

Peptide-mediated delivery of CRISPR enzymes for the efficient editing of primary human lymphocytes

In the format provided by the authors and unedited

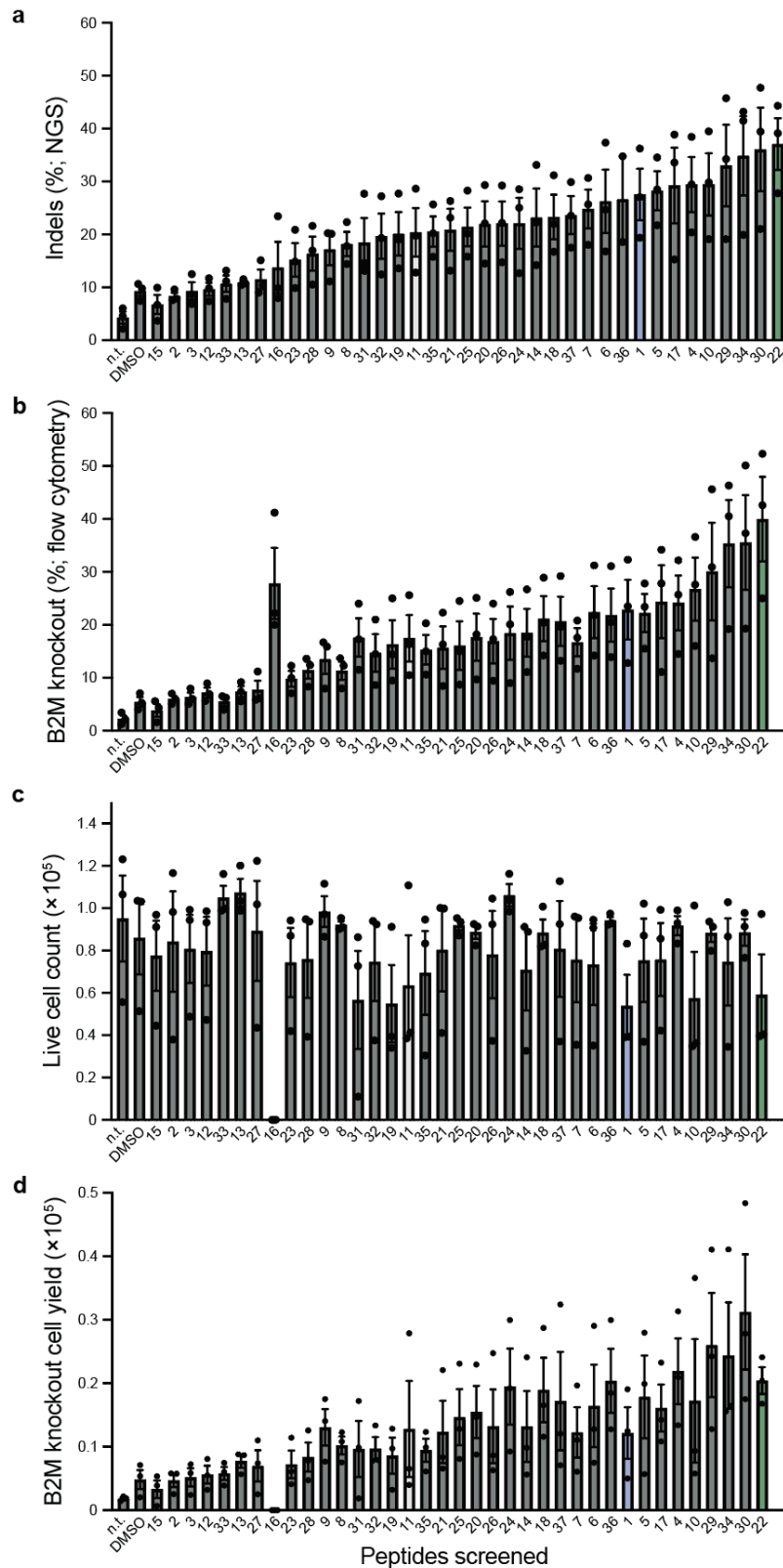
Contents

Supplementary figures

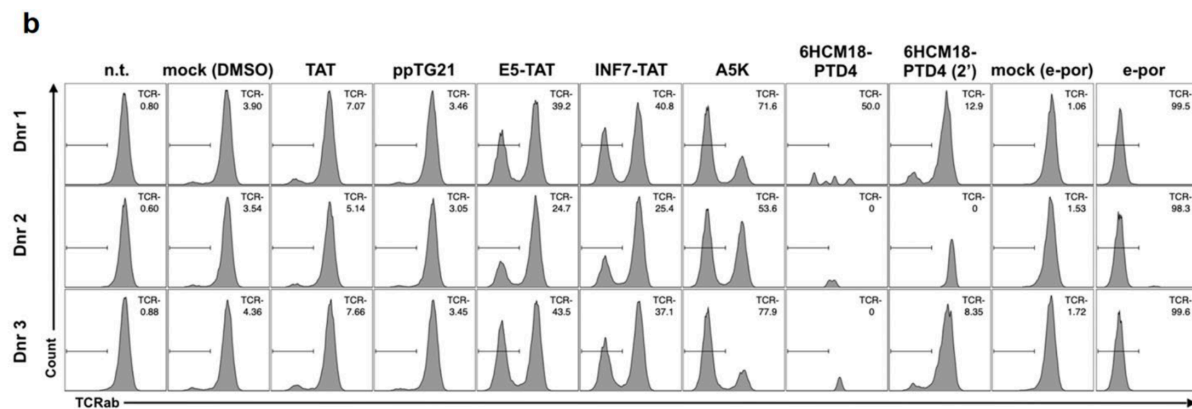
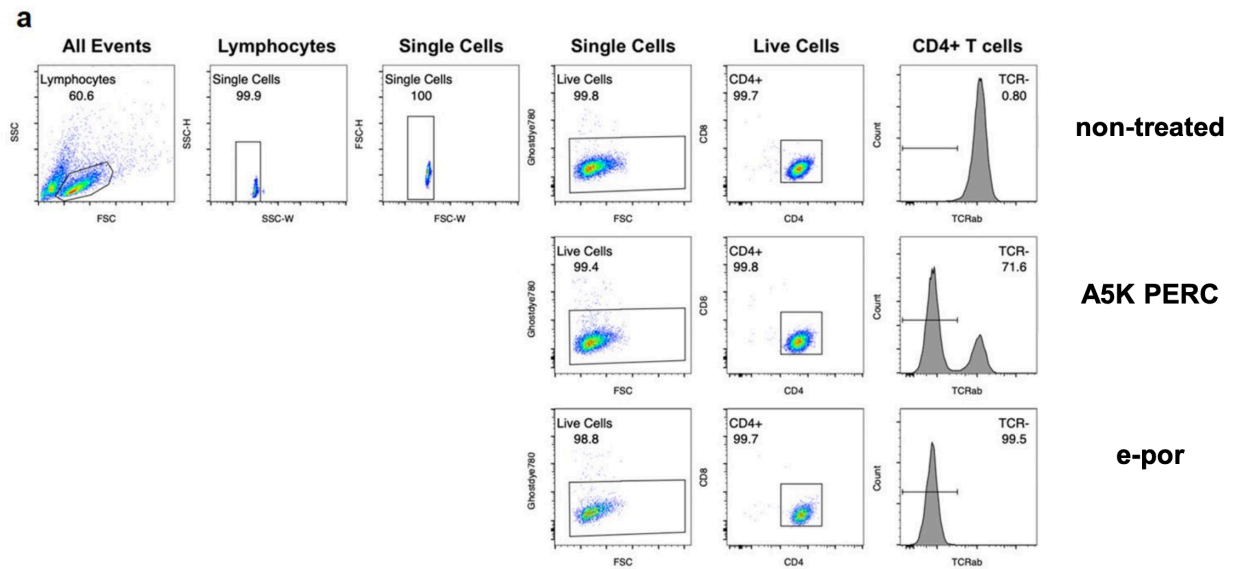
- 1 Arrayed screening of 37 peptides for Cas9 RNP delivery
- 2 Representative flow cytometry gating to assess knockout of TCR surface expression in CD4⁺ T cells
- 3 Peptide-enabled Cas9 *TRAC*-RNP delivery to CD4⁺ T cells
- 4 Dynamic light scattering analysis
- 5 Editing efficiency and viability following PERC or electroporation of various Cas9 enzymes
- 6 PERC in primary human B cells and primary human NK cells
- 7 Representative flow cytometry gating for assessing knockout of CD45 surface expression in B cells (related to Supplementary Fig. 6a)
- 8 Representative flow cytometry gating for assessing knockout of CD45 surface expression in B cells (related to Supplementary Fig. 6b)
- 9 Amino acid sequence of the ABE8e-SpCas9-NG adenine base editor
- 10 Peptide-enabled base editor RNP delivery to T cells to abrogate the start codon of *CCR5*
- 11 Base editing outcomes following PERC
- 12 Base editing outcomes following electroporation
- 13 Comparison of PERC and electroporation with an ssODN HDRT
- 14 Flow cytometry gating to assess CD5 knockout and FLAG-tag knock-in in CD4⁺ T cells
- 15 Gene expression analysis
- 16 A5K peptide does not promote an antigen-specific T cell response
- 17 Sequential vs. simultaneous editing (related to Fig. 1d and Fig. 3a,b)
- 18 Sequential vs. simultaneous editing (related to Fig. 1d and Fig. 3c,d)
- 19 Analysis of off-target effects
- 20 Schematic of sequential editing of three loci in CD3⁺ T cells
- 21 Representative flow cytometry gating for assessing TCR surface expression following Cas9 *TRAC*-RNP delivery to and CAR AAV transduction of CD3⁺ T cells (related to Fig. 4b)
- 22 Representative flow cytometry gating for assessing TCR surface expression following Cas9 *TRAC*-RNP delivery to CD3⁺ T cells (related to Fig. 4c)
- 23 Flow cytometry assessment of CD62L/CD45RA phenotypes in CD4⁺ and CD8⁺ T cells
- 24 Comparison of phenotypes in CD4⁺ T cells
- 25 Comparison of phenotypes in CD8⁺ T cells
- 26 Representative flow cytometry gating for assessing CD62L/CD45RA phenotypes
- 27 T cell editing for the extended culture experiment
- 28 T cell editing for the repetitive stimulation experiment
- 29 T cell editing for the *in vivo* tumor challenge experiment
- 30 Bioluminescence imaging trace for each mouse; associated with data in Fig. 5g

Supplementary tables

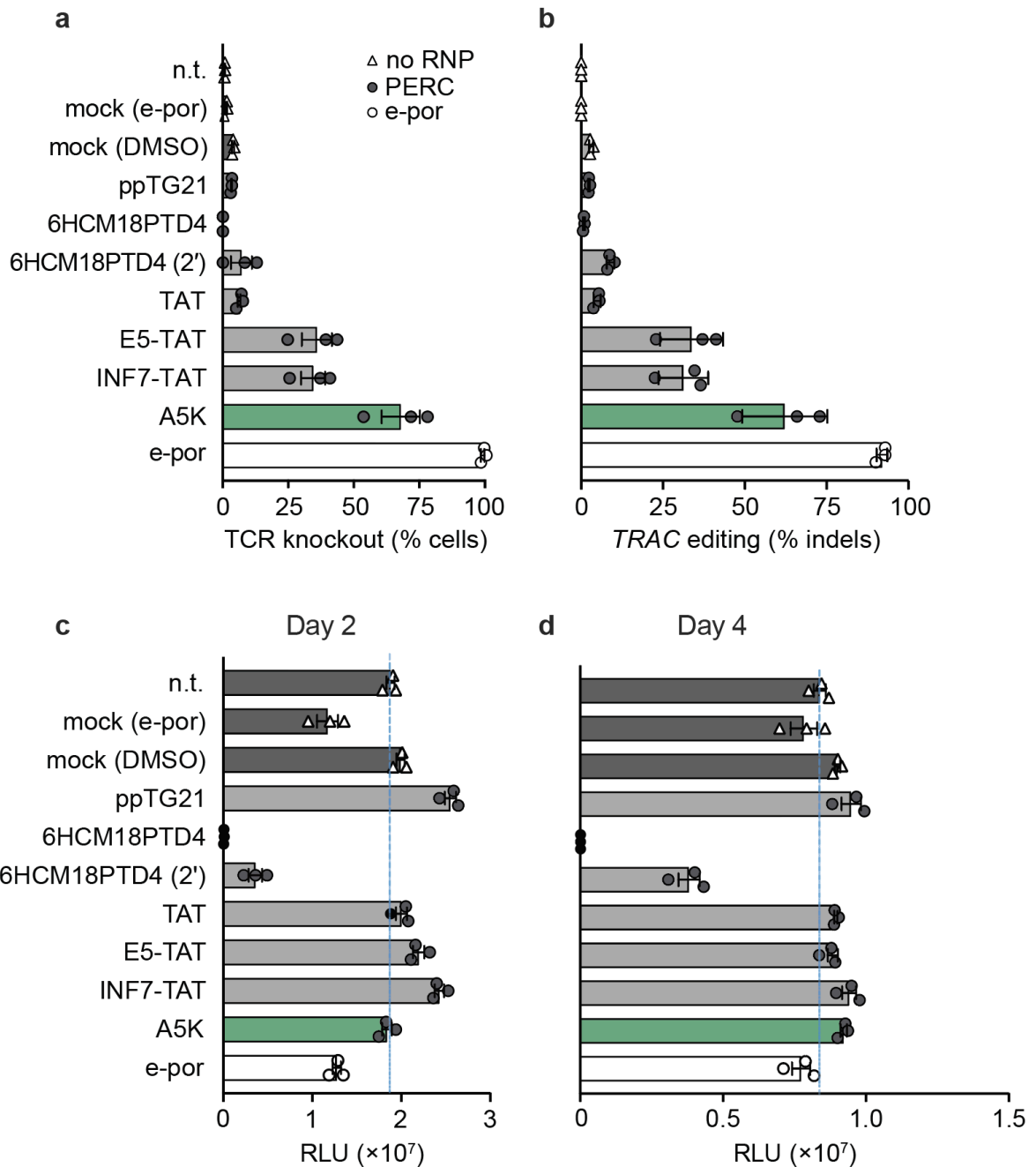
- 1 Peptides screened in Supplementary Fig. 1
- 2 Guide RNAs used
- 3 Antibodies used for flow cytometry analysis
- 4 Oligonucleotides used for ddPCR or for generating amplicons that were analyzed by NGS



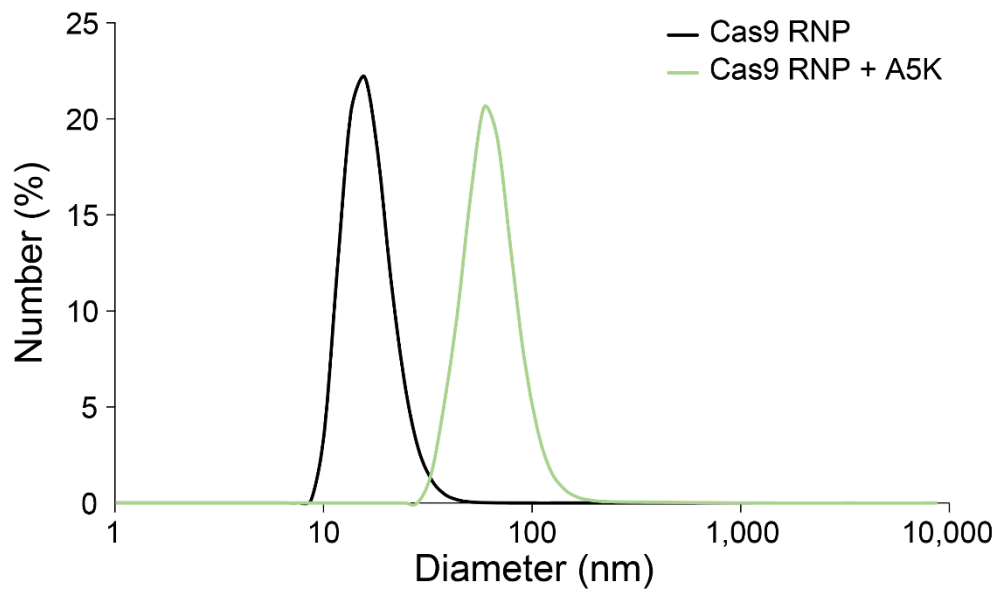
Supplementary Fig. 1 | Arrayed screening of 37 peptides for Cas9 RNP delivery. The bars in blue (#1, E5-TAT), light gray (#11, INF7-TAT), or green (#22, A5K) correspond to colors in **Supplementary Table 1**, and represent the peptides also tested in **Fig. 1c**. Peptides were evaluated three days after *B2M*-Cas9 RNP delivery to CD4⁺ T cells on the basis of **a**, indels produced at the *B2M* locus assessed by amplicon-based NGS (with efficiency being used to sort the peptides, an order maintained for the other plots), **b**, knockout of *B2M* surface expression assessed by flow cytometry, **c**, viability of cells evaluated by staining for dead cells with GhostDye780, and **d**, comparison of edited cell yield. Few cells survived PERC using the lysine-rich peptide 16, which likely underlies the incongruity between NGS and flow results (i.e. relative ranking).



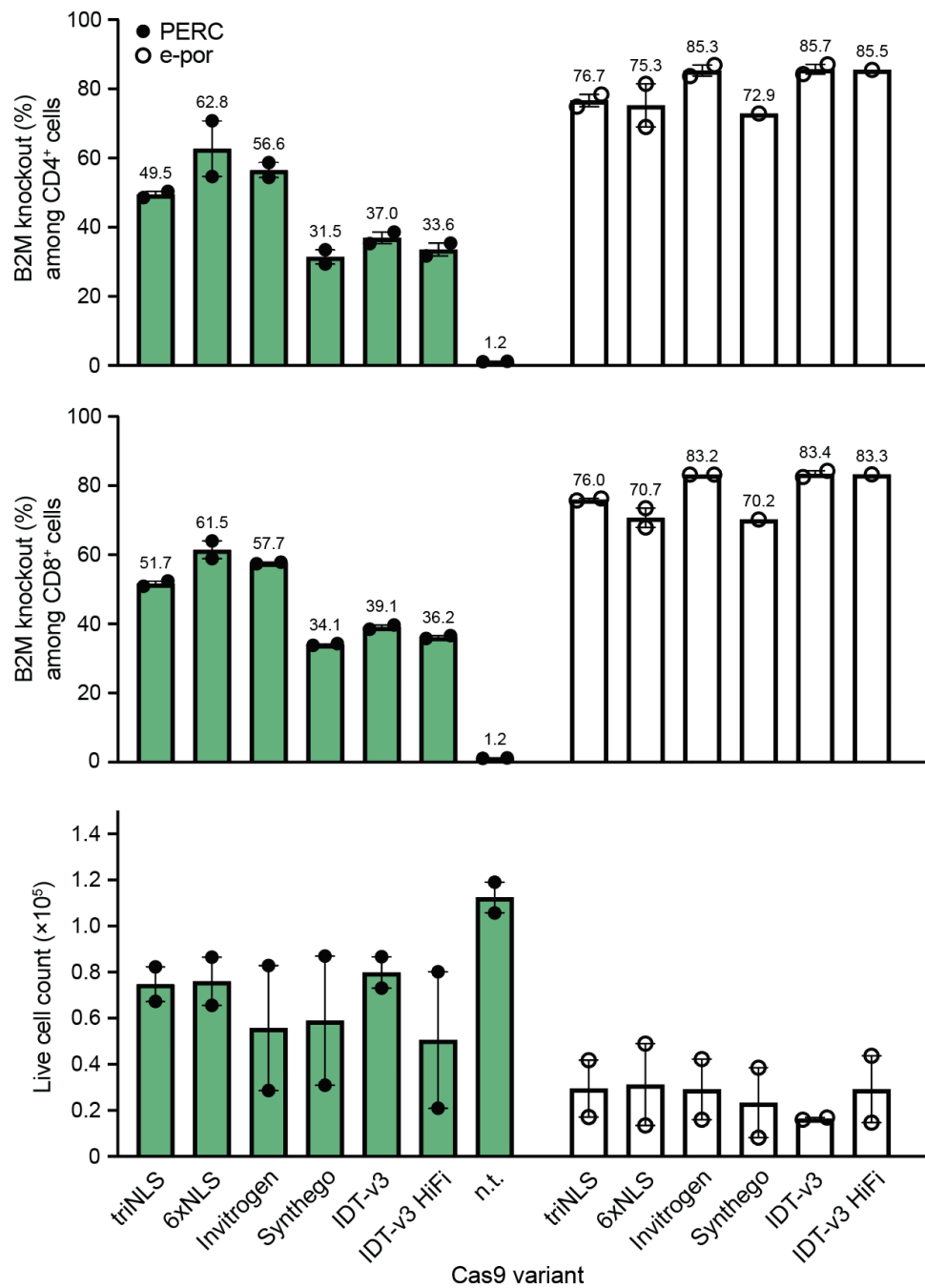
Supplementary Fig. 2 | a, Representative flow cytometry gating to assess knockout of TCR surface expression in CD4⁺ T cells. Data are related to **Fig. 1c**. **b**, Cas9 *TRAC*-RNP was delivered using various peptides or electroporation. Negative controls: mock peptide (DMSO only), mock electroporation, and non-treated (n.t.). Dnr = donor.



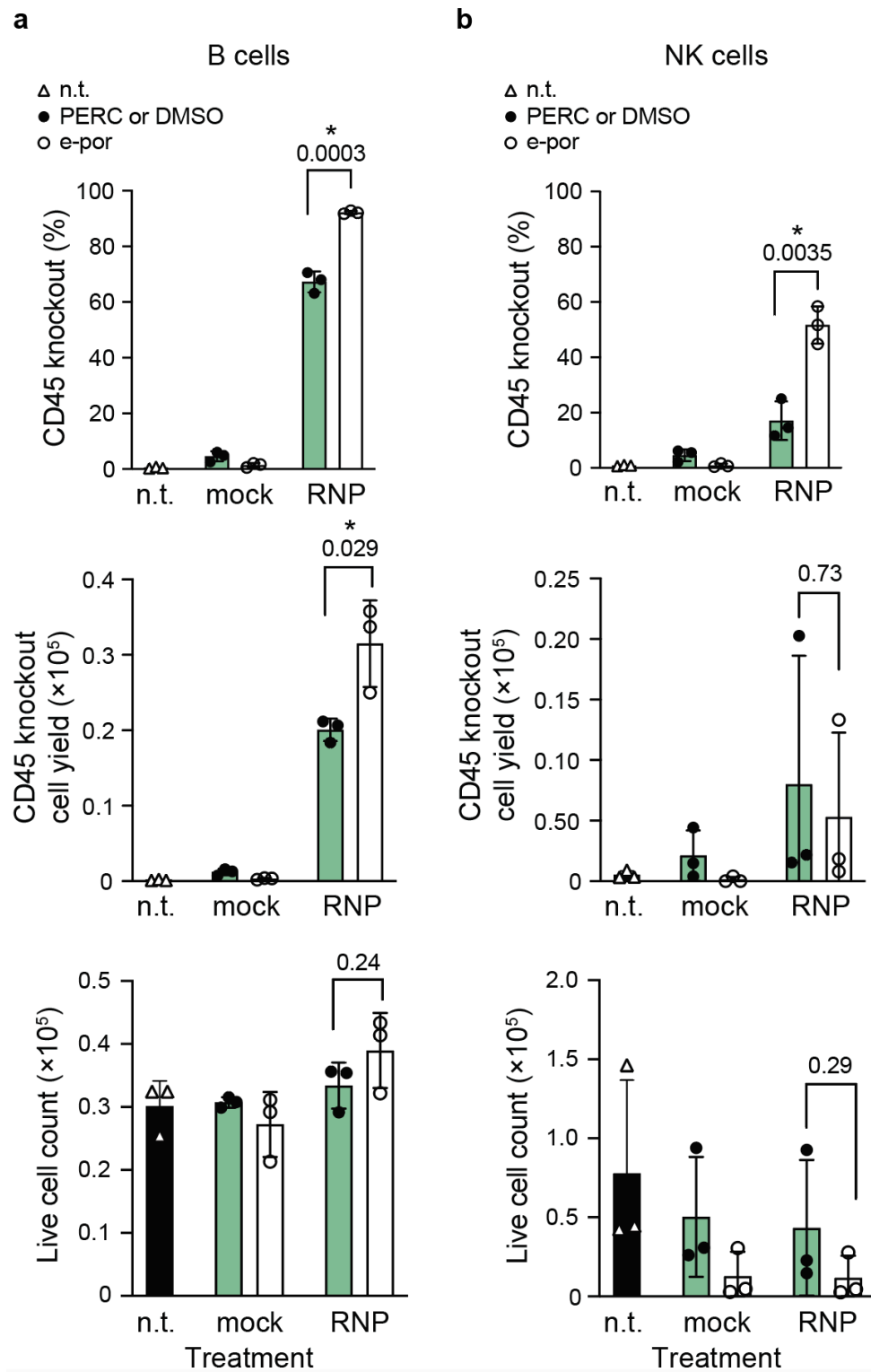
Supplementary Fig. 3 I Peptide-enabled Cas9 *TRAC*-RNP delivery to CD4⁺ T cells. Peptides' capacity for delivery was assessed based on **a**, flow cytometry for loss of TCR surface expression (also in **Fig. 1c**) and **b**, NGS for indels at the *TRAC* locus. **c,d**, Cell viability assay at **c**, two days after treatment (also in **Fig. 1c**) and **d**, four days after treatment. 2' = two-minute treatment and subsequent wash; all other treatments remained on cells. n.t. = non-treated, mock = DMSO only, or electroporated (no RNP). n=3 biological replicates from distinct human donors. Bars represent mean, and error bars represent \pm S.E.M.



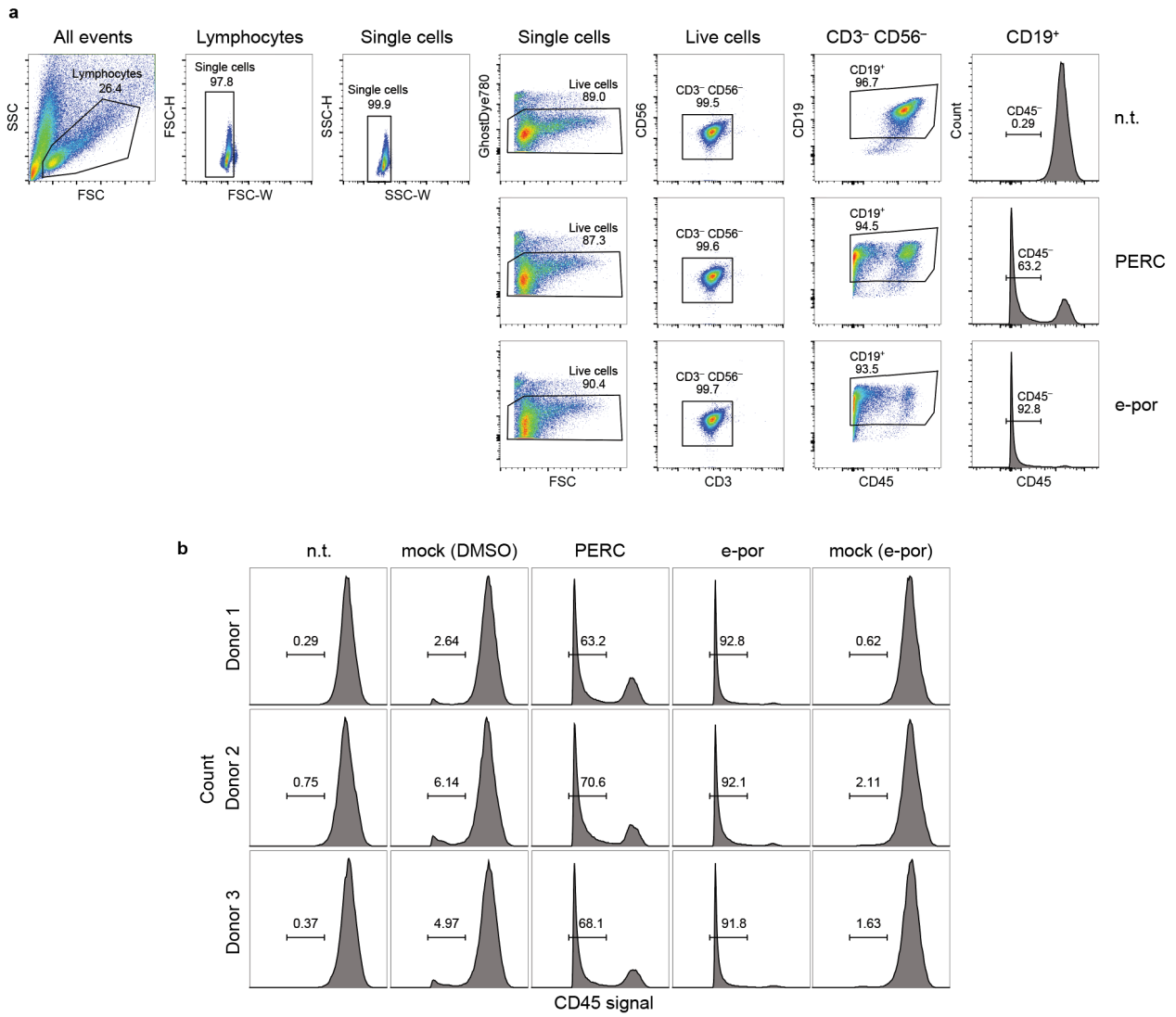
Supplementary Fig. 4 I Dynamic light scattering analysis. The analysis (performed “by number”) is of Cas9 *B2M*-RNP with and without the addition of A5K peptide in DMSO (1% v/v DMSO final). RNP alone is primarily ~20 nm particles: approximately the diameter expected based on prior 3D structures of CRISPR-Cas9 (~15 nm). In the presence of A5K peptide, ~60 nm particles are observed, most with a diameter <100 nm.



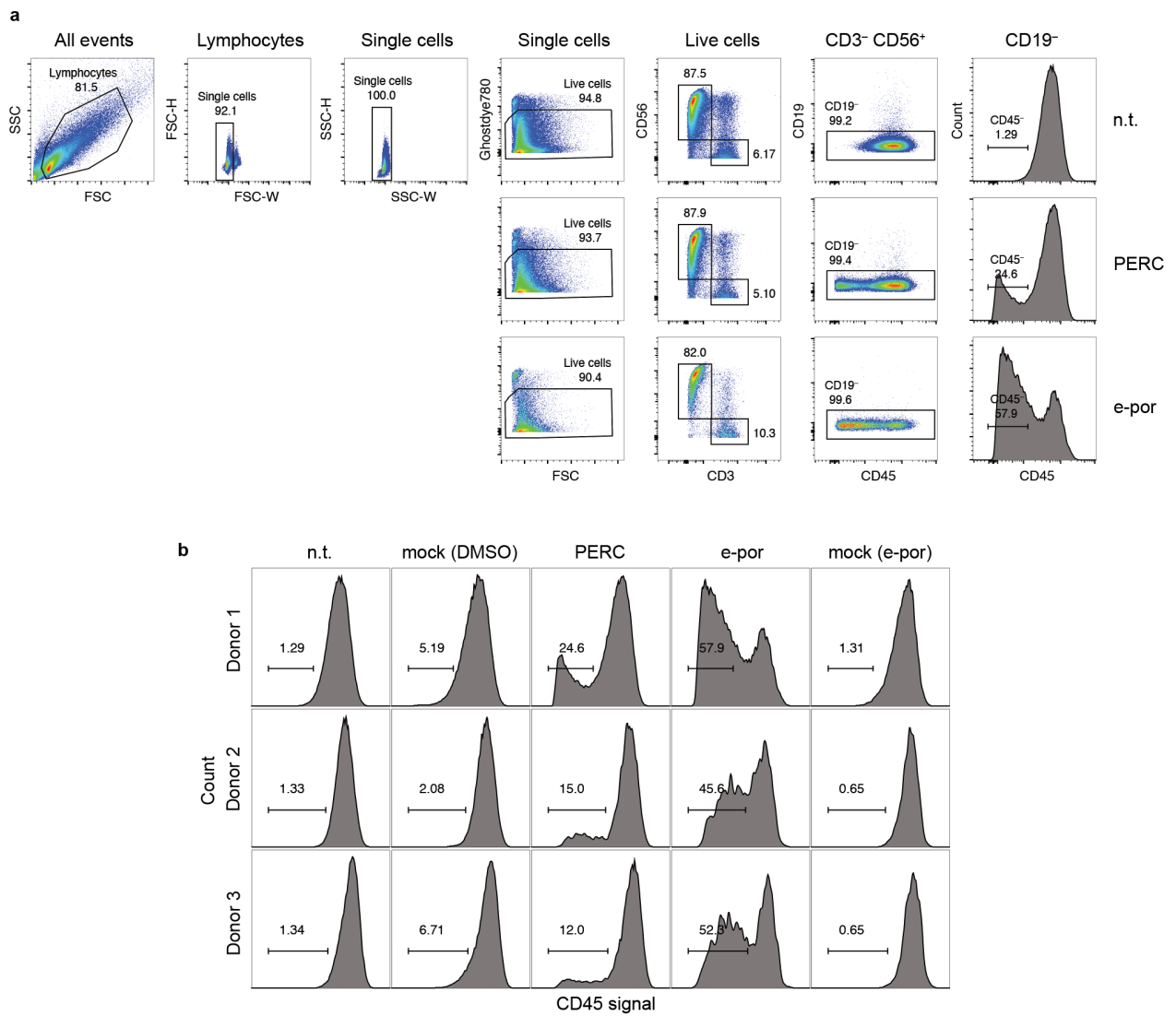
Supplementary Fig. 5 | Editing efficiency and viability following PERC or electroporation of various Cas9 enzymes. Two published NLS-rich constructs, triNLS and 6xNLS, were purified in academic labs (**Methods**). 6xNLS Cas9 was used in all other Cas9 nuclease experiments in this study. The other four constructs are from commercial vendors. Invitrogen: Invitrogen TrueCut Cas9 Protein v2 (ThermoFisher). Synthego: SpCas9 2NLS (Synthego). IDT-v3: Alt-R S.p. Cas9 Nuclease V3 (IDT). IDT-v3 HiFi: Alt-R S.p. HiFi Cas9 Nuclease V3 (IDT). n=2 biological replicates from distinct human donors. Bars represent mean, and error bars represent \pm S.E.M.



Supplementary Fig. 6 I PERC in primary human B cells and primary human NK cells. Peptide-enabled Cas9 *CD45*-RNP delivery to **a**, B cells and **b**, NK cells as measured by flow cytometry for CD45 surface expression. Results are presented as knockout frequency, edited cell yield, and total live cell count (independent of editing outcome). $n=3$ biological replicates from distinct human donors. Bars represent mean. Error bars represent S.D. P -values are from two-tailed unpaired t -tests with Holm-Sidak's correction method for multiple comparisons.



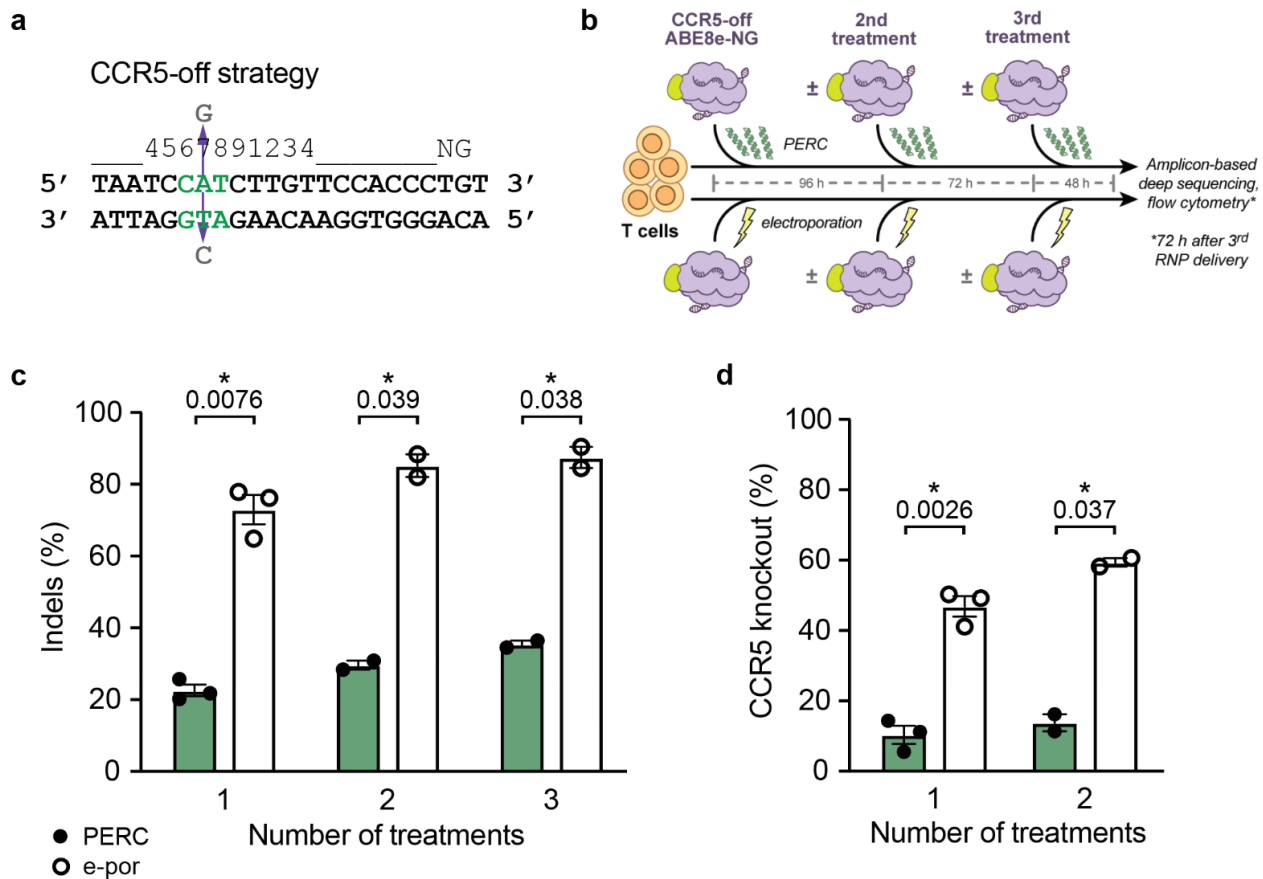
Supplementary Fig. 7 | a, Representative flow cytometry gating for assessing knockout of CD45 surface expression in B cells (related to **Supplementary Fig. 6a**). **b**, Cas9 *CD45*-RNP was delivered via PERC or electroporation (e-por), and frequencies of CD45 KO were assessed and compared to mock peptide (DMSO-treated), mock electroporated, and non-treated (n.t.) cells.



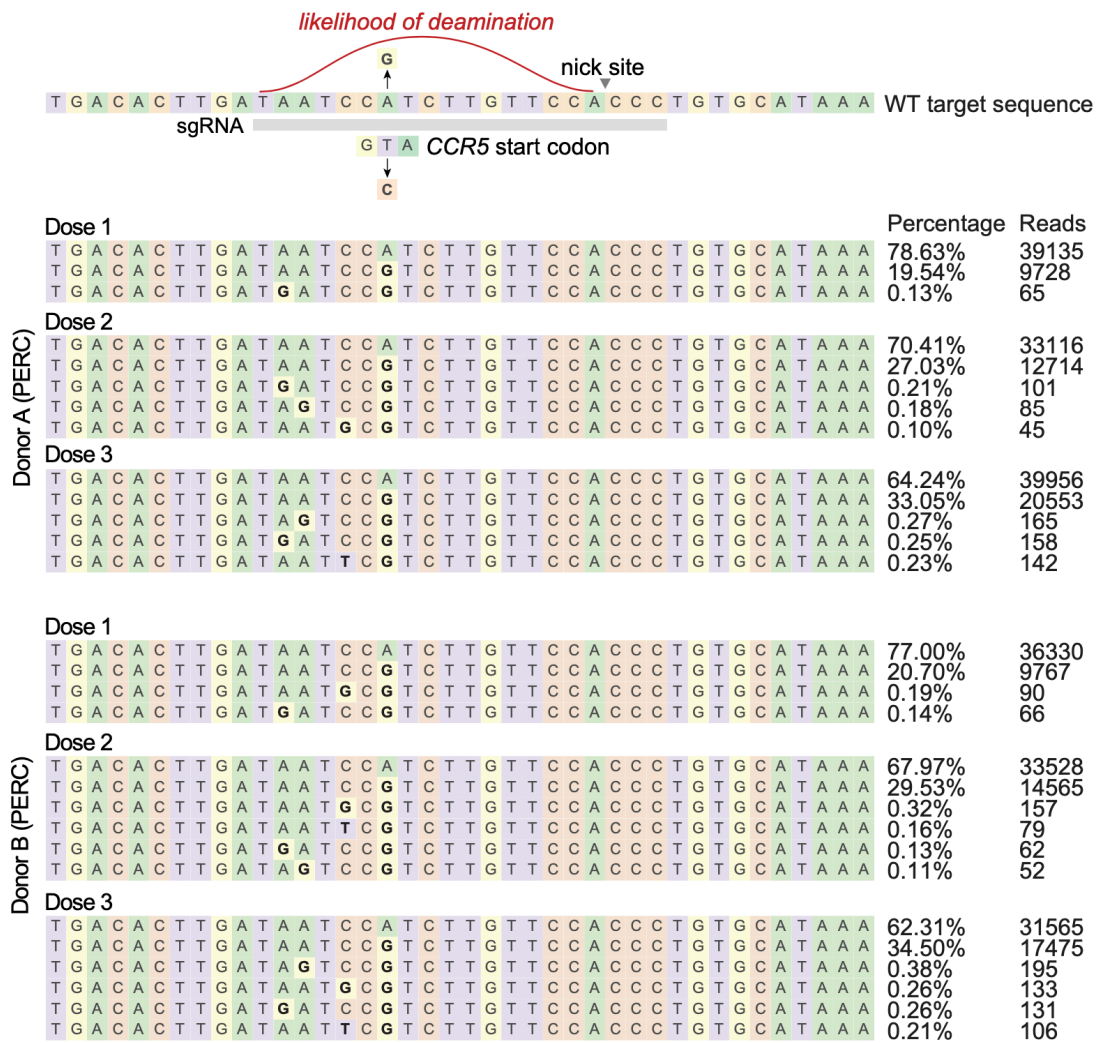
Supplementary Fig. 8 | a, Representative flow cytometry gating for assessing knockout of CD45 surface expression in NK cells (related to **Supplementary Fig. 6b**). **b**, Cas9 *CD45*-RNP was delivered via PERC or electroporation (e-por), and frequencies of CD45 KO were assessed and compared to mock peptide (DMSO-treated), mock electroporated, and non-treated (n.t.) cells.

MK**R**T**A**D**G**S**E**F**E**S**P**K**K**K**R**K**V**S**E**V**E**F**S**H**E**Y**W**M**R**H**A**L**T**L**A**K**R**A**W**D**E**R**E**V**P**V**G**A**V**L**V**H**N**N**R**V**I**G**E**G**W**N**R**P**I**G**R**H**D**P**T**A**H**A**A**I**M**A**L**
 R**Q**G**G**L**V**M**Q**N**Y**R**L**I**D**A**T**L**Y**V**T**L**E**P**C**V**M**C**A**G**A**M**I**H**S**R**I**G**R**V**V**F**G**A**R**D**A**K**T**G**A**A**G**S**L**M**D**V**L**H**H**P**G**M**N**H**R**V**E**I**T**E**G**I**L**A**D**E**C**A**A**L
 L**S**D**F**F**R**M**R**R**Q**E**I**K**A**Q**K**K**A**Q**S**S**T**D**S**G**G**S**S**G**G**S**S**G**S**S**E**T**P**G**T**S**E**S**A**T**P****E**S**S**G**G**S**S**G**G**S**S**E**V**E**F**S**H**E**Y**W**M**R**H**A**L**T**L**A**K**R**A**R**D**E**R**E
 V**P**V**G**A**V**L**V**L**N**N**R**V**I**G**E**G**W**N**R**A**I**G**L**H**D**P**T**A**H**A**E**I**M**A**L**R**Q**G**G**L**V**M**Q**N**Y**R**L**I**D**A**T**L**Y**V**T**F**E**P**C**V**M**C**A**G**A**M**I**H**S**R**I**G**R**V**V**F**G**V**R**N
 S**K**R**G**A**A**G**S**L**M**N**V**L**N**Y**P**G**M**N**H**R**V**E**I**T**E**G**I**L**A**D**E**C**A**A**L**L**C**D**F**Y**R**M**P**R**Q**V**F**N**A**Q**K**K**A**Q**S**S**I**N**S**G**G**S**S**G**G**S**S**G**S**S**E**T**P**G**T**S**E**S**A**T**P**
ES**S**G**G**S**S**G**G**S**D**K**K**Y**S**I**G**L**A**I**G**T**N**S**V**G**W**A**V**I**T**D**E**Y**K**V**P**S**K**K**F**K**V**L**G**N**T**D**R**H**S**I**K**K**N**L**I**G**A**L**L**F**D**S**G**E**T**A**E**A**T**R**L**K**R**T**A**R**R**R**Y**
 T**R**R**K**N**R**I**C**Y**L**Q**E**I**F**S**N**E**M**A**K**V**D**D**S**F**F**H**R**L**E**E**S**F**L**V**E**E**D**K**K**H**E**R**H**P**I**F**G**N**I**V**D**E**V**A**Y**H**E**K**Y**P**T**I**Y**H**L**R**K**K**L**V**D**S**T**D**K**A**D**L**R**L
 I**Y**L**A**L**A**H**M**I**K**F**R**G**H**F**L**I**E**G**D**L**N**P**D**N**S**D**V**D**K**L**F**I**Q**L**V**Q**T**Y**N**Q**L**F**E**E**N**P**I**N**A**S**G**V**D**A**K**A**I**L**S**A**R**L**S**K**S**R**R**L**E**N**L**I**A**Q**L**P**G**E**K**K
 N**G**L**F**G**N**L**I**A**L**S**L**G**L**T**P**N**F**K**S**N**F**D**L**A**E**D**A**K**L**Q**L**S**K**D**T**Y**D**D**D**L**D**N**L**L**A**Q**I**G**D**Q**Y**A**D**L**F**L**A**A**K**N**L**S**D**A**I**L**L**S**D**I**L**R**V**N**T**E**I**T**K**A
 P**L**S**A**S**M**I**K**R**Y**D**E**H**H**Q**D**L**T**L**L**K**A**L**V**R**Q**L**P**E**K**Y**K**E**I**F**F**D**Q**S**K**N**G**Y**A**G**Y**I**D**G**G**A**S**Q**E**E**F**Y**K**F**I**K**P**I**L**E**K**M**D**G**T**E**E**L**L**V**K**L**N**R**E**
 D**L**L**R**K**Q**R**T**F**D**N**G**S**I**P**H**Q**I**H**L**G**E**L**H**A**I**L**R**R**Q**E**D**F**Y**P**L**K**D**N**R**E**K**I**E**K**I**L**T**F**R**I**P**Y**V**G**P**L**A**R**G**N**S**R**F**A**W**M**T**R**K**S**E**E**T**I**T**P**W**N
 F**E**E**V**V**D**K**G**A**S**A**Q**S**F**I**E**R**M**T**N**F**D**K**N**L**P**N**E**K**V**L**P**K**H**S**L**L**Y**E**F**T**V**Y**N**E**L**T**K**V**K**Y**V**T**E**G**M**R**K**P**A**F**L**S**G**E**Q**K**A**I**V**D**L**L**F**K**T**N**R**K
 V**T**V**K**Q**L**K**E**D**Y**F**K**K**I**E**C**F**D**S**V**E**I**S**G**V**E**D**R**F**N**A**S**L**G**T**Y**H**D**L**L**K**I**I**K**D**K**D**F**L**D**N**E**E**N**E**D**I**L**E**I**V**L**T**L**T**L**F**E**D**R**E**M**I**E**E**R**L**K**T**Y**
 A**H**L**F**D**D**K**V**M**K**Q**L**K**R**R**R**Y**T**G**W**G**R**L**S**R**K**L**I**N**G**I**R**D**K**Q**S**G**K**T**I**L**D**F**L**K**S**D**G**F**A**N**R**N**F**M**Q**L**I**H**D**D**S**L**T**F**K**E**D**I**Q**K**A**Q**V**S**G**Q**G**D**S**L
 H**E**H**I**A**N**L**A**G**S**P**A**I**K**K**G**I**L**Q**T**V**K**V**V**D**E**L**V**K**V**M**G**R**H**K**P**E**N**I**V**I**E**M**A**R**E**N**Q**T**T**Q**K**G**Q**K**N**S**R**E**R**M**K**R**I**E**E**G**I**K**E**L**G**S**Q**I**L**K**E**H**P**V
 E**N**T**Q**L**Q**N**E**K**L**Y**L**Y**L**Q**N**G**R**D**M**Y**V**D**Q**E**L**D**I**N**R**L**S**D**Y**D**V**D**H**I**V**P**Q**S**F**L**K**D**D**S**I**D**N**K**V**L**T**R**S**D**K**N**R**G**K**S**D**N**V**P**S**E**E**V**V**K**M**K**N**Y
 W**R**Q**L**L**A**K**L**I**T**Q**R**K**F**D**N**L**T**K**A**E**R**G**L**S**E**L**D**K**A**G**F**I**K**R**Q**L**V**E**T**R**Q**I**T**K**H**V**A**Q**I**L**D**S**R**M**N**T**K**Y**D**E**N**D**K**L**I**R**E**V**K**V**I**T**L**K**S**K**L**V
 S**D**F**R**K**D**F**Q**F**Y**K**V**R**E**I**N**N**Y**H**H**A**D**A**Y**L**N**A**V**V**G**T**A**L**I**K**K**Y**P**K**L**S**E**F**V**Y**G**D**Y**K**V**Y**D**V**R**K**M**I**A**K**S**E**Q**E**I**G**K**A**T**A**K**Y**F**F**Y**S**N**I**M**N
 F**F**K**T**E**I**T**L**A**N**G**E**I**R**K**R**P**L**I**E**T**N**G**E**T**G**E**I**V**W**D**K**R**D**F**A**T**V**R**K**V**L**S**M**P**Q**V**N**I**V**K**K**T**E**V**Q**T**G**G**F**S**K**E**S**I**R**P**K**R**N**S**D**K**L**I**A**R**K**K**D**
 W**D**P**K**K**Y**G**G**F**V**S**P**T**V**A**Y**S**V**L**V**A**K**V**E**K**G**S**K**K**L**S**V**K**E**L**L**G**I**T**I**M**E**R**S**S**F**E**K**N**P**I**D**F**L**E**A**K**Y**K**E**V**K**D**L**I**I**K**L**P**K**Y**S**L**F**E**L**
 E**N**G**R**K**R**M**L**A**S**A**R**F**L**Q**K**G**N**E**L**A**L**P**S**K**Y**V**N**F**L**Y**L**A**S**H**E**K**L**K**G**S**P**E**D**N**E**Q**K**Q**L**F**V**E**Q**H**K**H**Y**L**D**E**I**E**Q**I**S**E**F**S**K**R**V**I**L**A**D**A**N**L
 D**K**V**L**S**A**Y**N**K**H**R**D**K**P**I**R**E**Q**A**E**N**I**I**H**L**F**T**L**T**N**L**G**A**P**R**A**F**K**Y**F**D**T**T**I**D**R**K**V**Y**R**S**T**K**E**V**L**D**A**T**L**I**H**Q**S**I**T**G**L**Y**E**T**R**I**D**L**S**Q**L**G**D**
 S**G**G**S**K**R**T**A**D**G**S**E**F**E**P**K**K**K**R**K**V**G**S**G**S**K**R**P**A**A**T**K**K**A**G**Q**A**K**K**K**

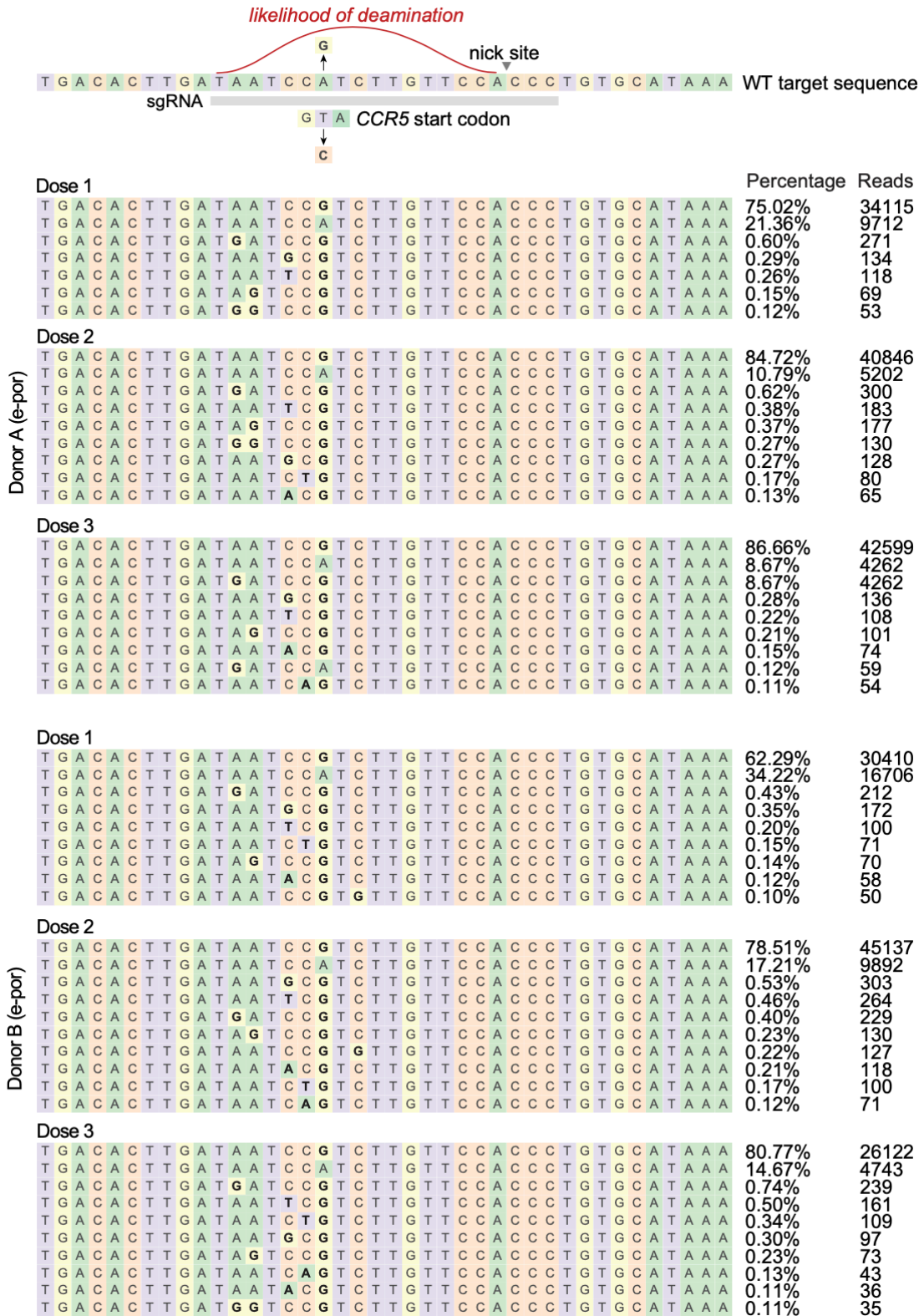
Supplementary Fig. 9 I Amino acid sequence of the ABE8e-SpCas9-NG adenine base editor. Each TadA domain is in cyan; inactivating alanine mutations for the N-terminal TadA domain and for one nuclease domain of Cas9 are in bold; bipartite SV40 NLS is in purple; nucleoplasmin NLS is in dark blue.



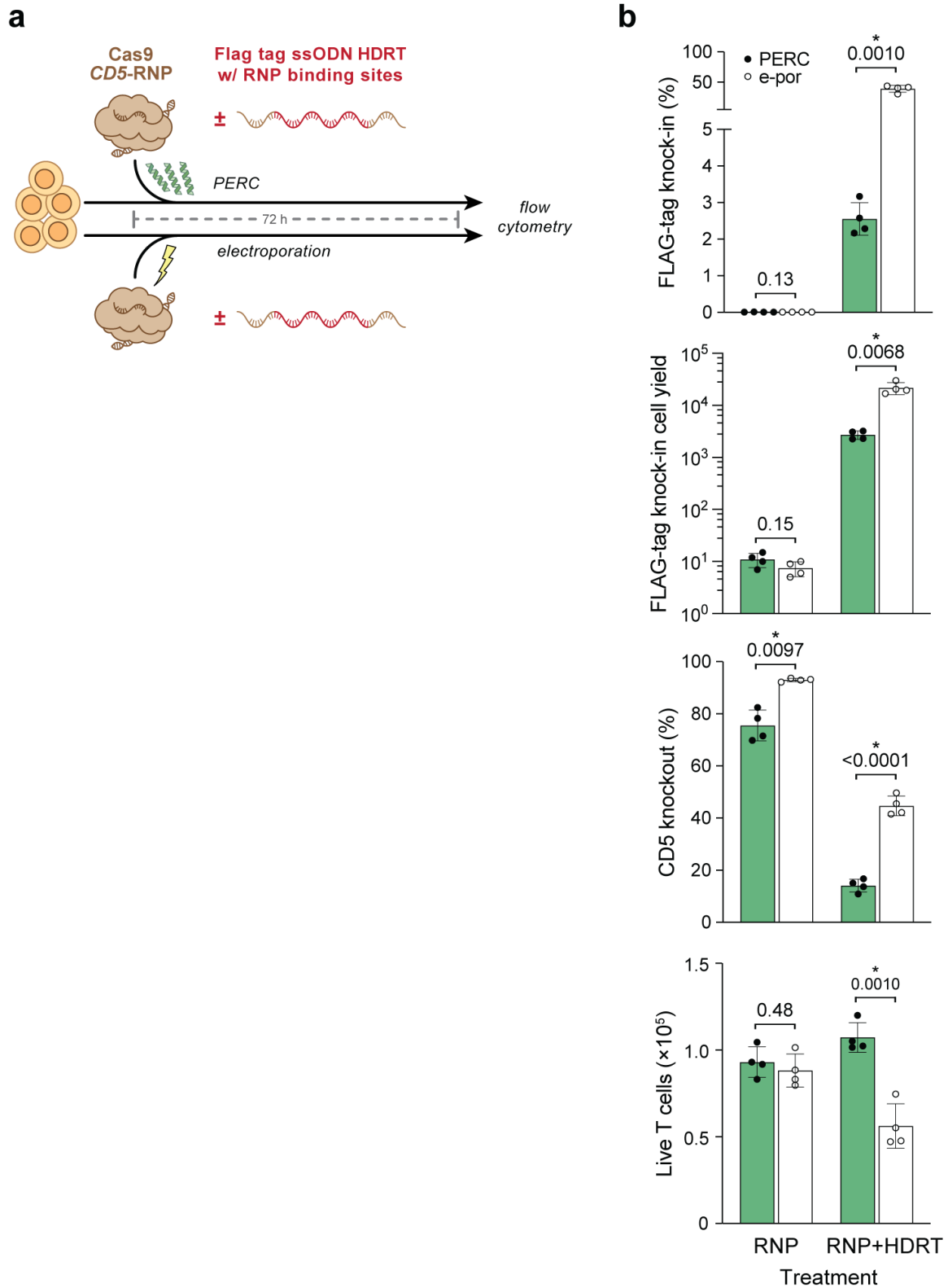
Supplementary Fig. 10 | Peptide-enabled base editor RNP delivery to T cells to abrogate the start codon of *CCR5*. **a**, Schematic for the base editing strategy. The spacer sequence is denoted by underscores, except for positions 4–14, which are represented by numerals to indicate the window of likely A-to-G base editing activity (shown as a red curve in the next two figures). The NG PAM is indicated to the right (3' side) of the spacer sequence and was used because there are no proximal canonical NGG PAM sites. Purple arrows indicate changes induced by ABE activity, which eliminates the start codon ATG (green, bottom strand). This strategy was shown to be capable of knocking out of CCR5 surface expression in T cells (Knipping, et al. *Molecular Therapy* 2022). **b**, ABE8e-Cas9-NG base editor RNP was delivered via PERC or electroporation to human CD4⁺ T cells. Cells were treated with a single dose or treated sequentially two or three times, with 3–4 days between treatments. Base editing outcomes were analyzed by **c**, NGS (non-treated cells had <0.05% base edits; data not shown) two days after the final dose, and **d**, flow cytometry was performed three days after the last dose to detect knockout of CCR5 surface expression. n=3 biological replicates from distinct human donors for the one-dose experiment; n=2 for the two-dose and three-dose experiments. Bars represent the mean; error bars represent S.E.M. *P*-values are from two-tailed Welch's unpaired *t*-tests.



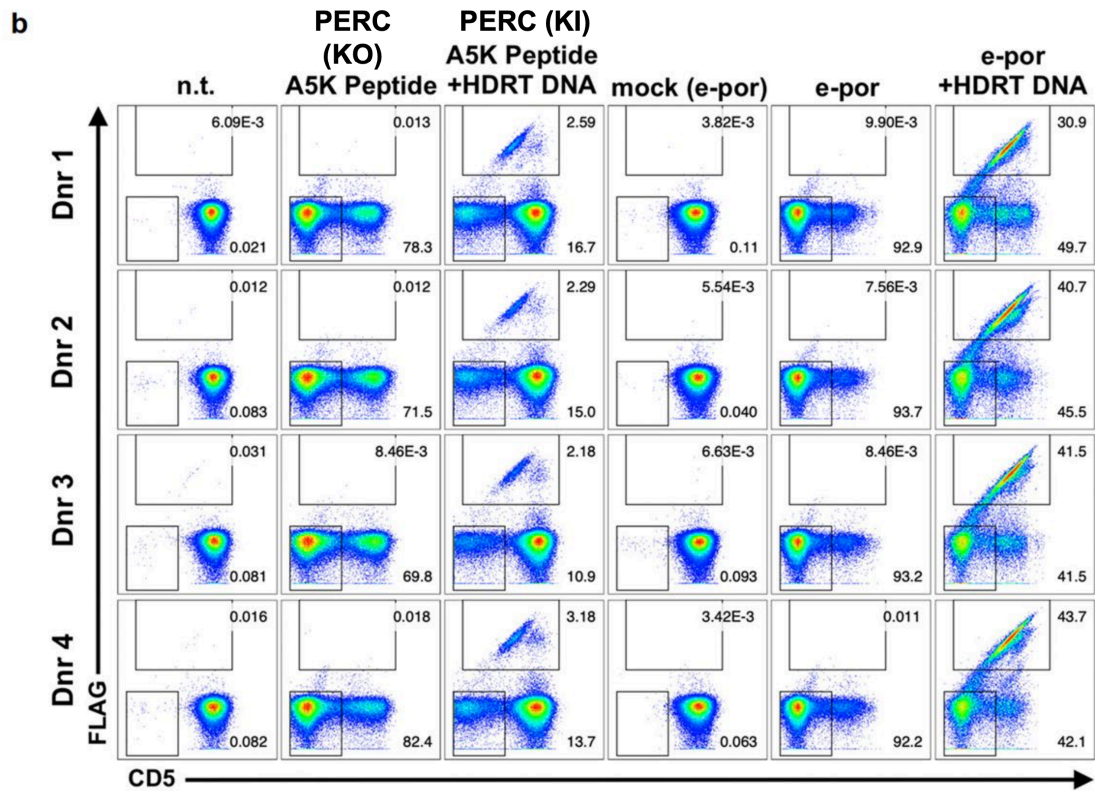
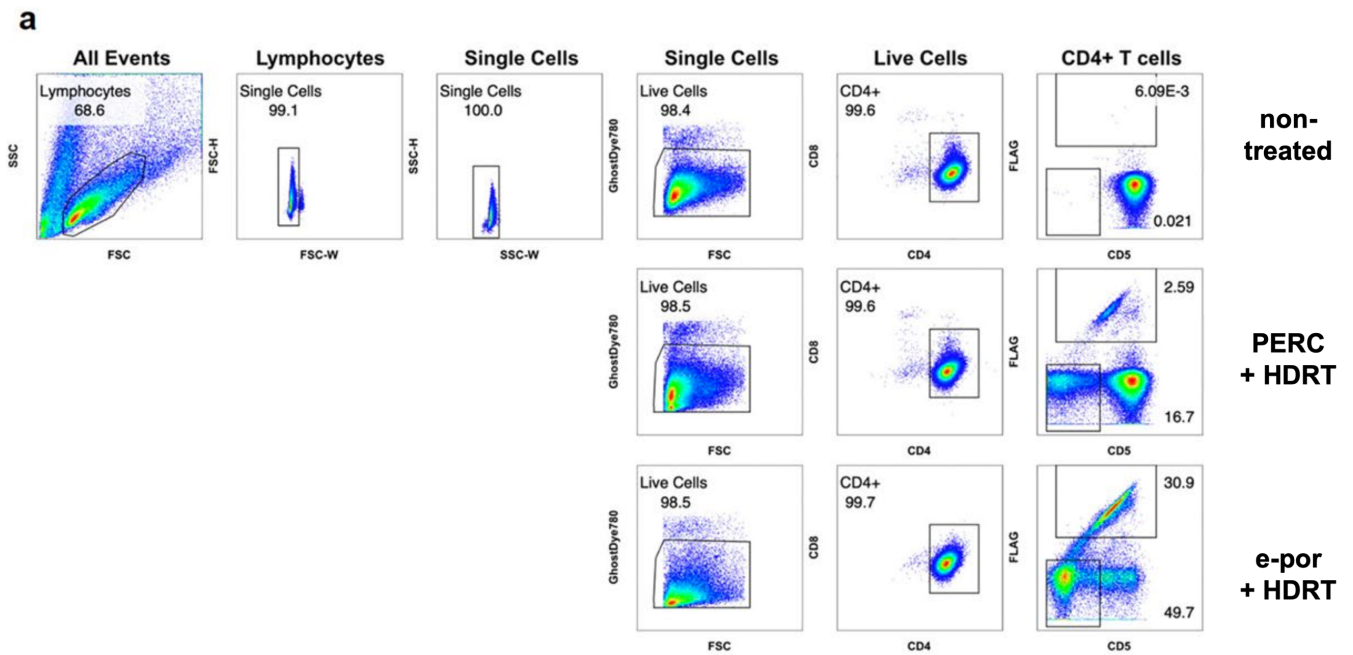
Supplementary Fig. 11 | Base editing outcomes following PERC. Allele plots showing NGS-based sequencing reads from T cells that received ABE8e-Cas9-NG RNP targeting the start codon of *CCR5*. RNP was delivered up to three times using PERC, and the reads resulting from each dosing strategy are reported. Because 0.1% is the approximate practical limit of detection for our analysis, only reads with frequency $\geq 0.1\%$ are shown. Related to **Supplementary Fig. 10, 12**. The intended A→G conversion is outlined in red, and these reads contributed to the editing efficiency reported in **Supplementary Fig. 10**. Unintended C→T and C→G conversions were detected at the two C nucleotides 5' (left) of the targeted A. These unintended conversions were detected only in reads that also contained the intended A→G conversion that ablates the *CCR5* start codon.



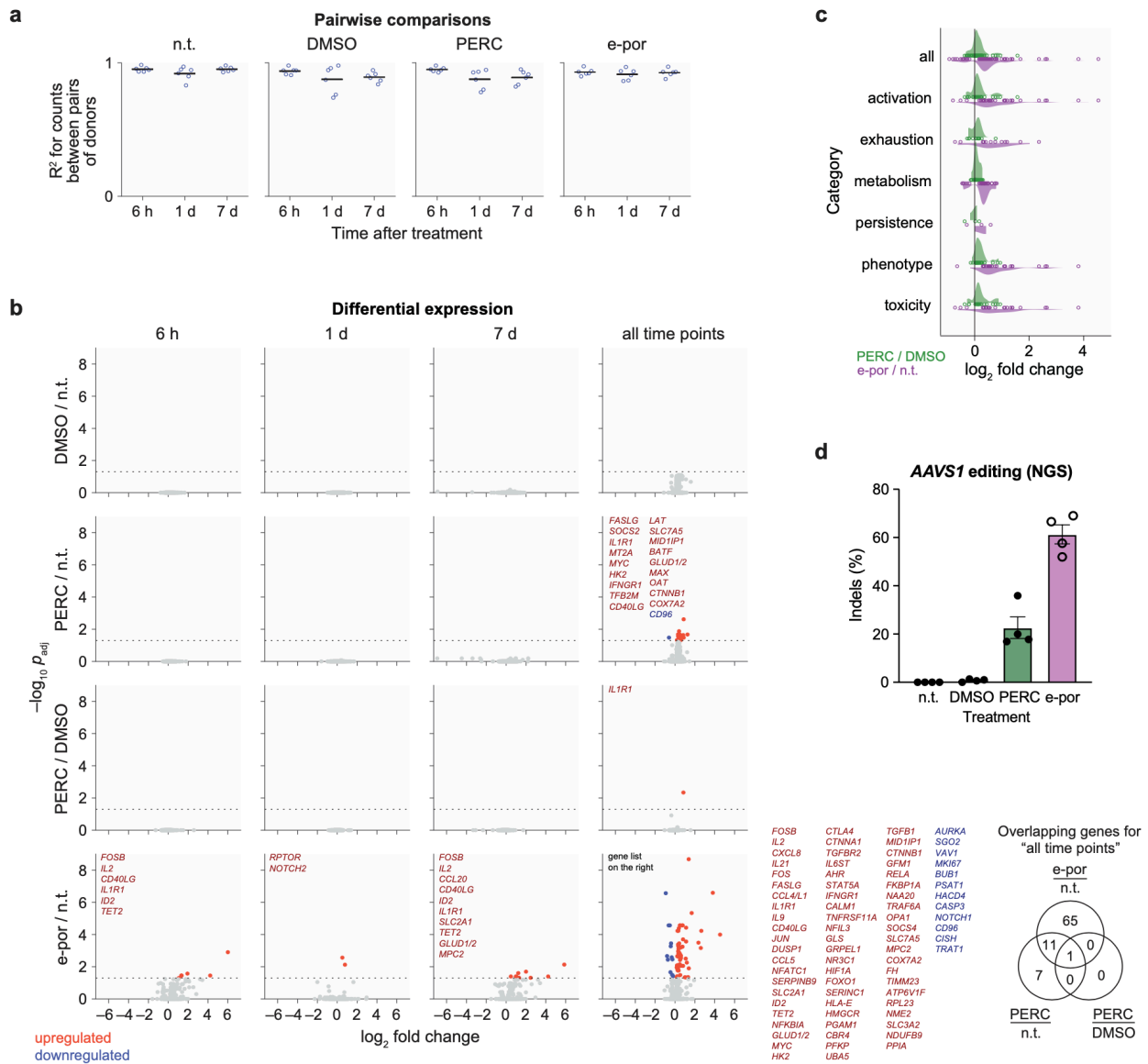
Supplementary Fig. 12 I Base editing outcomes following electroporation. Allele plots showing NGS-based sequencing reads from T cells that received ABE8e-Cas9-NG RNP targeting the start codon of *CCR5*. RNP was delivered up to three times using electroporation, and the reads resulting from each dosing strategy are reported. Because 0.1% is the approximate practical limit of detection for our analysis, only reads with frequency $\geq 0.1\%$ are shown. Related to **Supplementary Fig. 10, 11**. The intended A→G conversion is outlined in red, and these reads contributed to the editing efficiency reported in **Supplementary Fig. 10**. Unintended C→T and C→G conversions were detected at the two C nucleotides 5' (left) of the targeted A. These unintended conversions were detected only in reads that also contained the intended A→G conversion that ablates the *CCR5* start codon.



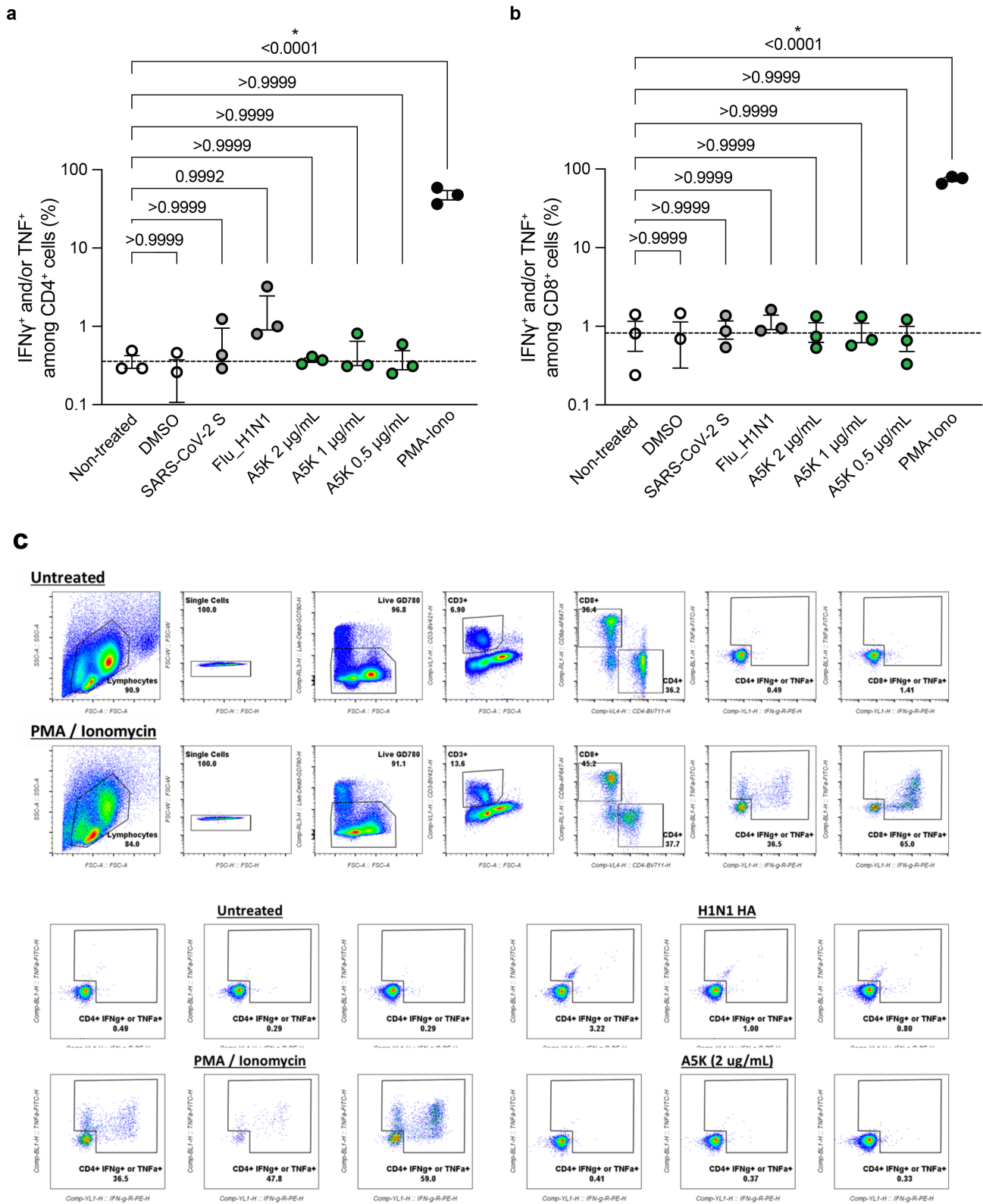
Supplementary Fig. 13 | Comparison of PERC and electroporation with an ssODN HDRT. a, Schematic representing the experimental protocol for delivery of Cas9 *CD5*-RNP with or without DNA HDRT for FLAG-tag knock-in, via either PERC using A5K (30 μ M) or electroporation, in *CD4*⁺ T cells. **b**, Knockout and knock-in editing were assessed by flow cytometry. Results are presented as knock-in frequency, edited cell yield (FLAG-tag⁺), knockout frequency (*CD5*⁻), and total number of live cells in each treatment. *n*=4 biological replicates from distinct human donors. Bars represent the mean, and error bars represent \pm S.D. *P*-values are from two-tailed Welch's unpaired *t*-tests.



Supplementary Fig. 14 | a,b, Flow cytometry gating to assess CD5 knockout and FLAG-tag knock-in in CD4⁺ T cells. Cas9 *CD5*-RNP was delivered via PERC or electroporation, with or without DNA HDRT for FLAG-tag knock-in.

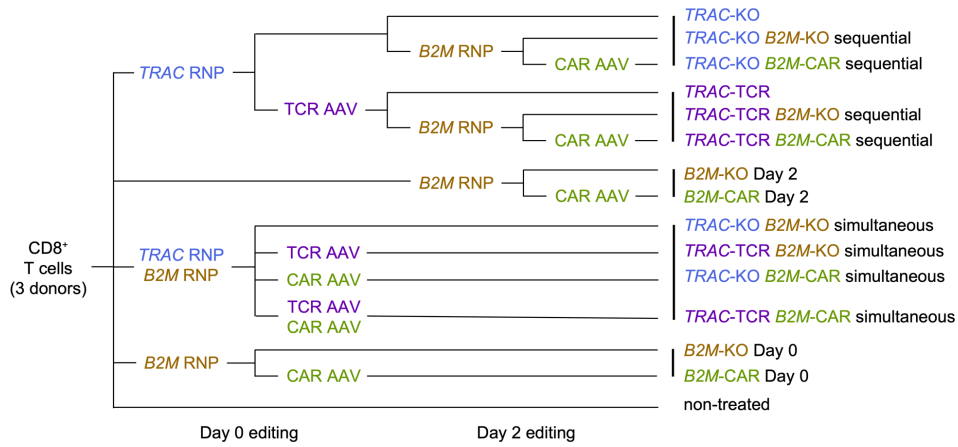


Supplementary Fig. 15 | Gene expression analysis. a, Donor-to-donor correlations (R^2) in gene expression. For $n=4$ biological replicates from distinct donors, there are six pairwise comparisons. **b**, The volcano plots depict gene expression fold changes and adjusted p -values at each time point and for all time points incorporated. Red denotes significant upregulation and blue denotes significant downregulation. The Venn diagram depicts the number of and overlap in differentially expressed genes. **c**, Comparison of fold changes across gene categories, considering the $n = 77$ genes that are differentially expressed in either PERC/DMSO or e-por/n.t. **d**, Indel percentages for each condition, as determined by NGS.

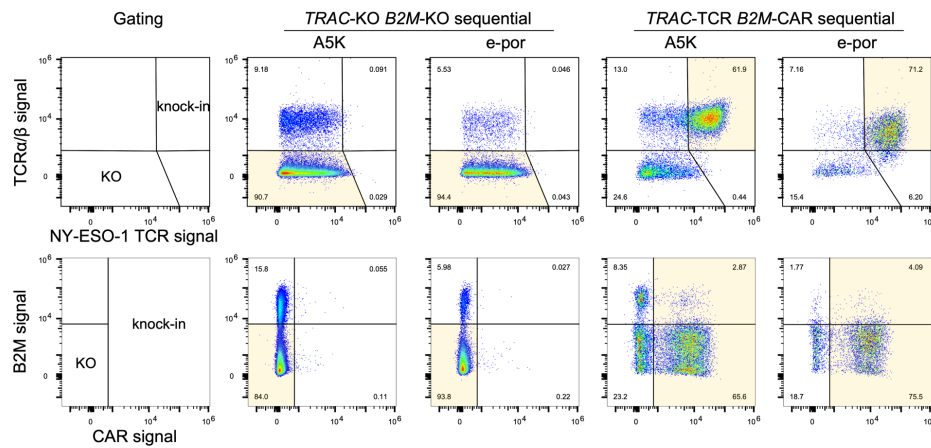


Supplementary Fig. 16 | A5K peptide does not promote an antigen-specific T cell response. PBMCs were isolated and cultured in the presence of either A5K peptide, peptide pools derived from SARS-CoV-2 or influenza virus H1N1, or controls as indicated. **a**, CD4 $^+$ T cell and **b**, CD8 $^+$ T cell subsets were assessed for production of IFN- γ or TNF. *P*-values are from an ANOVA and Holm-Šidák multiple comparisons test. **c**, Intracellular staining and flow cytometry with representative flow analysis gating. *n*=3 biological replicates from distinct human donors. Bars represent the mean, and error bars represent \pm S.E.M.

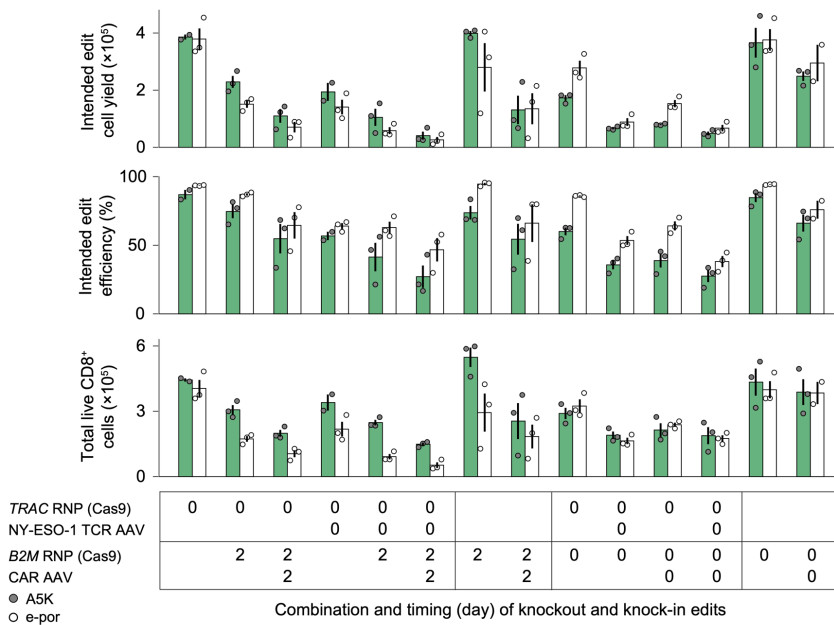
a



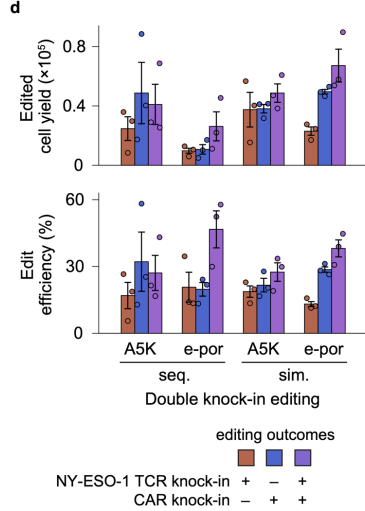
b



c

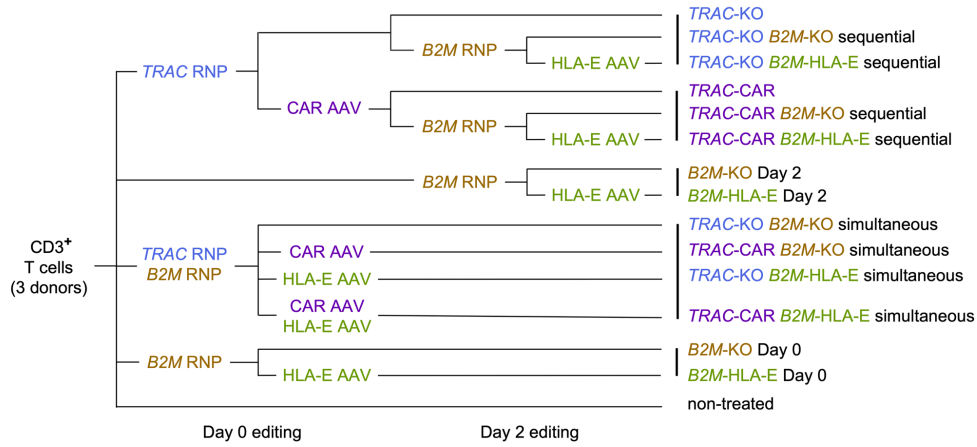


d

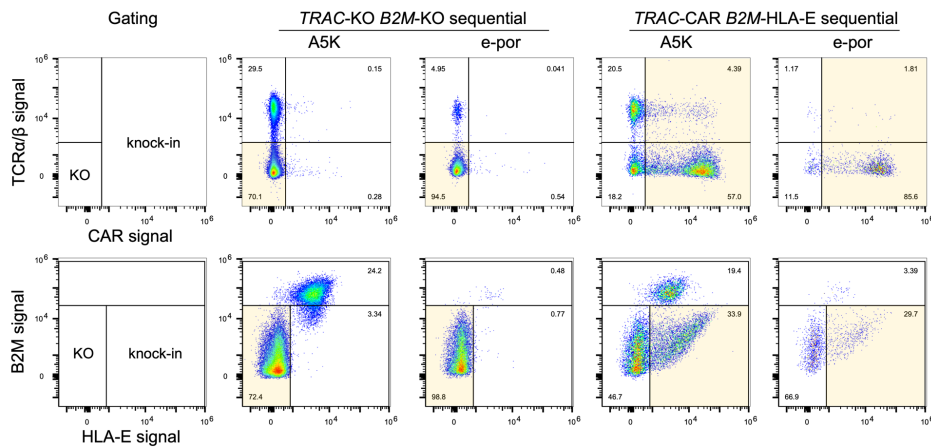


Supplementary Fig. 17 | Sequential vs. simultaneous editing (related to Fig. 1d and Fig. 3a,b). a, Experiment schematic. **b**, Gating on representative samples. **c,d**, Editing outcomes. Dotted boxes in **c** indicate conditions that were investigated further in Fig. 3e (translocation analysis). CD8⁺ T cell editing involved Cas9 TRAC-RNP & NY-ESO-1 TCR AAV and Cas9 B2M-RNP & CAR AAV. Non-treated cells express TCRα/β and B2M. Double knockout cells are defined as TCRα/β⁻ NY-ESO-1 TCR⁻ B2M⁻ CAR⁻. Double knock-in cells are defined as TCRα/β⁺ NY-ESO-1 TCR⁺ CAR⁺. n=3 biological replicates from distinct human donors. Bars represent the mean, and error bars represent \pm S.E.M.

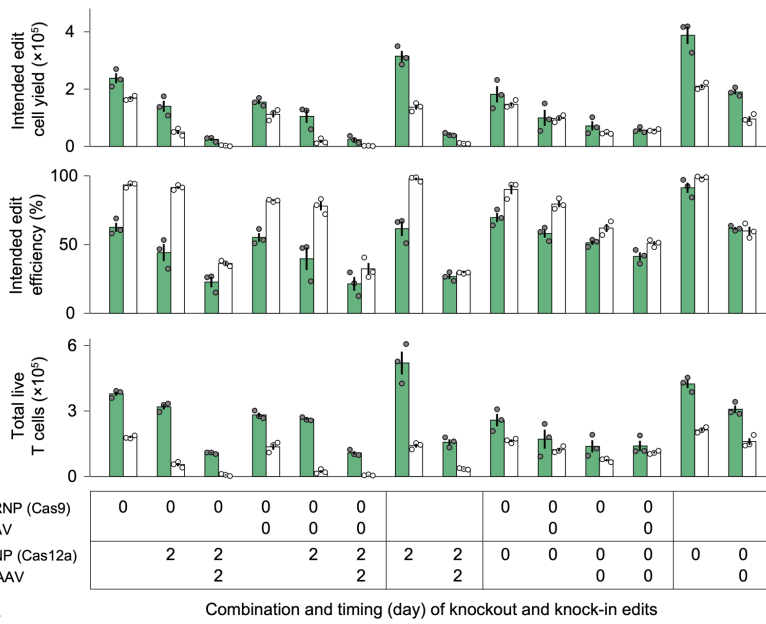
e



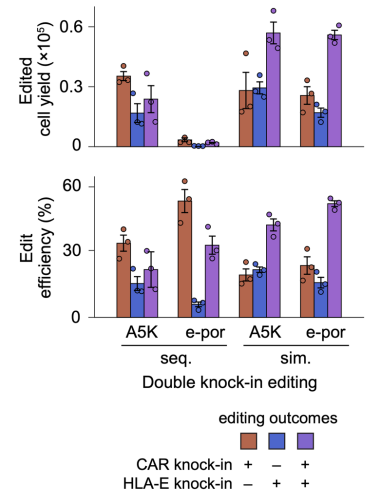
f



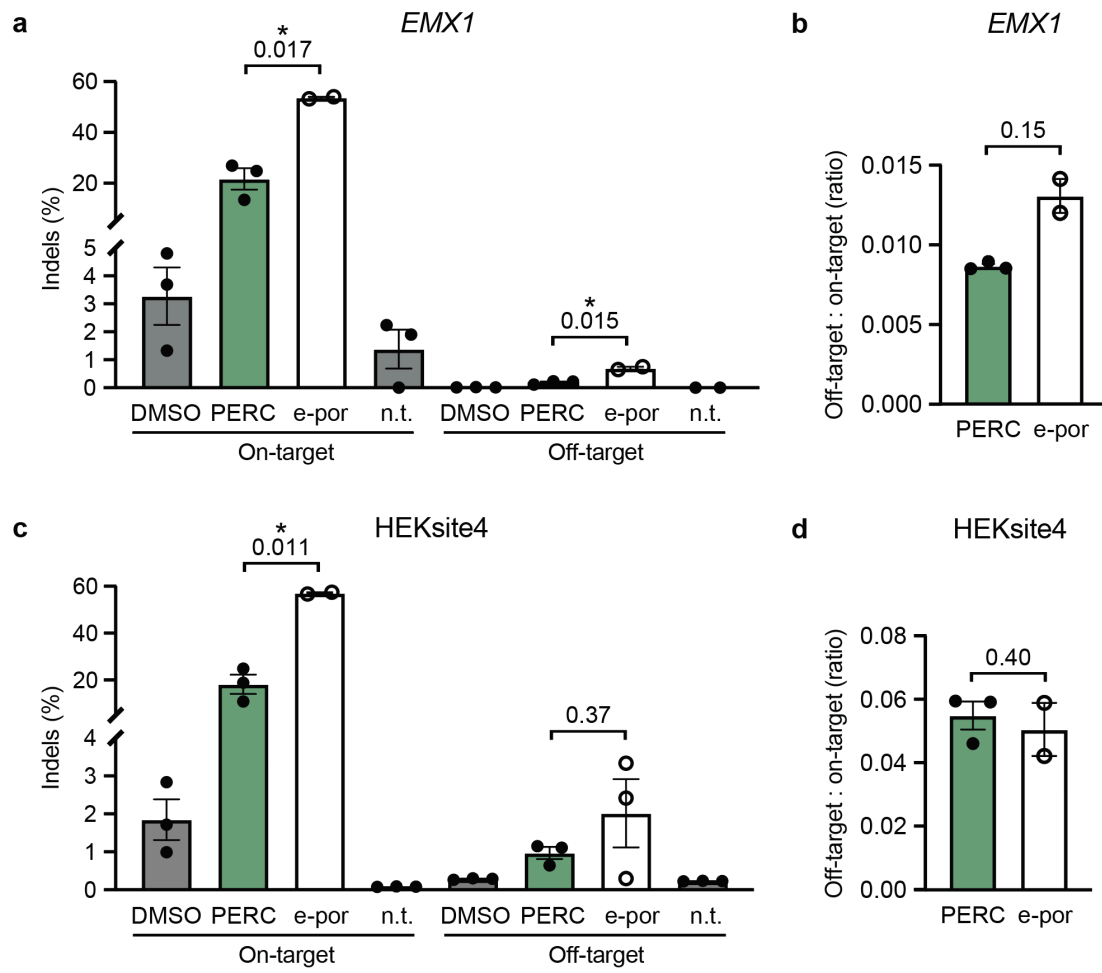
g



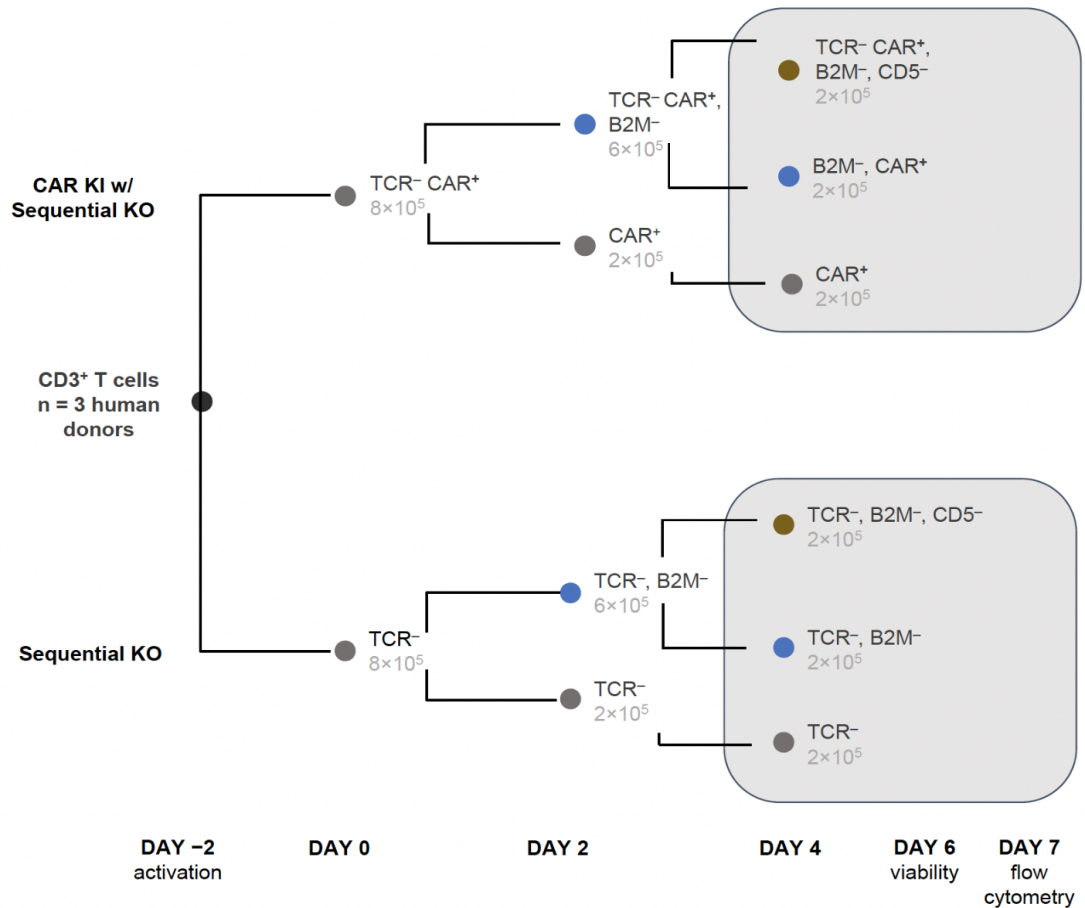
h



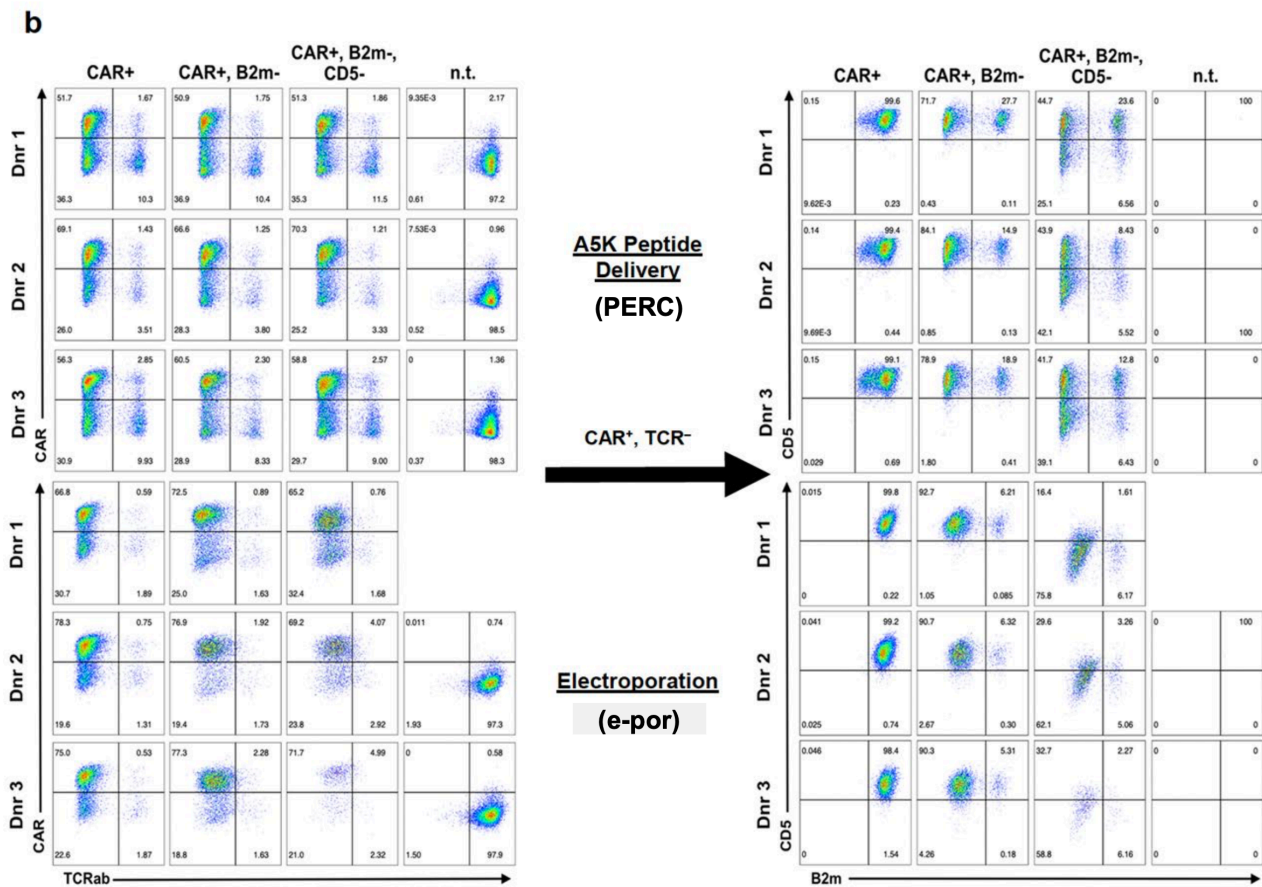
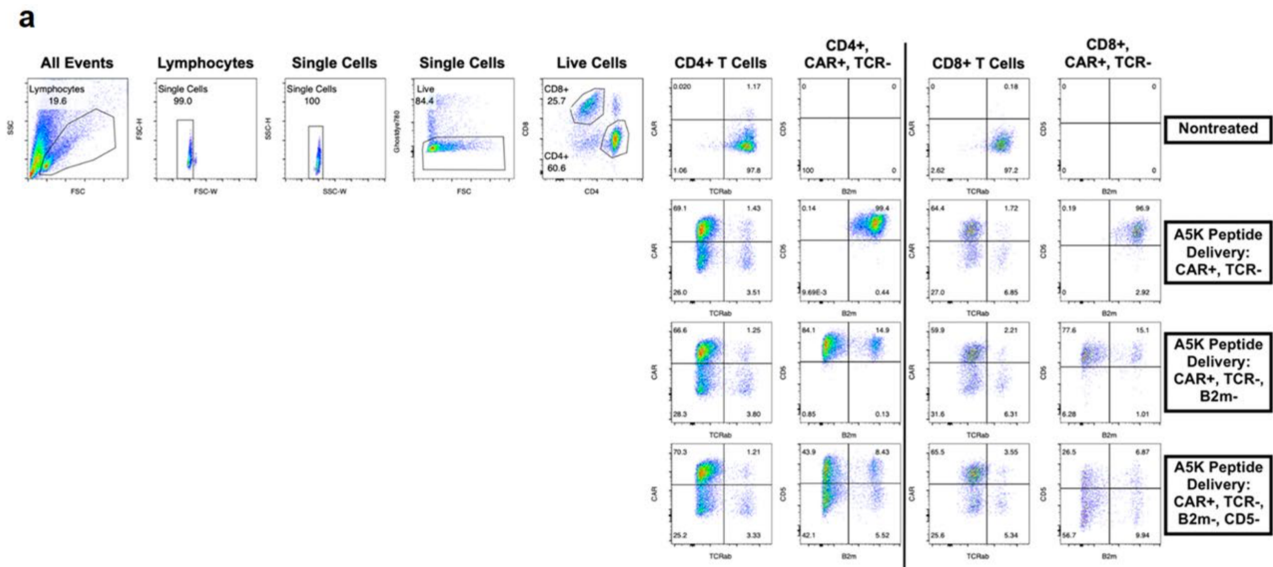
Supplementary Fig. 18 | Sequential vs. simultaneous editing (related to Fig. 1d and Fig. 3c,d). a, Experiment schematic. b, Gating on representative samples. c,d, Editing outcomes. CD3⁺ T cell editing involved Cas9 TRAC-RNP & CAR AAV and Cas12a B2M-RNP & HLA-E AAV. Non-treated cells express TCRα/β, B2M at a high level, and HLA-E at a low level. Double knockout cells are defined as TCRα/β⁻ CAR⁻ B2M⁻ HLA-E⁻. Double knock-in cells are defined as CAR⁺ B2M^{lo} HLA-E⁺. Compared to non-treated cells, cells with HLA-E knock-in express HLA-E at a high level. n=3 biological replicates from distinct human donors. Bars represent the mean, and error bars represent \pm S.E.M.



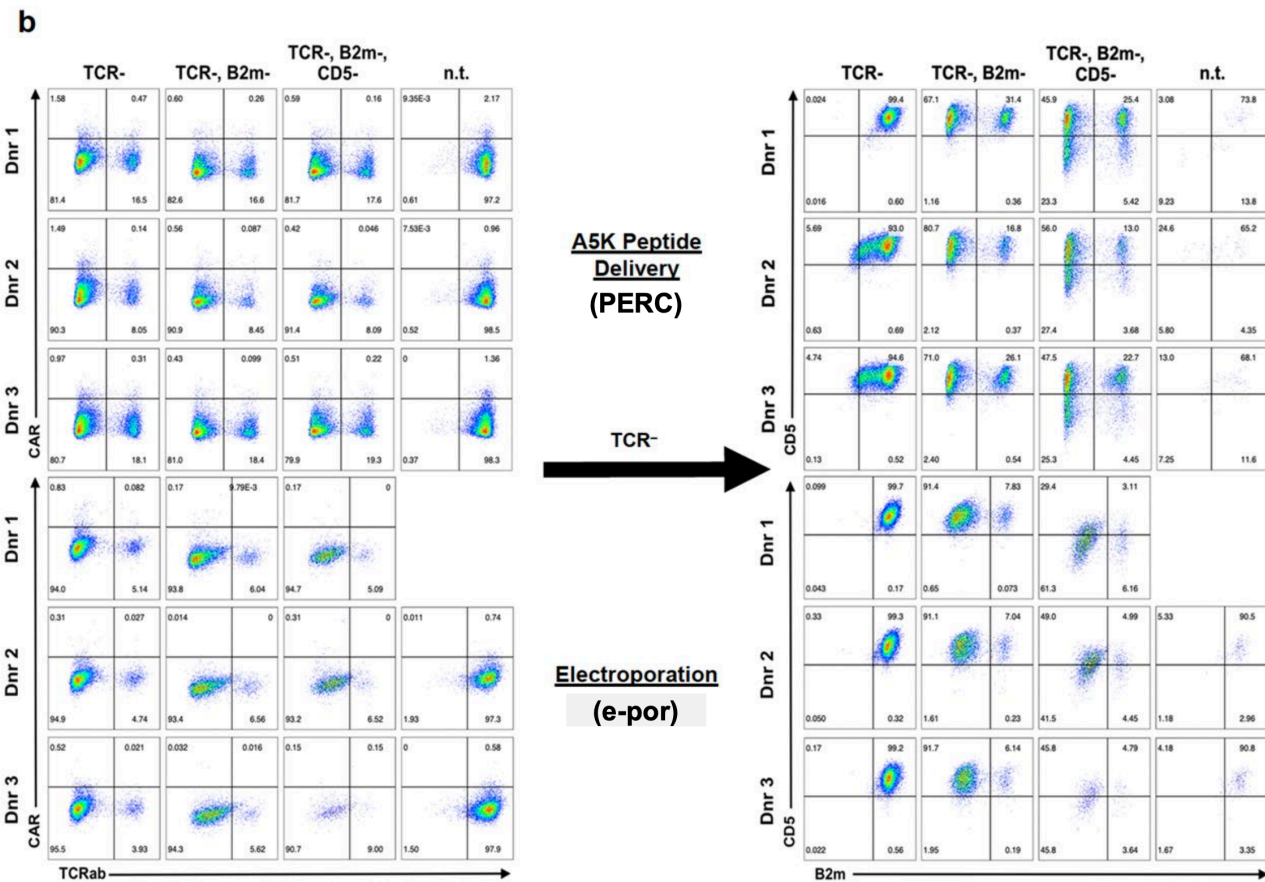
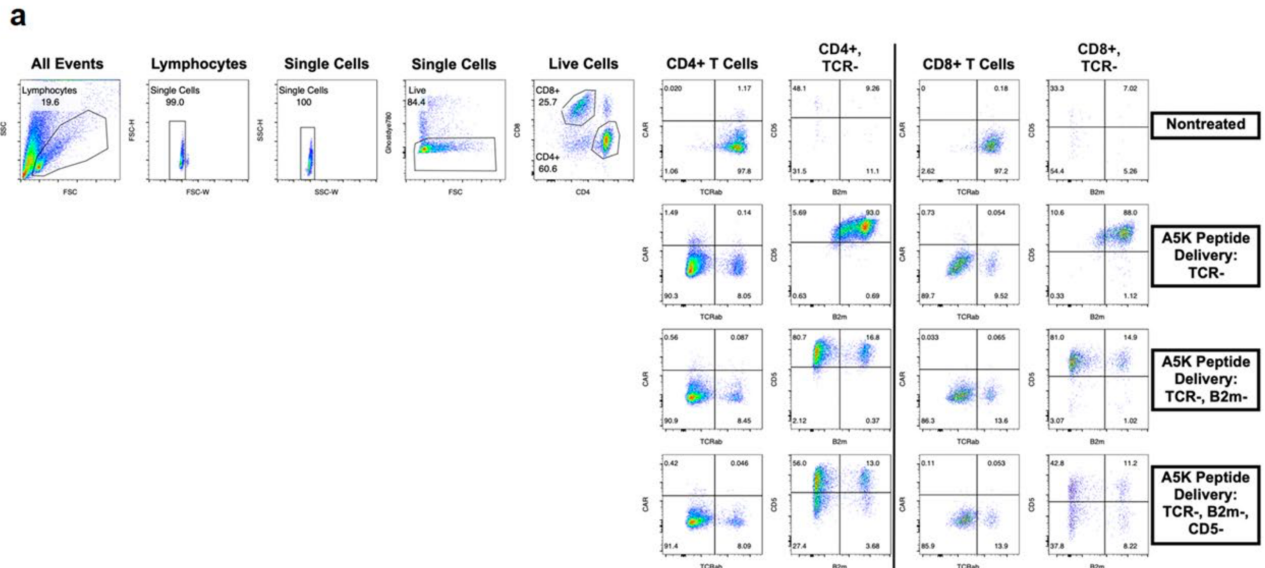
Supplementary Fig. 19 | Analysis of off-target effects. On-target and off-target editing efficiencies were assessed by amplicon-based NGS in CD4⁺ T cells treated with Cas9 RNP targeting two loci, **a,b**, *EMX1* and **c,d**, HEKsite4. Bars represent the mean, and error bars represent \pm S.E.M. *P*-values are from two-tailed Welch's unpaired *t*-tests. *n*=3 biological replicates from distinct human donors. Guide RNA sequences are listed in **Supplementary Table 2**.



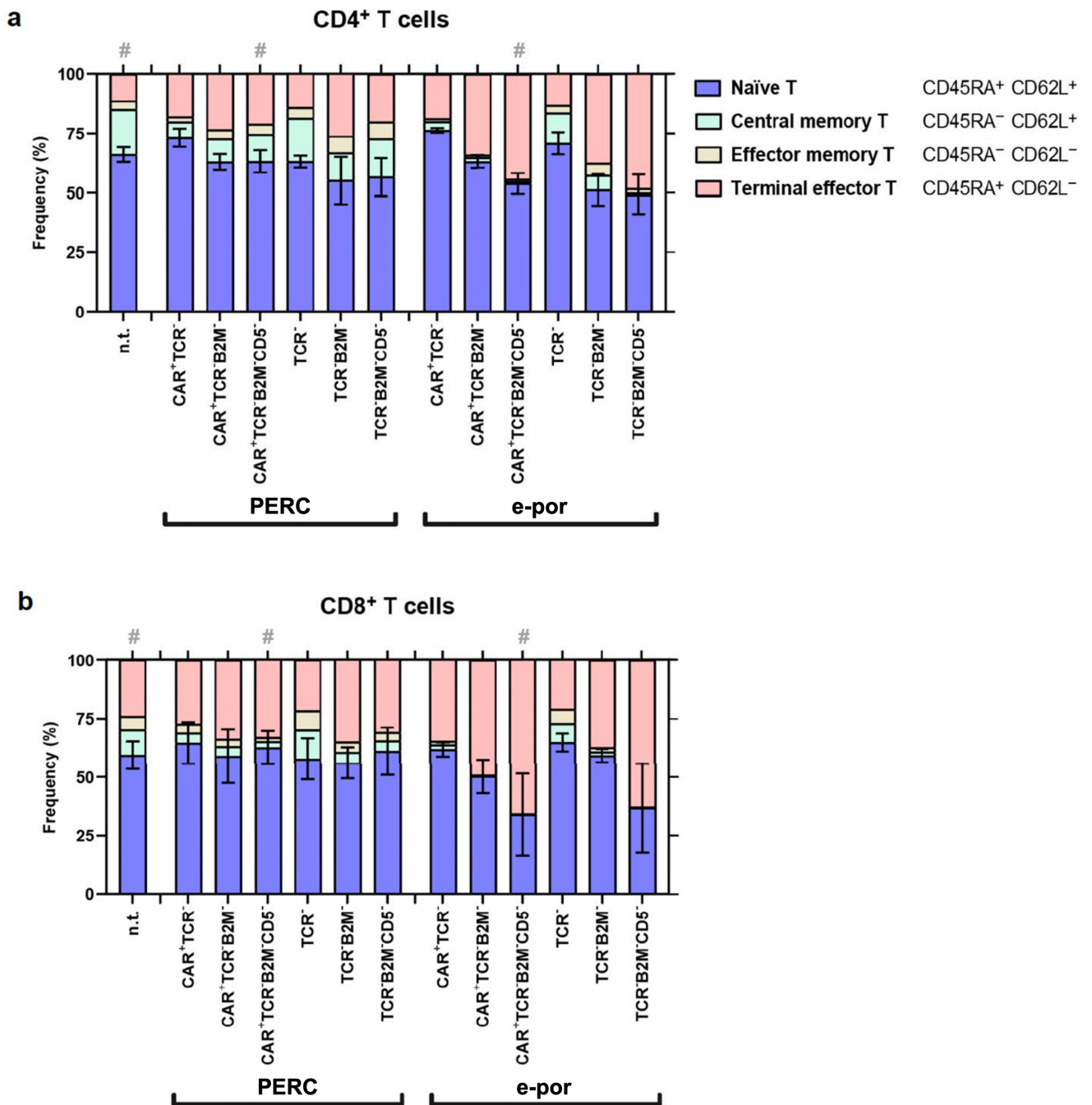
Supplementary Fig. 20 | Schematic of sequential editing of three loci in CD3⁺ T cells. Upper branch: Cas9 *TRAC*-RNP and CAR AAV on Day 0, Cas9 *B2M*-RNP on Day 2, and Cas9 *CD5*-RNP on Day 4 (related to **Fig. 4b**). Lower branch: Cas9 RNPs without CAR AAV (related to **Fig. 4c**). Cell numbers in each condition were scaled up if cells needed to be subsequently split for sequential editing, with the treatment appropriately scaled for the number of cells. Cell density was returned to 1 × 10⁶ cells/mL at each branch point. To compare edited cell counts between conditions at different branch points, data were normalized to assume the same starting cell number. Flow cytometry was conducted on Day 7.



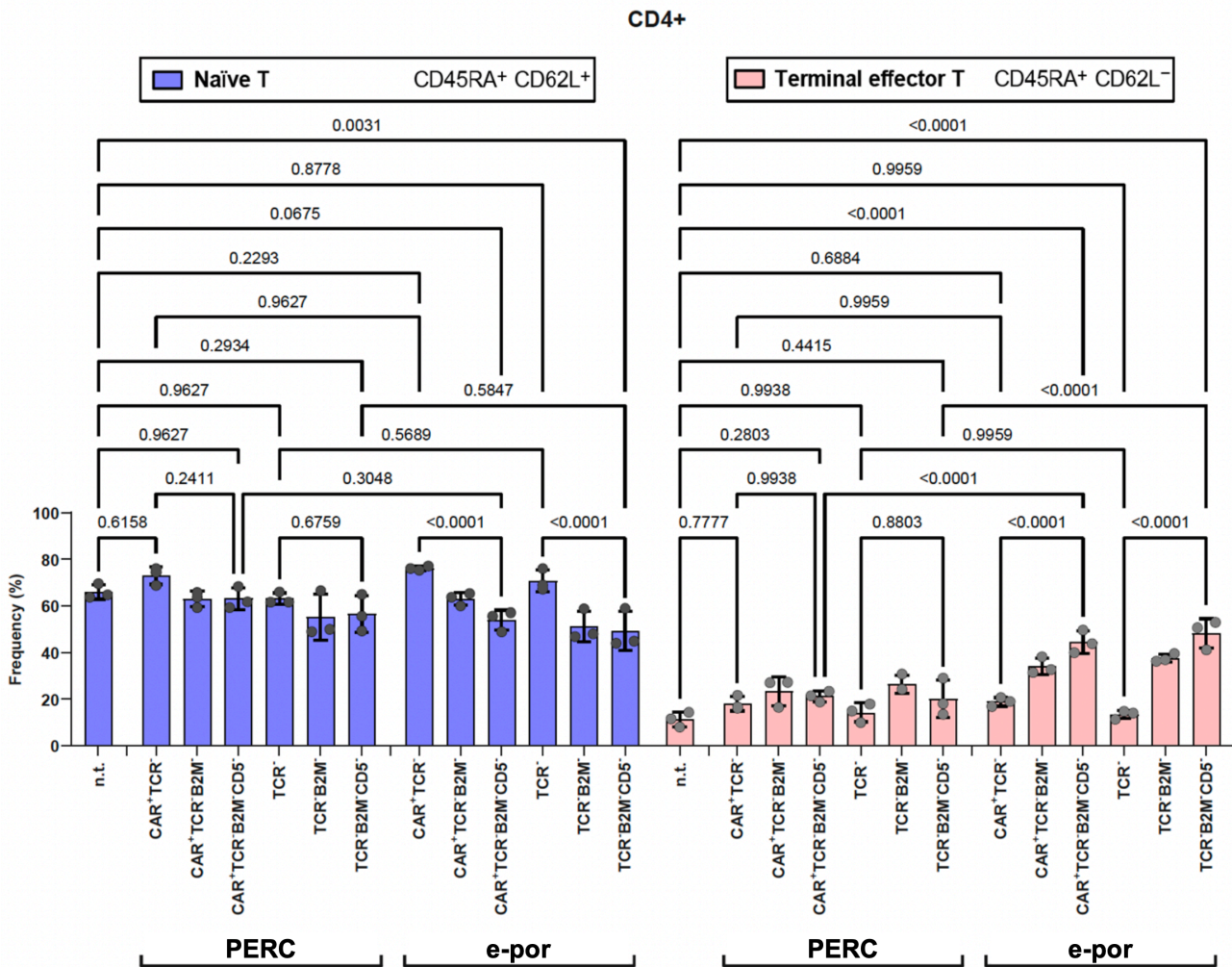
Supplementary Fig. 21 | a, Representative flow cytometry gating for assessing TCR surface expression following Cas9 *TRAC*-RNP delivery to and CAR AAV transduction of CD3⁺ T cells (related to **Fig. 4b**). **b**, Representative gating for assessing knockout of B2M and CD5 in sequentially edited CAR⁺ T cells (related to **Fig. 4b**).



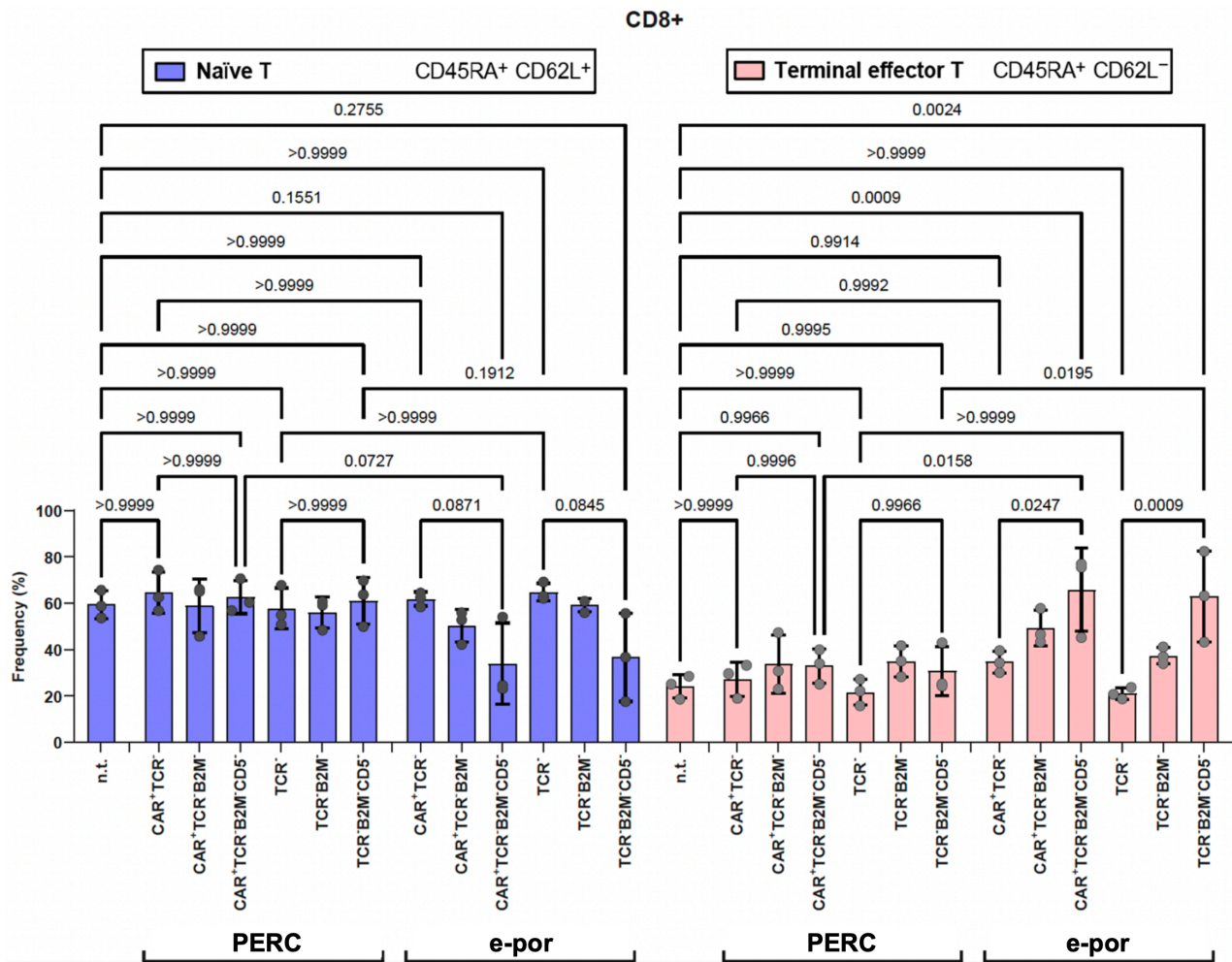
Supplementary Fig. 22 | a, Representative flow cytometry gating for assessing TCR surface expression following Cas9 *TRAC*-RNP delivery to CD3⁺ T cells (related to Fig. 4c). **b**, Representative gating for assessing knockout of B2M and CD5 in sequentially edited T cells (related to Fig. 4c).



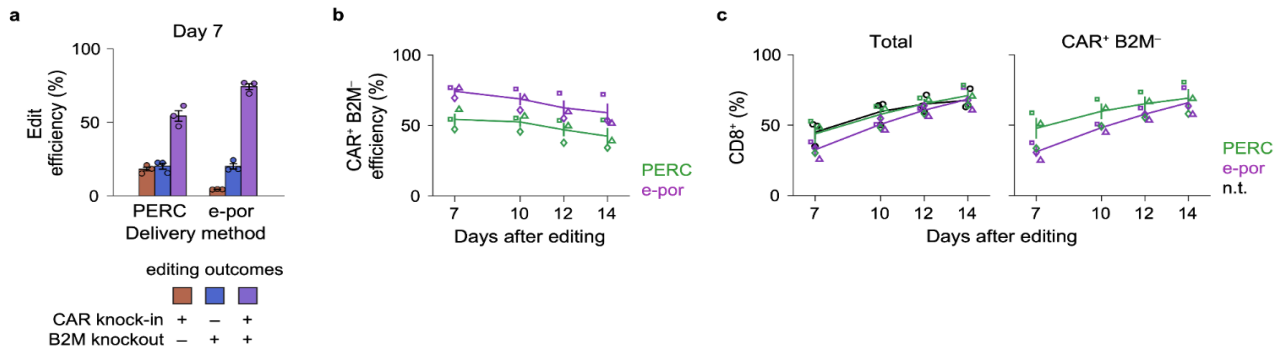
Supplementary Fig. 23 | a,b, Flow cytometry assessment of CD62L/CD45RA phenotypes in CD4⁺ and CD8⁺ T cells. For each sequential editing treatment via PERC or electroporation (outlined in **Supplementary Fig. 20**), phenotypes were assessed independent of editing outcome. Representative flow cytometry gating shown in **Supplementary Fig. 26**. “#” indicates that data are also represented in pie charts in **Fig. 4d**. Bars represent a mean of n=3 biological replicates from distinct human donors. Error bars are displayed for the naïve populations and represent S.E.M.



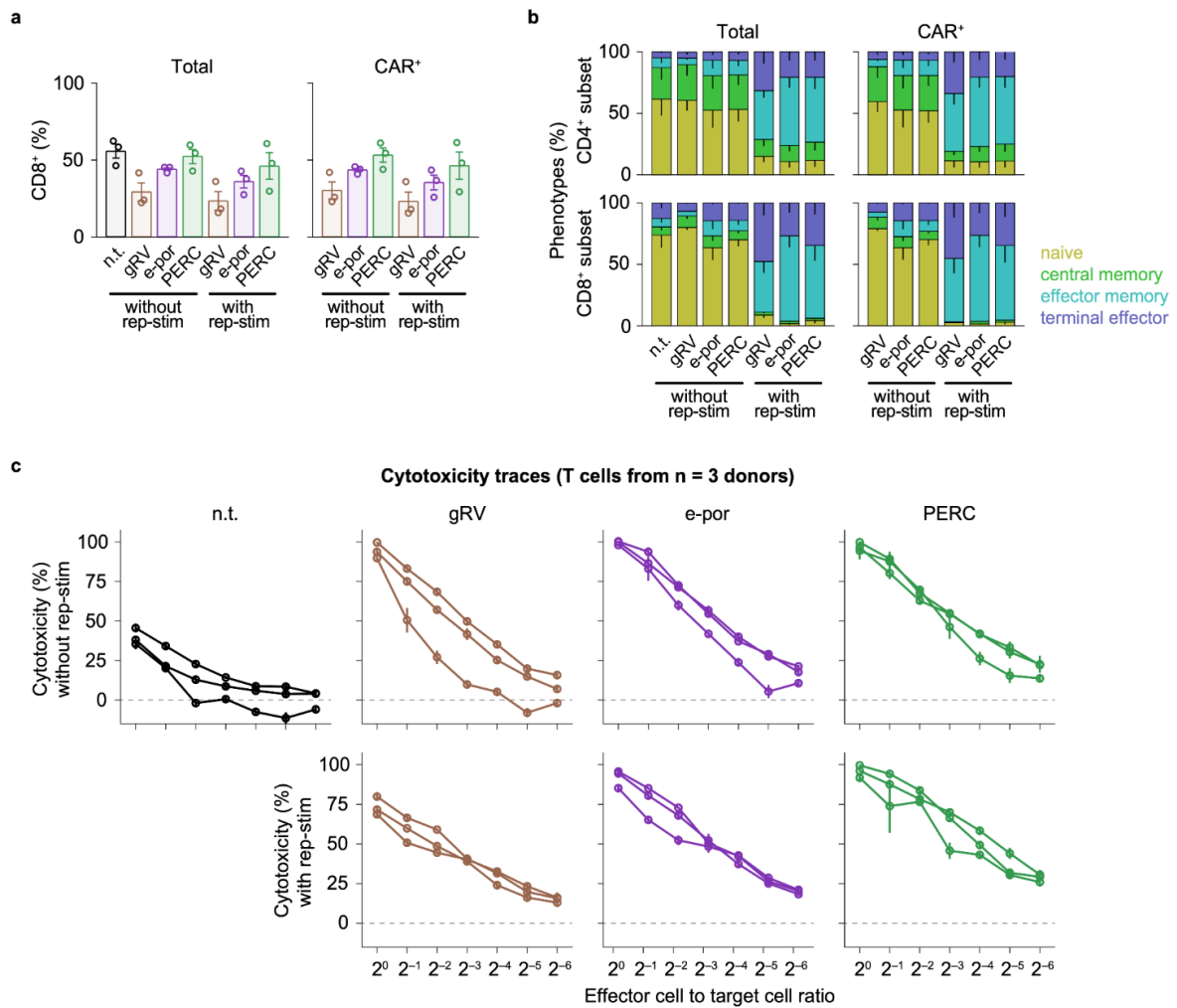
Supplementary Fig. 24 | Comparison of phenotypes in CD4⁺ T cells. Proportions of naïve (CD45RA⁺ CD62L⁺) and terminal effector (CD45RA⁺ CD62⁻) CD4⁺ T cells were assessed by flow cytometry. For each sequential editing treatment via PERC or electroporation (outlined in **Supplementary Fig. 20**), phenotypes were assessed independent of editing outcome. Representative flow cytometry gating shown in **Supplementary Fig. 26**. Same data as in **Supplementary Fig. 23**. Brackets indicate each comparison between two populations with adjusted *p*-values from a two-way ANOVA with Holm-Sidak's correction method for multiple comparisons. Bars represent a mean of *n*=3 biological replicates from distinct human donors. Error bars represent S.E.M.



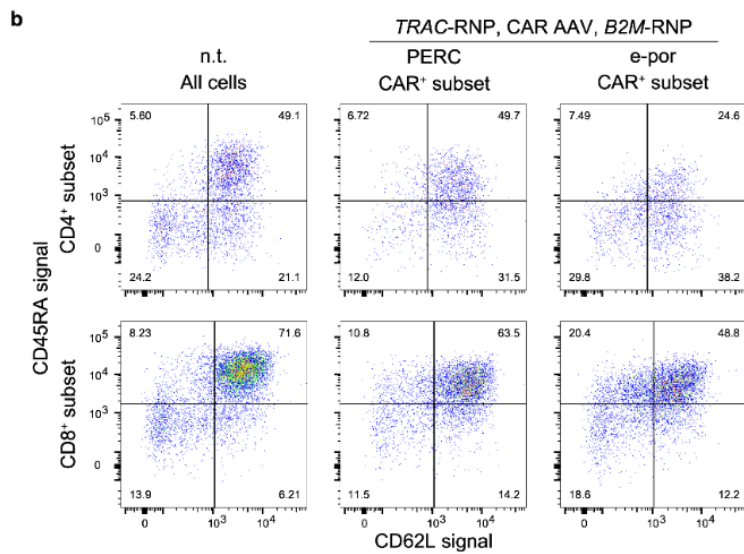
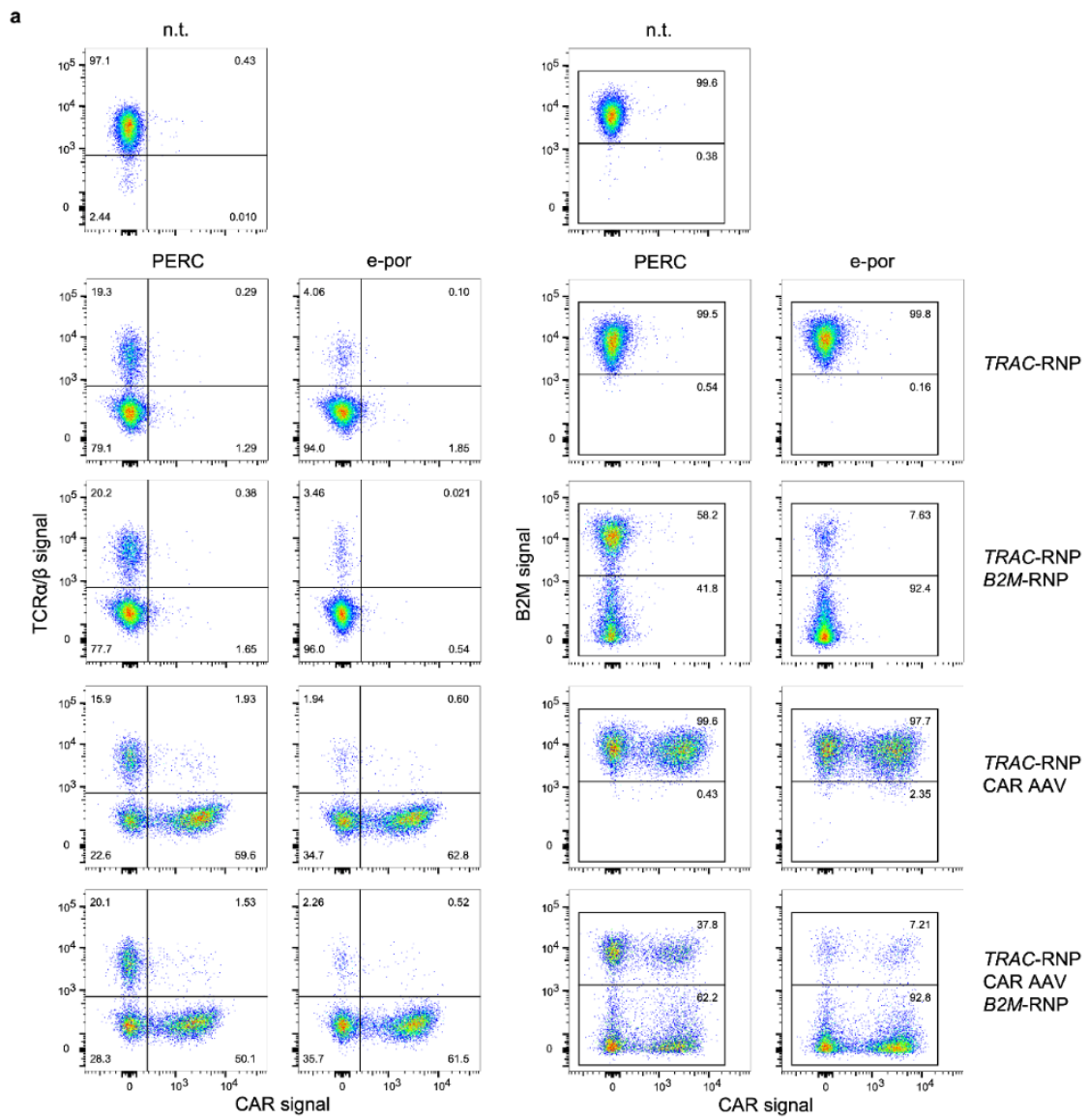
Supplementary Fig. 25 | Comparison of phenotypes in CD8⁺ T cells. Proportions of naive (CD45RA⁺ CD62L⁺) and terminal effector (CD45RA⁺ CD62L⁻) CD8⁺ T cells were assessed by flow cytometry. For each sequential editing treatment via PERC or electroporation (outlined in **Supplementary Fig. 20**), phenotypes were assessed independent of editing outcome. Representative flow cytometry gating shown in **Supplementary Fig. 26**. Same data as in **Supplementary Fig. 23**. Brackets indicate each comparison between two populations with adjusted *p*-values from a two-way ANOVA with Holm-Sidak's correction method for multiple comparisons. Bars represent a mean of n=3 biological replicates from distinct human donors. Error bars represent S.E.M.



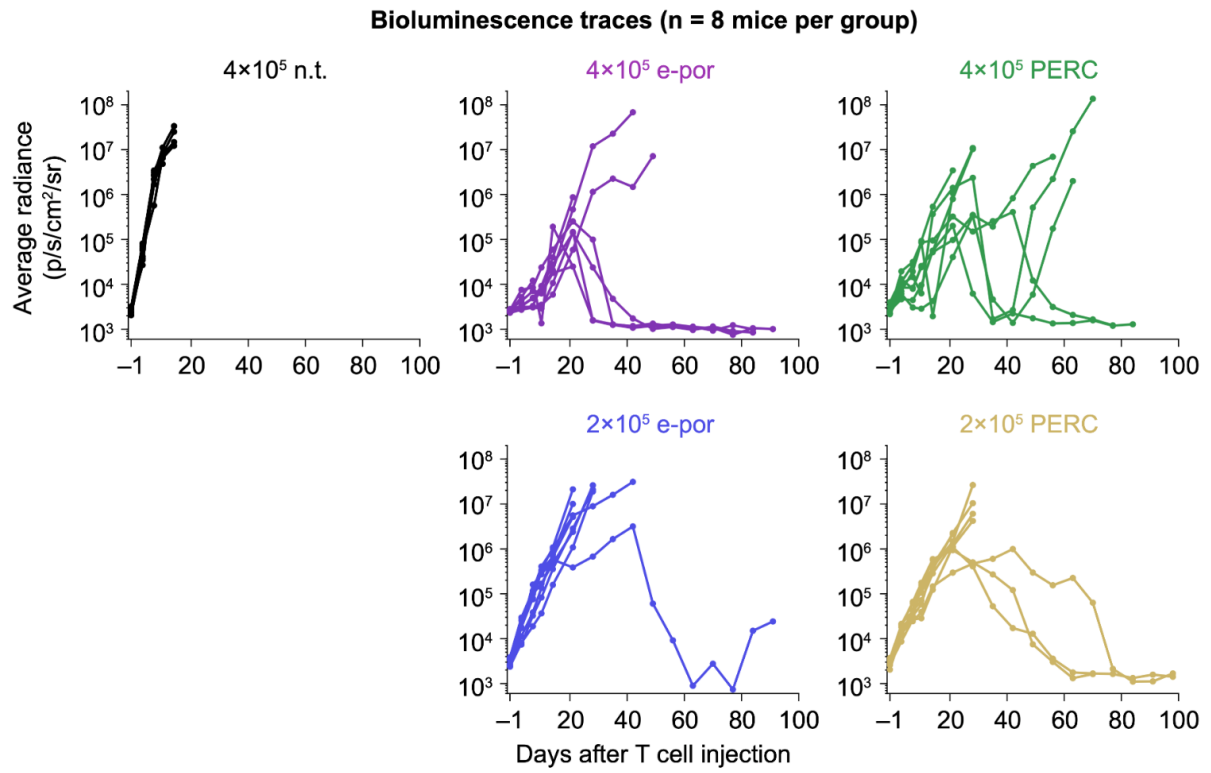
Supplementary Fig. 27 I T cell editing for the extended culture experiment. **a**, Percentages of cells that underwent CAR knock-in and/or B2M knockout. **b**, Percentages of edited cells over time. **c**, Percentages of CD8⁺ cells over time, considering all cells or the CAR⁺ subset.



Supplementary Fig. 28 | T cell editing for the repetitive stimulation experiment. **a**, Percentages of CD8⁺ cells in each condition, considering all cells or the CAR⁺ subset. **b**, CD62L/CD45RA phenotypes of CD4⁺ and CD8⁺ cells, considering all cells or the CAR⁺ subset. **c**, Cytotoxicity traces for T cells from each of three donors (related to Fig. 5c). Error bars represent S.E.M from three technical replicates.



Supplementary Fig. 29 | T cell editing for the *in vivo* tumor challenge experiment. **a,b**, Flow cytometry characterization of editing outcomes and phenotype prior to injection. Edited cells that were injected were treated with Cas9 *TRAC-RNP*, CAR AAV, and Cas9 *B2M-RNP*.



Supplementary Fig. 30 | Bioluminescence imaging trace for each mouse; associated with data in **Fig. 5g**.

#	Name	Sequence
1	E5-TAT	GLFEAIAEFIENGWEGLIEGWYG GRKKRRQRRR
2	E5-TAT-Cys	GLFEAIAEFIENGWEGLIEGWYG GRKKRRQRRR C
3	Cys-E5-TAT	C GLFEAIAEFIENGWEGLIEGWYG GRKKRRQRRR
4	E5-TAT_YGYG	GLFEAIAEFIENGWEGLIEGWYG Y GRKKRRQRRR
5	E5-TAT_YGYG_R3	GLFEAIAEFIENGWEGLIEGWYG Y GRKKRRQRRR R
6	E5-TAT_YGAG	GLFEAIAEFIENGWEGLIEGWYG A GRKKRRQRRR
7	E5-TAT_YGGG	GLFEAIAEFIENGWEGLIEGWYG G GRKKRRQRRR
8	E5-TAT_YG	GLFEAIAEFIENGWEGLIEGWY Y GRKKRRQRRR
9	E5-R8Q_YGYG	GLFEAIAEFIENGWEGLIEGWYG Y GRRRRRQRRR
10	E4D-TAT	GLFEAIAEFIENGWEGLI D GWYG GRKKRRQRRR
11	INF7-TAT	GLFEAIEGFIENGWEGMIDGWYGYGRKKRRQRR
12	I7T-Cys	GLFEAIEGFIENGWEGMIDGWYGYGRKKRRQRR C
13	Cys-I7T	C GLFEAIEGFIENGWEGMIDGWYGYGRKKRRQRR
14	I7T_YGG	GLFEAIEGFIENGWEGMIDGWYG GRKKRRQRR
15	H5WYG-TAT	GLFHAI AHFIHGWHGLIHGWYGYGRKKRRQRR
16	K5WYG-TAT	GLFKAI AKFIKGGWGLIKGWYGYGRKKRRQRR
17	E5WYG-TAT	GLFEAIAEFIEGGWEGLIEGWYGYGRKKRRQRR
18	INF7-G1R_TAT	R LFEAIEGFIENGWEGMIDGWYGYGRKKRRQRR
19	INF7-G1K_TAT	K LFEAIEGFIENGWEGMIDGWYGYGRKKRRQRR
20	INF7-L2F_TAT	F LFEAIEGFIENGWEGMIDGWYGYGRKKRRQRR
21	INF7-E4G_TAT	GLF G AIEGFIENGWEGMIDGWYGYGRKKRRQRR
22	INF7-A5K_TAT	GLFE K IEGFIENGWEGMIDGWYGYGRKKRRQRR
23	INF7-E7W_TAT	GLFEAI W GFIENGWEGMIDGWYGYGRKKRRQRR
24	INF7-G8E_TAT	GLFEAIE E FIENGWEGMIDGWYGYGRKKRRQRR
25	INF7-F9L_TAT	GLFEAIE L IENGWEGMIDGWYGYGRKKRRQRR
26	INF7-E11W_TAT	GLFEAIEGFI W NGWEGMIDGWYGYGRKKRRQRR
27	INF7-W14E_TAT	GLFEAIEGFIENG E EGMIDGWYGYGRKKRRQRR
28	INF7-E15R_TAT	GLFEAIEGFIENG R G MIDGWYGYGRKKRRQRR
29	INF7-G16A_TAT	GLFEAIEGFIENG A MIDGWYGYGRKKRRQRR
30	INF7-G20L_TAT	GLFEAIEGFIENGWEGMID L WYGYGRKKRRQRR
31	INF7-Y22N_TAT	GLFEAIEGFIENGWEGMIDG N GYGRKKRRQRR
32	INF7_TAT-R33del	GLFEAIEGFIENGWEGMIDGWYGYGRKKRR Q R
33	INF7_TAT-4RH	GLFEAIEGFIENGWEGMIDGWYGY G H K K H H Q H R
34	ret-inv-INF7_TAT	rrqrkrkrgygywgdimgewgneifgeiaeflg ("D" amino acids)
35	INF7-E4G_M17L_TAT	GLF G AIEGFIENGW E LIDGWYGYGRKKRRQRR
36	INF7-G1R_E7W_TAT-Y24G_R26G	R LFEAI W GFIENGWEGMIDGWY G G G YGRKKRRQRR
37	E5-R8Q	GLFEAIAEFIENGWEGLIEGWYG GRRRRRQRRR

Supplementary Table 1 | Peptides screened in **Supplementary Fig. 1**. Highlighted peptides have corresponding colored bars in **Supplementary Fig. 1** and were also compared as part of **Fig. 1c**. Amino acid differences (from “founder” peptides, #1 or #11) are indicated in red. The retro/inverse form of INF7-TAT (peptide #34) is a reversed peptide sequence and used non-canonical D-amino acids.

Gene target or gRNA name	Spacer sequence [PAM]	Enzyme	Cell type
<i>AAVS1</i>	GGGGCCACTAGGGACAGGAT [TGG]	SpCas9	T cells
<i>B2M</i>	GAGTAGCGCGAGCACAGCTA [AGG]	SpCas9 (for KO)	T cells
<i>B2M</i>	GGCCACGGAGCGAGACATCT [CGG]	SpCas9 (for KI)	T cells
<i>B2M</i>	[TTTC] AGTGGGGGTGAATTCAGTGT	AsCas12a Ultra	T cells
<i>CD5</i>	CAGTCGCTTCCTGCCTCGGA [CGG]	SpCas9	T cells
<i>CD45</i>	AGTACATGAATTATGAGATA [TGG]	SpCas9	B cells, NK cells
CCR5off-1	TAATCCATCTTGTTCCACCC [TGT]	ABE8e-NG-SpCas9	T cells
<i>EMX1</i>	GAGTCCGAGCAGAAGAAGAA [GGG]	SpCas9	T cells
HEKsite4	GGCACTGCGGCTGGAGGTGG [GGG]	SpCas9	T cells
<i>TRAC</i>	CAGGGTTCTGGATATCTGT [GGG]	SpCas9	T cells
<i>TRAC</i>	[TTTA] GAGTCTCTCAGCTGGTACAC	AsCas12a Ultra	T cells

Supplementary Table 2 | Guide RNAs used.

Target	Fluorophore	Dilution	Manufacturer	Clone	Catalog #
B2M	FITC	1:200	BioLegend	2M2	316304
B2M	PE	1:200	BioLegend	2M2	316306
CAR (scFv)	AF647	1:100	Jackson ImmunoResearch	polyclonal	115-606-072
CCR5	AF647	1:10	BioLegend	HEK1/85a	313712
CD19	APC	1:50	BioLegend	HIB19	302211
CD3ε	BV421	1:50	BioLegend	UCHT1	300434
CD3ε	FITC	1:300	BioLegend	UCHT1	300405
CD3ε	PE	1:300	BioLegend	UCHT1	300407
CD4	APC	1:300	BioLegend	OKT4	317415
CD4	BUV395	1:200	BD Biosciences	SK3	563550
CD4	BV421	1:300	BioLegend	OKT4	317433
CD4	BV711	1:50	BioLegend	SK3	344648
CD4	PeCy7	1:300	BioLegend	OKT4	317413
CD45	BV421	1:25	BioLegend	2D1	368521
CD45RA	BB515	1:25	BD Biosciences	HI100	564552
CD45RA	PerCP	1:25	BioLegend	HI100	304155
CD5	APC	1:300	BioLegend	UCHT2	300611
CD5	PE	1:200	BioLegend	UCHT2	300607
CD62L	BUV395	1:25	BD Biosciences	DREG-56	740301
CD62L	BV421	1:25	BD Biosciences	DREG-56	563862
CD8	AF647	1:50	BioLegend	SK1	344726
CD8	BV711	1:300	BioLegend	SK1	344733
CD8	FITC	1:300	BioLegend	SK1	344703
n/a	GhostDye Red 780	1:1000	Tonbo Biosciences	n/a	13-0865-T500
HLA-E	PE	1:50	Invitrogen	3D12HLA-E	12-9953-42
IFN-γ	PE	1:50	BioLegend	4S.B3	502509
NY-ESO-1 TCR	PE	1:50	Immudex	HLA-A*02:01 SLLMWITQV dextramer	WB3247
TCRα/β	BV421	1:50	BioLegend	IP26	306721
TCRα/β	PE-Cy7	1:50	BioLegend	IP26	306720
TCRα/β	PerCP-Cy5.5	1:50	BioLegend	IP26	306723
TNF	FITC	1:50	BioLegend	MAB11	502906
n/a	TruStain FcX	1:25	BioLegend	n/a	422301

Supplementary Table 3 | Antibodies used for flow cytometry analysis.

Gene	Forward primer sequence	Reverse primer sequence	Probe sequence	Assay
AAVS1	TTCCCAGGGCCGGTTAATGTG	TCTGCCTAACAGGAGGTGGGG	-	NGS
B2M	ATATAAGTGGAGGCGTCGCG	GAAGTCACGGAGCGAGAGAG	-	NGS
CCR5	TTTGCAATTCATGGAGGGCACTAA	GGTTGAGCAGGTAGATGTCAGT	-	NGS
TRAC	CATCACTGGCATCTGGACTCCA	CGGTGAATAGGCAGACAGAC	-	NGS
EMX1 on-target	CATCTGTGCCCCCTCC	GTTGCCACCCCTAGTCATTGGA	-	NGS
EMX1 off-target	AATGTGCTTCAACCCATCACGGCCTA	CATCAGTGTGGCTTTCACAAGGATGG	-	NGS
HEKsite4 on-target	CTGAGATCCTGTCCTTAGTTTACTG	TTTCAACCCGAACGGAGAC	-	NGS
HEKsite4 off-target	GCAAGGCGGGAAAAGAGAAA	CTAAGCTCTCATGCCCAGGG	-	NGS
5'B2M-3'TRAC	GCTGGGCACGCGTTTAATAT	TCGGTGAATAGGCAGACAGA	CATTCCCTGAAGCTGACAGCATTCGGGC	ddPCR
5'TRAC-5'B2M	ATCACTGGCATCTGGACTCC	GCTGGGCACGCGTTTAATAT	CATTCCCTGAAGCTGACAGCATTCGGGC	ddPCR

Supplementary Table 4 | Oligonucleotides used for ddPCR or for generating amplicons that were analyzed by NGS.