome editing of CD4+ T cells. We were able to ablate a target gene with the random insertion and deletion mutations that likely result from nonhomologous end joining (NHEJ) repair of a Cas9-induced double-stranded DNA break (DSB). Cells with genomic edits in CXCR4 could be enriched by sorting based on low CXCR4 expression. We were also able to introduce precisely targeted nucleotide replacements in primary T cells at CXCR4 and PD-1 by homology-directed repair (HDR) using Cas9 RNPs and exogenous single-stranded DNA templates. This technology enabled Cas9-mediated generation of “knock-in” primary human T cells. Deep sequencing of a target site confirmed that Cas9 RNPs promoted knock-in

Significance

T-cell genome engineering holds great promise for cancer immunotherapies and cell-based therapies for HIV, primary immune deficiencies, and autoimmune diseases, but genetic manipulation of human T cells has been inefficient. We now have achieved efficient genome editing by delivering Cas9 protein pre-assembled with guide RNAs. These active Cas9 ribonucleoproteins (RNPs) enabled successful Cas9-mediated homology-directed repair in primary human T cells. Cas9 RNPs provide a programmable tool to replace specific nucleotide sequences in the genome of mature immune cells—a longstanding goal in the field. These studies establish Cas9 RNP technology for diverse experimental and therapeutic genome engineering applications in primary human T cells.


Conflict of interest statement: J.A.D. is a co-founder of Caribou Biosciences Inc. and Editas Medicine and is on the scientific advisory board of Caribou Biosciences Inc. The A. M. laboratory receives sponsored research support from Epimics. A patent has been filed based on the findings described here.

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Data deposition: The sequence reported in this paper has been deposited in the database (accession no. •••••).

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Results

We aimed to overcome long-standing challenges in genetic manipulation of primary T cells and establish an efficient genome engineering toolkit. Recent reports in mammalian cell lines suggest that Cas9 RNPs can accomplish efficient and specific genome editing (15–18). Given the significant challenges of efficient genome editing of T cells with DNA delivery of Cas9, we tested the efficacy of Cas9 RNP delivery for targeted genome editing in primary human T cells (Fig. 1L).

Ablation of HIV Coreceptor CXCR4 with Cas9 RNPs. A major goal in T-cell engineering is targeted ablation of specific cell-surface receptors, including coreceptors for HIV infection and coinhibitory immune checkpoints that impair tumor immune response. Here, we programmed the Cas9 RNPs to target the exonic sequence of CXCR4, which encodes a chemokine receptor with multiple roles in hematopoiesis and cell homing that is expressed on CD4+ T cells and serves as a coreceptor for HIV entry (19–21). We purified recombinant Streptococcus pyogenes Cas9 carrying two nuclear localization signal sequences fused at the C terminus. This Cas9 protein was incubated with in vitro-transcribed sgRNA designed to uniquely recognize the human CXCR4 genomic sequence (Fig. 1B). These preassembled Cas9 RNP complexes were electroporated into human CD4+ T cells isolated from healthy donors.

Electroporation of CXCR4 Cas9 RNPs caused efficient, site-specific editing of genomic DNA. The Cas9 RNP-induced DBSs in the CXCR4 gene were likely repaired by NHEJ, a predominant DNA repair pathway in cells that gives rise to variable insertions and deletions (indels) and often results in frameshift mutations and loss of gene function (22). Flow cytometry revealed a Cas9 RNP dose-dependent increase in the percentage of T cells expressing low levels of CXCR4, consistent with mutation of the CXCR4 gene (Fig. 1C). The T7 endonuclease 1 (T7E1) assay is a convenient method to assess editing at specific sites in the genome. Here, T7E1 assay confirmed genomic DNA editing at the CXCR4 locus in cells treated with CXCR4 Cas9 RNPs, but not in control cells treated with Cas9 protein alone (no sgRNA; CTRL). Cas9 RNP-treated cells were separated based on CXCR4 expression with fluorescence-activated cell sorting (FACS). Using the T7E1 assay, we found an enrichment of editing in the CXCR4hi cells (15–17%) compared with CXCR4lo cells (4–12% with varying doses of Cas9 RNP) (Fig. 1D). Sanger sequencing of the target CXCR4 genomic site, performed to directly identify editing events, suggested that the T7E1 assay may have underestimated editing efficiency. The T7E1 assay uses denaturation and hybridization of the wild-type and mutant sequences to create a mismatch DNA duplex, which is then digested by T7 endonuclease. However, hybridization of the mismatch duplex may be inefficient, especially when the indel mutation is drastically different from the wild-type sequence, making self-hybridization an energetically more favorable product. Other potential reasons for observed underestimation of editing efficiency with endonuclease assays include incomplete duplex melting, inefficient cleavage of single-base-pair indels, and deviation from the expected 300- and 600-bp products on the agarose gel as a result of large genome edits (23). Sequencing of the CXCR4 gene in CXCR4hi cells showed that 5/6 clones had mutations/deletions whereas such mutations/deletions were observed in only 4/10 clones and 0/9 clones in CXCR4lo and CTRL-treated CXCR4lo cells, respectively. Importantly, none of the observed edits in the CXCR4lo population terminated the coding sequence (one missense mutation and three in-frame deletions), consistent with the maintenance of protein expression. By contrast, the CXCR4hi population was enriched for cells with a more extensive mutational burden in the...
Efficient homology-directed repair allows 3% of all NHEJ events (defined as the sum of all NHEJ events in a given cell) to be repaired by Cas9 RNPs, indicating that a high fraction of editing resulted from HDR. The nearly complete loss of CXCR4 staining with addition of the HDR template suggests that the mutation introduced by HDR (84DLLFV88 → S4ESLDP88) strongly affected the cell-surface expression of CXCR4 or its recognition by the antibody (Fig. 2B and C). The editing efficiency was reduced with 200 pmol HDR template, perhaps as a result of cellular toxicity.

Both total editing and HDR could be enriched by sorting the CXCR4+ population, although the effect was less pronounced than in Fig. 1, consistent with the larger fraction of CXCR4 cells in the unsorted population. Note that in these experiments a more stringent gate was applied to separate the cells with the highest expression of CXCR4, and this CXCR4 population no editing was observed. These studies collectively demonstrated the power of Cas9 RNPs coupled with single-stranded oligonucleotide HDR template to precisely replace targeted DNA sequences in primary human T cells.

Deep Sequencing of Target Genomic DNA. Deep sequencing of the targeted CXCR4 locus allowed more detailed and quantitative analysis of genome-editing events. The results highlighted in Fig. 3 show the frequency of insertions, deletions, and HDR-mediated nucleotide replacement in CXCR4 Cas9 RNP-treated cells with or without CXCR4 HDR template compared with control-treated cells. In CXCR4 Cas9 RNP-treated cells, we found 55% of reads overlapping the CXCR4 target site containing at least one indel within a 200-nucleotide window centered around the expected cut site (Fig. 3A and B and Dataset S1). As discussed above, the T7E1 assays are useful for identifying edited loci, but may underestimate actual editing efficiency (quantitation of the T7E1 assay in Fig. 2D suggested 33% editing efficiency).

**Fig. 2.** Efficient homology-directed repair allows targeted DNA replacement in primary human T cells. (A) Schematic representation of single-stranded oligonucleotide HDR template with 90-nt homology arms designed to replace 12 nt, including the PAM sequence, to precisely replace targeted DNA sequences in primary human T cells. (B) Histograms of CXCR4 cell-surface expression in primary human T cells. (C) FACS plots (corresponding to histograms in B) show maximal ablation of CXCR4 with Cas9 RNP treatment and 100 pmol of HDR template. (D) T7E1 assay was used to estimate the “% Total Edit” (defined as the sum of all NHEJ and HDR events that gives rise to indels at the Cas9 cleavage site) was observed in the presence of 50 pmol CXCR4 HDR template, as estimated by T7E1 assays. At this concentration, 14% HDR was estimated by HindIII digest of the target locus, indicating that a high fraction of editing resulted from HDR (see results below for further quantification).
compared with the 55% editing efficiency computed by deep sequencing. We also sequenced the two top predicted "off-target" sites for the CXCR4 Cas9 RNP (Fig. 3B). Rare indels were observed at both off-target sites (∼1–2%), but at a rate comparable to that observed for those sites in the control cells treated with Cas9 protein only (∼1–2%) (Dataset S1).

The deep-sequencing results allowed quantitative analysis of observed indel mutations and their spatial distribution in the target region. Consistent with reports that S. pyogenes Cas9 cuts about three nucleotides upstream from the PAM sequence, we found the highest frequency of indels at four nucleotides upstream of the PAM (Fig. 3A). Indels were distributed throughout the sequenced region (Fig. 3C and D) with the majority of events near cut sites (>94% within 40 nucleotides). In CXCR4 Cas9 RNPs treated with 100 pmol of HDR template and 21% with 100 pmol HDR template (Fig. 3A). Of the reads with HDR template sequence incorporated, ∼14% of the detected HDR template reads contained additional nonspecific indels surrounding the incorporated HindIII site or other imperfect forms of editing within the 200-nucleotide window centered at the predicted cut site. However, the frequency of indels in reads with the HindIII site incorporated was reduced compared with reads where the HindIII site was not detected (Fig. 3C and D and Fig. S2). Interestingly, there was a consistent pattern of deletion events between CXCR4 Cas9 RNP with and without CXCR4 HDR template with an enrichment of deletions of 5 nucleotides (11%) and 22 nucleotides (5.4%) (Fig. S2). Replacement of the PAM sequence likely helped to limit re-cutting of knock-in sequence. Overall, 18–22% of reads (with varying concentrations of HDR template) had correctly replaced nucleotides throughout the sequenced genomic target site, suggesting that this approach could prove useful for generation of experimental and therapeutic nucleotide knock-in primary human T cells.

Specific Knock-In Targeting of Key Cell-Surface Receptors. To confirm that Cas9 RNPs mediate HDR at other genomic sites, we designed a guide RNA and HDR template to target the PD-1 (PDCD1) locus. PD-1 is an “immune checkpoint” cell-surface receptor found on the surface of chronically activated or exhausted T cells that can inhibit effective T-cell–mediated clearance of cancers. Monoclonal antibody blockade of PD-1 is approved for treatment of advanced malignancy, and genetic deletion of PD-1 may prove useful in engineering T cells for cell-based cancer immunotherapies (12). Primary human T cells were electroporated with a PD-1 Cas9 RNP and a PD-1 HDR template designed to generate a frameshift mutation and a knock-in HindIII restriction site in the first exon of PD-1, thereby replacing the PAM sequence (Fig. 4).

To examine the specificity of Cas9 RNP-mediated targeting, we compared PD-1 cell-surface expression following treatment with PD-1 Cas9 RNP vs. CXCR4 Cas9 RNP (which should not target
the PD-1 locus) or scrambled guide Cas9 RNP (no predicted cut within the human genome). We performed replicate experiments side by side with two different blood donors and with sgRNAs generated with two different in vitro transcription protocols (SI Materials and Methods). PD-1 Cas9 RNPs electroporated with PD-1 HDR template significantly reduced the percentage of cells with high PD-1 cell-surface expression relative to both CXCR4 Cas9 RNPs and scrambled guide Cas9 RNPs delivered with PD-1 HDR template (Fig. 4B). Similarly, CXCR4 Cas9 RNPs and CXCR4 HDR template caused a decrease in the CXCR4\textsuperscript{hi} cell population relative to both PD-1 and scrambled guide Cas9 RNP treatments with CXCR4 HDR template (Fig. 4C). Loss of CXCR4 was not a nonspecific effect of single-stranded DNA delivered along with CXCR4 Cas9 RNP; we observed a higher percentage of CXCR4-expressing cells after treatment with CXCR4 Cas9 RNP and scrambled HDR template than with CXCR4 Cas9 RNP and CXCR4 HDR template (Fig. S1A). These findings confirmed the target-specific modulation of cell-surface receptor expression in primary T cells with the programmable Cas9 RNP and HDR template treatments.

We then tested the specificity of HDR templates for nucleotide replacement (Fig. 4 D; examples of corresponding cell-surface expression data are shown in Fig. S1B). As expected, we observed efficient PD-1 editing by PD-1 Cas9 RNPs regardless of whether they were delivered with PD-1 HDR template, CXCR4 HDR template, or HDR template; in contrast, the HindIII site was incorporated into PD-1 only in the presence of both PD-1 Cas9 RNP and PD-1 HDR template, but not with CXCR4 HDR template, which should not be recombined at PD-1 locus due to the lack of sequence homology. Similarly, a HindIII site was incorporated only into CXCR4 following treatment with CXCR4 Cas9 RNP and CXCR4 HDR template; HDR was not observed at the CXCR4 locus with PD-1 HDR template, control scrambled HDR template (with a HindIII site), or without HDR template (Fig. 4D). Taken together, these studies established that specific pairing of a programmed Cas9 RNP and corresponding HDR template is required for targeted nucleotide replacement in primary human T cells.

**Discussion**

Cas9-mediated genome engineering has enormous potential to experimentally and therapeutically target DNA elements crucial for T-cell function. We report here successful genome engineering in human CD4\textsuperscript{+} T cells by delivery of in vitro-assembled Cas9 RNPs. Electroporation of Cas9 RNPs allowed targeted knock-out of the CXCR4 cell-surface receptor. Cas9 RNPs also promoted successful Cas9-mediated genetic knock-in of specific nucleotides to CXCR4 and PD-1 in primary human T cells. The efficient targeted DNA replacement in mature immune cells achieves a longstanding goal in the immunology field to enable diverse research and therapeutic applications. These studies collectively establish a broadly applicable toolkit for genetic manipulation of human primary T cells.

There are notable advantages to genome engineering with transient Cas9 RNP delivery compared with other CRISPR/Cas9 delivery methods. Recent work reported ablation of cell-surface markers in human CD4\textsuperscript{+} T cells by transfection of plasmid carrying the cas9 gene and guide RNA-coding sequence (3). Although successful, efficiency was notably low in CD4\textsuperscript{+} T cells compared with other cell types, possibly due to suboptimal levels of Cas9 or sgRNA, suboptimal nuclear translocation, or suboptimal intracellular Cas9 RNP complex formation (or some combination of these factors). Cas9 RNP-based delivery circumvents these challenges. Delivery of Cas9 RNPs offers fast editing action and rapid protein turnover in the cells as they are reportedly degraded within 24 h of delivery (15). This limited temporal window of Cas9 editing may make Cas9 RNPs safer for therapeutic applications than other

**Fig. 4.** Cas9 RNPs can be programmed for knock-in editing of PD-1 or CXCR4. (A) Schematic representation of the single-stranded PD-1 HDR template with 90-nt homology arms designed to replace 12 nt with 11 nt, introducing a novel HindIII restriction enzyme cleavage site to replace the PAM sequence (red), sgRNA target (blue) and PAM (green) sequences are indicated. (B) Histograms of PD-1 cell-surface expression levels assessed by flow cytometry. All cells were treated with 100 pmol of PD-1 HDR template. PD-1 Cas9 RNP-treated cells are shown in blue, CXCR4 Cas9 RNP-treated cells in light gray, and scrambled guide (no predicted cut within the human genome) Cas9 RNP-treated cells in dark gray. (C) Histograms of CXCR4 cell-surface expression levels assessed by flow cytometry. All cells were treated with 100 pmol of CXCR4 HDR template. CXCR4 Cas9 RNP-treated cells are shown in red, PD-1 Cas9 RNP-treated cells in light gray, and scrambled guide Cas9 RNP-treated cells in dark gray. (D) Results of four experiments with two different in vitro-transcribed and purified CXCR4 and PD-1 sgRNAs (SI Materials and Methods) tested in two different blood donors. For each blood donor, experiments done with phenolchloroform-extracted sgRNAs are shown on Top and experiments with PAGE-purified sgRNAs are shown at the Bottom; scrambled guides were prepared for both experiments with phenolchloroform extraction. Dotted line indicates gating on PD-1 high-expressing or CXCR4 high-expressing cells, respectively. The percentage of PD-1 high-expressing cells was significantly lower with PD-1 Cas9 RNP treatment compared either CXCR4 Cas9 HDR treatment (P < 0.001) or scrambled guide Cas9 RNP treatment (P < 0.001). The percentage of CXCR4 high-expressing cells was significantly lower with CXCR4 Cas9 HDR treatment compared with either PD-1 Cas9 HDR treatment (P < 0.001) or scrambled guide Cas9 RNP treatment (P < 0.001) (Pearson’s χ\textsuperscript{2}). (D) Genome editing was analyzed by T7E1 assay, whereas HDR was detected by HindIII digestion, which specificity, or without the newly integrated HindIII site; cleavage products for both assays are indicated with arrowheads. Concentrations of various HDR templates are indicated above the agarose gels. CTRL HDR template refers to a scrambled version of the original CXCR4 HDR template, including a HindIII restriction site. A nonspecific second gel band of unclear significance was noted in the T7E1 of the T7E1 digest.
delivery modes where cells are exposed to Cas9 for a longer time frame. Further testing will be needed to assess the purity of Cas9 RNPs and potential off-target effects before any clinical use. Our findings now suggest that Cas9 RNPs are able to rapidly and efficiently edit human T cells.

We were able to achieve remarkably efficient HDR here with almost complete loss of the CXCR4 \(^\text{681}\) cell populations with Cas9 RNPs and an HDR template targeting CXCR4 in one experiment. Up to 25% of the reads showed incorporation of HDR template sequence in the CXCR4 locus, and ∼20% of the reads showed correctly replaced sequence throughout the target site with no additional indels. Future studies will optimize remaining variables that affect editing and HDR efficiency in primary T cells. We recently demonstrated in cultured cell lines that variation in cell type and cell cycle dynamics significantly alter Cas9 RNP efficiency (16). In primary human T cells, editing efficiency could also be affected by T-cell donor-specific factors (e.g., genetics, recent infection), in vitro T-cell activation status, and characteristics of the targeted genomic locus (e.g., DNA sequence, chromatin state). Characterizing these variables and further optimizing genome engineering efficiency will accelerate the experimental and therapeutic editing of T cells using Cas9 RNPs.

The ability to edit specific DNA sequences in human T-cell subsets will enable experimental investigation of transcription factors, regulatory elements, and target genes implicated in T-cell inflammation and suppressive functions. Extensive efforts have mapped key gene regulatory circuitry controlling the development and function of diverse and specialized T-cell subsets (24). We recently reported that most causal genetic variants contributing to risk of human autoimmune diseases map to key regulatory elements in T cells (25). Looking forward, genome editing of primary T cells will now provide a powerful perturbation test to assess the function of regulatory elements and characterize the effects of disease-associated coding and noncoding variation.

Therapeutic editing requires improved techniques to identify successfully edited cells in a population. Selection of edited cells is notably challenging in primary cells that cannot be maintained in vitro indefinitely in culture, unlike transformed cell lines. Here we demonstrate FACS enrichment of edited cells based on expected phenotypic changes in cell-surface receptor expression. The success of Cas9 RNP-mediated HDR should also allow introduction of genetic markers to purify homogeneously edited cells for further functional characterization and potentially also for therapeutic applications.

Therapeutic T-cell engineering requires efficient and precisely targeted genome editing in primary cells. The Cas9 RNP technology reported here should accelerate efforts to correct genetic variants and engineer human T-cell function for the treatment of infection, autoimmunity, and cancer.

**Materials and Methods**

**Human T-cell isolation and culture.** Human primary T cells were either isolated from fresh whole blood or buffy coats. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll gradient centrifugation. CD4 \(^{+}\) T cells were pre-enriched with an EasySep Human CD4 \(^{+}\) T-cell enrichment kit (Stemcell Technologies) according to the manufacturer’s protocol. Pre-enriched CD4 \(^{+}\) T cells were stained with following antibodies: cCD4-PerCP (SK3; Becton Dickinson), cCD25-APC (BC96; TonBioscience), cCD127-PE (RS3-44; TonBioscience), cCD45RA-violet-Fluor450 (H100; TonBioscience), and cCD45RO-FITC (UCHL1; TonBioscience). CD4 \(^{+}\)CD25\(^{hi}\)/CD127\(^{lo}\) T effectors (Teffs) were isolated using a FACS Aria IIlu (Becton Dickinson).

**Cas9 RNP Assembly and Electroporation.** Cas9 RNP was prepared immediately before experiments by incubating 20 μM Cas9 with 20 μM sgRNA at 1:1 ratio in 20 μM Hepes (pH 7.5), 150 mM KCl, 1 mM MgCl\(_2\), 10% glycerol and 1 μM O\(_{6}\)-methyl-2′-deoxyadenosine (2′-dC) 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).

**Analysis of Genome Editing.** Editing efficiency was estimated by T7 endonuclease I assay. HDR templates were designed to introduce a HindIII restriction site into the targeted gene loci; successful HDR was confirmed with HindIII restriction enzyme digestion. The genomic region flanking the Cas9 target site for HDR was amplified by the two-step PCR method. Sequencing libraries were sequenced with the Illumina HiSeq 2500. See SI Materials and Methods for further details.

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Supporting Information

Schumann et al. 10.1073/pnas.1512503112

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 vacuum 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 CaCl₂ and MgCl₂ free HBSS. Buffoy 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 pre-enriched with an 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 (SK; Becton Dickinson), αCD25-APC (BC96; TONBO Biosciences), αCD127-PE (R34-34; TONBO Biosciences), αCD45RA-PEC (HL100; TONBO Biosciences), and αCD45RO-Flt3L (UCHL1; TONBO Biosciences). CD4⁺CD25⁺CD127⁻ Teffs were isolated using a FACS Aria III (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. Buffoy 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 (Gibco), 50 μg/mL penicillin/streptomycin (Corning), 5 μmol/L sodium pyruvate (UCSF CCF), and 10% FBS (Atlanta Biologicals). After electroporation the medium was supplemented with 40 μM 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 the nuclear membrane. The protein was expressed with a N-terminal hexahistidine tag and maltose binding protein in the nuclear membrane.

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 (Ulitranger) sequences: CXC4R1_1: 5′-TATA TAC GAC TCA CTA TAG GAA GCG TGA TGA CAA AGA AGG TTT TAG AGC TAT GCT GTA AGC AGC AGA ATA GCA GAT TAA AAT AAG G -3′ and SLKSI (5′-GCA CGT CCA TGG TGC CAC TAT TTT TTC AAG AAG) 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:isoamyl alcohol and then once with chloroform before isopropanol precipitation overnight at −20 °C. The DNA pellet was washed three times with 70% ethanol, dried by vacuum, and dissolved in DEPC-treated water. The PD-1 sgRNA template was assembled from T25, SLKSI, SLK2O, and SLKSI15 (5′-TAA TAC GAC TCA CTA TAG GAA GCG TGA TGA CAA AGA AGG TTT TAG AGC TAT GCT GGA AAC AGC ATA GCA AGT TAA AAT AAG 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 MgCl₂, 0.01% Triton X-100, 2 μM 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 2x STOP solution (95% deionized formamide, 0.05% bromophenol blue, and 20 mM EDTA) at 60 °C for 5 min. The RNA was purified by electrophoresis in 10% 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% 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% glycerol, and 1 μM TCEP. The sgRNA was heated to 70 °C for 5 min and cooled to room temperature. MgCl₂ 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 OD₂₆₀nm using Nanodrop and adjusted to 100 μM using 20 mM Hepes (pH 7.5), 150 mM KCl, 10% glycerol, 1 μM TCEP, and 1 mM MgCl₂. The sgRNA was stored at −80 °C.

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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 Optimization 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 DNase I (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. S14. Phenol/chloroform extraction was performed after addition of 190 µL RNA-free H₂O. sgRNA was precipitated with 80 µL 3 M sodium acetate and 420 µL isopropanol and incubation at −20 °C for 4 h. The RNA pellet was washed twice with 70% EtOH and once with 100% 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 RNP was prepared immediately before experiments by incubating 20 µM Cas9 with 20 µg sgRNA at a 1:1 ratio in 20 µL HEPES (pH 7.5), 150 mM KCl, 1 mM MgCl₂, 10% 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 × 10⁶ T cells was washed three times with PBS before resuspension in 8 µL 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, 20 µF, 100 Ω, 35 °C at 75 °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 reassayed DNA. After 1 h of incubation at 37 °C, the reaction was quenched with 6x 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 measured using Image Lab. The percentage of editing was calculated using the following equation: [1 - (1 - 1)/(1 + b + a + b + c)] × 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. 4D), 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

FACS Analysis of Edited T Cells. CXCR4 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 III (Becton Dickinson).

PCR Amplification of Target Region. A total of 5 × 10⁴ to 2 × 10⁵ cells were resuspended in 100 µL of Quick Extraction solution (Ep-center) 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 GAA GAT GTG ACT TTG AAA CC-3′ and reverse 5′-GGA CAG GAT GAC ATT ACC AGG CAG GAT AAG GCC-3′ (938 bp); and for PD-1—forward 5′-GGG GCT CAT CCC ATC TTC AG-3′ and reverse 5′-GCC ACA GGA 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 1 cycle of 72 °C for 1 min. The PCR products were purified on 2% 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. 1E, 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 reassayed DNA. After 1 h of incubation at 37 °C, the reaction was quenched with 6x 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 - 1)/(1 + b + a + b + c)] × 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. 4D), 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.
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% 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 region flanking the Cas9 target site for the CXCR4 on-target and two off-target sites was 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 ACT GAT GAA GGC CAG GAT GAC GAC-3'); off-target #1 [POU domain, class 2, transcription factor 1 isofom #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 ACT GCT GAT GTA GCG GTC GGC GTC CAG ACT GAT GAA GGC CAG GAT GAC GAC-3'); off-target #2 glutamate receptor 1 isofom #2 (GRIAI) locus; 5'-ACA CTC TTT CCC TAC ACG ACG CTC TTC CGA TCT NNN NNC CTG GTT GTC CAA GCC CAG CCC CAG TTA TTC ATC ACG TGT GCT CTT CCG ATC TNN NNN ACT CTG CAC TGG TAT ATC AAT ACA 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% agarose gel, stained with SYBR Gold, and gel-extracted using Qiagen gel extraction kit.

Illumina TrueSeq Universal adapter (5'-AAAT GAT AGC GCG ACC ACC GAG TAT ACT CCT TCC CTA CAC GAC GCT TTT CCG ATC T-3') and modified Illumina RNA PCR barcode primer (5'-CAAG GCA GAA GAC GGC ATGA CGA GAT-Index GTG ACT GGA GTT CAG ACG TGT GCT CTT CCG ATC T-3') were attached to the ampiclon 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% 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 BWA. 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 did not contain the 12-nt replacement derived from HDR (these are the fractions shown in Fig. 3B). 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.
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 on Bottom.

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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).
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.
Dataset S1. Summary of editing frequencies based on deep sequencing

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).
Q: 1_In sentence beginning "The DNA pellet was washed..." please replace DEPC with its definition.
Q: 2_Please clarify "(- strand)" in sentence beginning "The HDR templates for..."
Q: 3_In sentence beginning "The hybridization reaction contained...." is "°C/s" as intended without number of seconds indicated here and below?
Q: 4_In sentence beginning "All reads that did not contain...." please replace "BWA" with its definition.