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Rapid and efficient generation of antigen-specific isogenic T cells from cryopreserved blood samples

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Keywords

Antigen-specific immune responses, clinical trials, CRISPR/Cas9, gene editing, immune monitoring, knockout, proliferation, T cells, viability

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Abstract

Clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9 (CRISPR/Cas9)-mediated gene editing has been leveraged for the modification of human and mouse T cells. However, limited experience is available on the application of CRISPR/Cas9 electroporation in cryopreserved T cells collected during clinical trials. To address this, we aimed to optimize a CRISPR/Cas9-mediated gene editing protocol compatible with peripheral blood mononuclear cells (PBMCs) samples routinely produced during clinical trials. PBMCs from healthy donors were used to generate knockout T-cell models for interferon- γ , Cbl proto-oncogene B (CBLB), Fas cell surface death receptor (Fas) and T-cell receptor (TCR $\alpha\beta$) genes. The effect of CRISPR/Cas9-mediated gene editing on T cells was evaluated using apoptosis assays, cytokine bead arrays and *ex vivo* and *in vitro* stimulation assays. Our results demonstrate that CRISPR/Cas9-mediated gene editing of *ex vivo* T cells is efficient and does not overtly affect T-cell viability. Cytokine release and T-cell proliferation were not affected in gene-edited T cells. Interestingly, memory T cells were more susceptible to CRISPR/Cas9 gene editing than naïve T cells. *Ex vivo* and *in vitro* stimulation with antigens resulted in equivalent antigen-specific T-cell responses in gene-edited and untouched control cells, making CRISPR/Cas9-mediated gene editing compatible with clinical antigen-specific T-cell activation and expansion assays. Here, we report an optimized protocol for rapid, viable and highly efficient genetic modification in *ex vivo* human antigen-specific T cells, for subsequent functional evaluation and/or expansion. Our platform extends CRISPR/Cas9-mediated gene editing for use in gold-standard clinically used immune-monitoring pipelines and serves as a starting point for development of analogous approaches, such as those including transcriptional activators and/or epigenetic modifiers.

INTRODUCTION

Clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9 (CRISPR/Cas9)-mediated gene targeting has been used to significantly improve our understanding of T-cell biology and discover regulators of, for example, T-cell proliferation and differentiation.^{1–3} Most of this work in this regard has been performed using cultured peripheral blood mononuclear cells

(PBMCs) from healthy donors, or using patient T cells after an initial *ex vivo* expansion.³ In this setting, cell numbers are not limited and there is generally no need to maintain the clonal repertoire of T cells. By contrast, samples obtained within clinical trials in immune oncology are generally limited and antigen specificity is paramount in understanding the underlying immune biology.^{4–6} Expanding the CRISPR/Cas9 toolbox to antigen-specific T cells from clinical samples would

therefore enable further dissection of anticancer immune responses and discovery of novel targets for combination immunotherapy.¹

Herein, special consideration should be given to maintaining the *ex vivo* T cells' phenotype and function during the process of gene engineering.^{4,7} In addition, a CRISPR/Cas9-mediated gene targeting protocol for clinical samples should ideally be compatible with the timelines used in best-practice immunomonitoring protocols. As such, viral-mediated transfection models, such as lentivirus, adenovirus and retrovirus with variable knockout efficiencies and T-cell toxicity are unsuitable.^{8–10} Ribonucleoprotein (RNP) complexes of Cas9 and a guide RNA (gRNA), when introduced by electroporation, have been reported as suitable for *ex vivo* modification of T cells, but whether a high level of viability and clonality can be maintained for incorporation into immunomonitoring protocols remains unclear.^{8,9}

Here, we report on an optimized protocol for rapid, viable and highly efficient genomic manipulation in *ex vivo* human antigen-specific T cells. We demonstrate the efficacy of this gene editing system within common immunomonitoring pipelines and across gRNAs. This system is robust across donors and usable for gene editing in both memory and naïve T cells for studying recall and *de novo* responses, respectively.

RESULTS

CRISPR/Cas9-mediated gene editing of *ex vivo* T cells does not overtly affect T-cell viability

We set out to optimize a CRISPR/Cas9-mediated gene editing protocol compatible with PBMC samples analogous to those routinely procured during clinical trials. PBMCs were processed according to best practice operating protocols for PBMC isolation, cryopreservation and cell density used in storage. Using these standardized PBMC aliquots, we first optimized RNP complex electroporation using efficiency and viability as main parameters. As proof of concept, we targeted interferon- γ (IFN γ) as it is not expressed *ex vivo* in T cells, readily induced upon activation and easily assessable by ELISA or intracellular flow cytometry. Electroporation of Cas9/IFN γ gRNA RNP complexes into T cells resulted in an almost complete abrogation of IFN γ expression in both CD8 and CD4 T cells (Figure 1a, c; $P < 0,0001$). Electroporation, inclusion of Cas9/IFN γ gRNA RNP complexes, Cas9/trans-activating CRISPR RNA (tracrRNA) complexes and/or electroporation alone had no effect on T-cell viability as measured using amine-reactive dye staining (Figure 1b, e). By varying experimental parameters, we observed that while Cas9

protein concentration did not affect T-cell viability, the size of the electroporation cuvette was critically important, resulting in a reduced relative viability when using 20- μ L cuvettes compared with 100- μ L cuvettes (Figure 1d). IFN γ knockout efficiency was similar for all experimental conditions and in both CD4 and CD8 T cells (Figure 1d). As lower doses of Cas9 protein were equally effective, we chose 100 μ g mL⁻¹ in 100- μ L cuvettes as optimal concentration in all subsequent experiments. Analysis of additional parameters of cell viability: 7-aminoactinomycin D, phosphatidyl serine exposure and caspase activation confirmed the minimal loss of cell viability under these conditions (Figure 1e). Non-T cells in the PBMC fraction were more sensitive to the T-cell optimized protocol, as evident from increased cell death in the total PBMC fraction (Figure 1f).

CRISPR/Cas9-mediated gene editing of *ex vivo* T cells does not overtly affect T-cell function

Next, we tested whether our optimized protocol affected T-cell function by evaluating cytokine production and proliferation of gene-edited T cells. While release of IFN γ into the extracellular space was markedly reduced in IFN γ knockout T cells, no differences were observed in the release of other cytokines, such as tumor necrosis factor alpha (TNF α) and interleukin (IL)-2 (Figure 2a, b, Supplementary figure 1). Atypical induction of IL-4, IL-6 or IL-10 was also not observed under these conditions (Figure 2a, b). We also analyzed whether *ex vivo* gene-engineered T cells required recovery following electroporation by activating T cells using anti-CD3/CD28 beads at 0, 30, 60, 120 and 240 min after RNP nucleofection, followed by a 2-day culture and restimulation using phorbol 12-myristate 13-acetate (PMA)/ionomycin. Under all conditions, production of IFN γ , but not TNF α or IL-2, was specifically abrogated (Figure 2c, d). Accordingly, anti-CD3/CD28 bead- or phytohemagglutinin-based expansion of T cells directly following electroporation did not impair expansion capacity over a period of 14 days (Figure 2e). Furthermore, gene-edited T cells that expanded over this period remained IFN γ negative, suggesting the optimized protocol did not negatively affect T-cell fitness when compared with unedited T cells (Figure 2f and Supplementary figure 1). We reasoned that the direct *ex vivo* gene editing, followed by immediate T-cell activation, could even be leveraged to knockout genes critical for anti-CD3/CD28 bead-based activation, as the steady-state protein levels in the absence of transcription are likely sufficient to drive signaling during the initial 24–48 h of culture. To test this hypothesis, we disrupted expression of the T-cell receptor (TCR $\alpha\beta$) complex. T

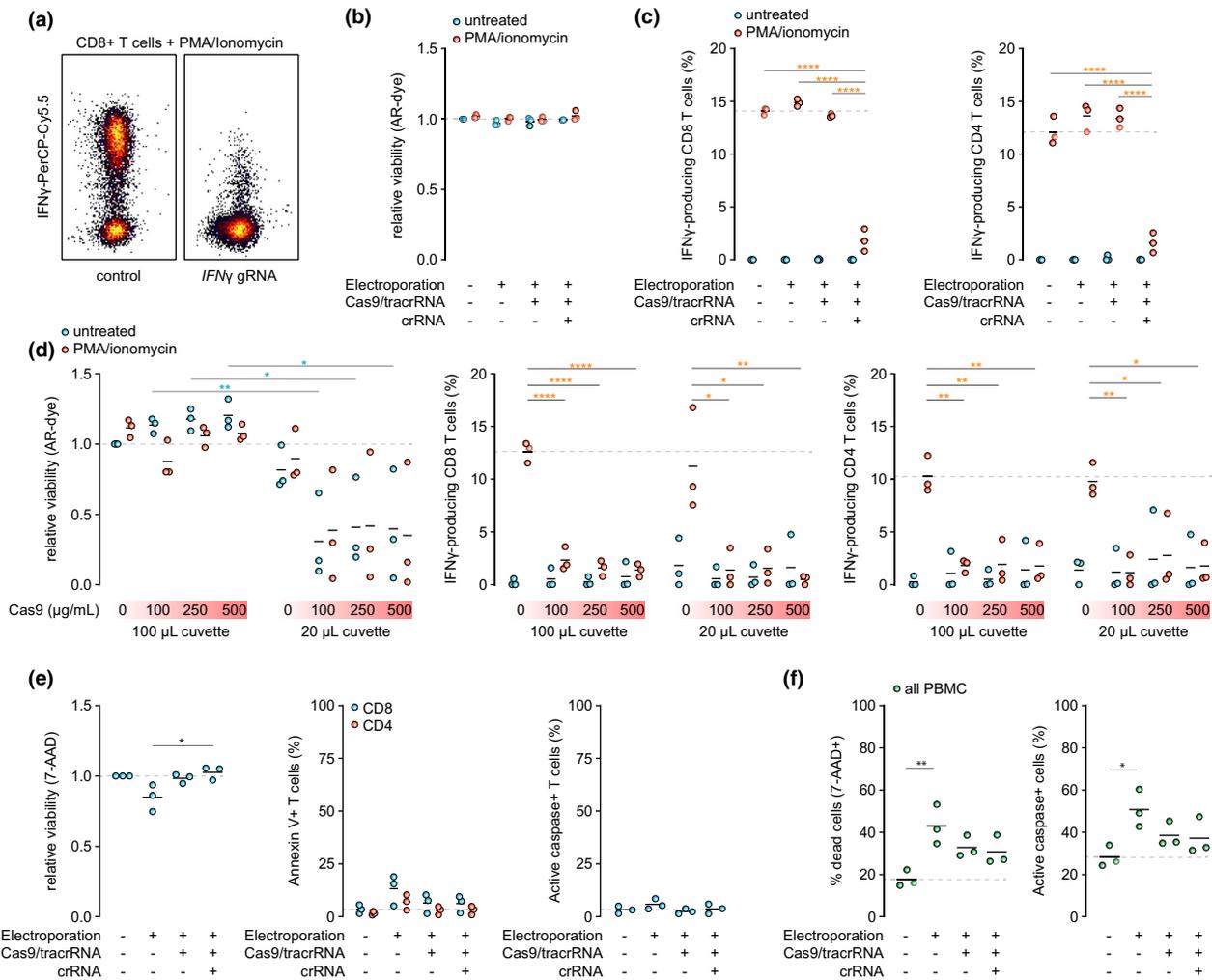


Figure 1. CRISPR/Cas9 electroporation of *ex vivo* T cells does not affect T-cell viability. Electroporation of PBMCs with Cas9/IFN γ RNP complexes results in almost complete abrogation of IFN γ expression in both CD8 and CD4 T cells. About 16 h after electroporation, PBMCs were stimulated for 4 h with PMA/ionomycin. **(a, c)** Representative flow cytometry contour plot showing the significantly reduced expression of IFN γ production in Cas9/IFN γ gRNA RNP-electroporated CD8 and CD4 T cells. **(b)** Scatterplots demonstrating the viability of PBMCs after electroporation alone, with Cas9/tracrRNA complexes and/or Cas9/IFN γ gRNA RNP complexes. Viability was measured using amine-reactive dye. **(d)** Effect of different cuvette sizes (100 and 20 μL) and different Cas9 protein concentrations (0, 100, 250 and 500 $\mu\text{g mL}^{-1}$) on cell viability showing the maintenance of optimal viability when using a 100- μL cuvette. **(e, f)** Analysis of additional parameters of cell viability was performed using 7-AAD, PS exposure and caspase activation in **(e)** CD8 and CD4 T cells and **(f)** in the total PBMC fraction. All experiments were performed in triplicate. Blue dots represent unstimulated PBMCs; orange dots represent PBMCs stimulated with PMA/ionomycin for 4 h; green dots represent all PBMCs; **(e)** in the middle scatterplot, blue dots represent CD8 T cells and orange dots CD4 T cells. One-way ANOVA or unpaired *t*-tests were performed where appropriate. * $P \leq 0.05$, ** $P \leq 0.01$ and **** $P \leq 0.0001$. 7-AAD, 7-aminoactinomycin D; AR, amine reactive; crRNA, CRISPR RNA; CRISPR/Cas9, clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9; gRNA, guide RNA; IFN, interferon; PBMC, peripheral blood mononuclear cells; PMA, phorbol 12-myristate 13-acetate; PS, phosphatidyl serine; RNP, ribonucleoprotein; tracrRNA, trans-activating CRISPR RNA.

cells were activated using anti-CD3/CD28 beads directly after RNP electroporation. Again, the CRISPR Cas9/TCR $\alpha\beta$ -gRNA RNP complex electroporation resulted in high TCR $\alpha\beta$ knockout efficiency in both CD8 and CD4 T cells at 48 h (Figure 2g). As anticipated, TCR $\alpha\beta$

knockout T cells proliferated comparably to non-edited T cells over a period of 14 days (Figure 2h). As for IFN γ , no detrimental effects of gene editing were observed and near-complete loss of TCR $\alpha\beta$ was maintained at day 14 (Figure 2i).

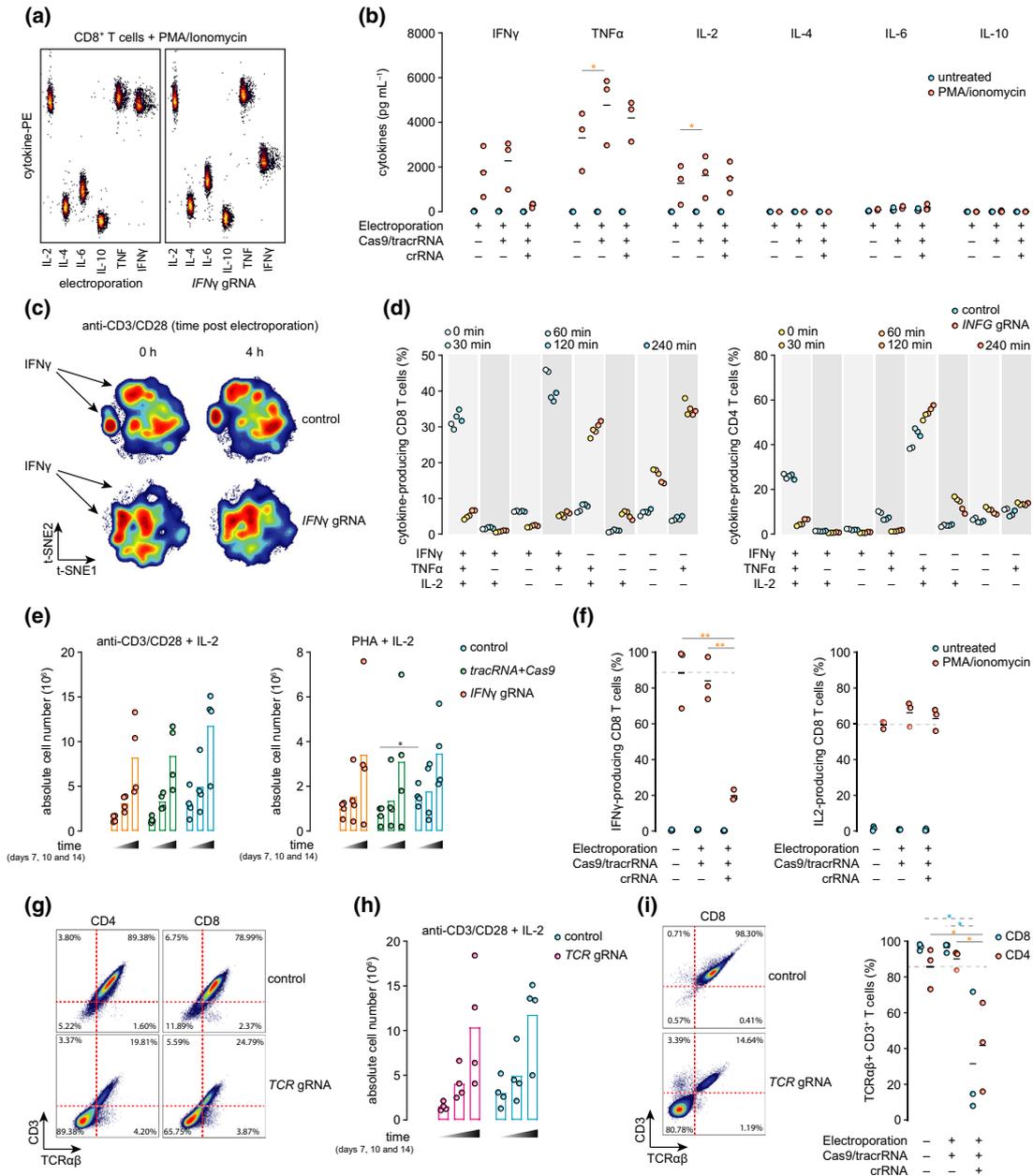


Figure 2. CRISPR/Cas9-mediated gene editing of *ex vivo* T cells does not affect T-cell function. IFN γ , TNF α , IL-2, IL-4, IL-6 and IL-10 release was evaluated using a cytokine bead array kit. **(a, b)** Electroporation with Cas9/IFN γ gRNA RNP complexes results in reduced IFN γ release, whereas no differences are observed in the release of TNF α and IL-2 1 day after electroporation upon stimulation with PMA/ionomycin for 4 h. Atypical induction of IL-4, IL-6 or IL-10 was also not observed. **(c)** t-SNE plots showing the reduced IFN γ release after stimulation with beads at $t = 0$ and $t = 4$ h after electroporation. **(d)** IFN γ , TNF α and IL-2 cytokine-producing CD8 T cells after activation with anti-CD3/CD28 beads at 0, 30, 60, 120 and 240 min after RNP nucleofection, followed by a 2-day culture and restimulation using PMA/ionomycin. **(e)** Scatterplot showing the anti-CD3/CD28 bead- or PHA-based expansion of T cells directly following electroporation over 14 days. **(f)** IFN γ and IL-2 production of gene-edited CD8 T cells after 14 days of expansion. **(g)** Flow cytometry contour plot showing the TCR $\alpha\beta$ knockout in CD8 and CD4 T cells 48 h after electroporation. **(h)** Expansion capacity of anti-CD3/CD28-activated TCR $\alpha\beta$ knockout cells over 14 days. **(i)** Flow cytometry contour plot (left) and scatterplot (right) showing the TCR $\alpha\beta$ knockout in CD8 and CD4 T cells after 14 days. All experiments were performed in triplicate or quadruplicate. One-way ANOVA was performed where appropriate. * $P \leq 0.05$ and ** $P \leq 0.01$. crRNA, CRISPR RNA; CRISPR/Cas9, clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9; gRNA, guide RNA; IFN, interferon; IL, interleukin; PE, phycoerythrin; PHA, phytohemagglutinin; PMA, phorbol 12-myristate 13-acetate; RNP, ribonucleoprotein; TCR, T-cell receptor; TNF, tumor necrosis factor; tracrRNA, trans-activating CRISPR RNA; t-SNE, t-distributed stochastic neighbor embedding.

CRISPR/Cas9-mediated gene editing of *ex vivo* T cells preferentially targets memory T cells

We next determined whether memory or naïve T cells were more amenable to CRISPR/Cas9-mediated gene editing in the *ex vivo* setting. PBMCs were subjected to TCR $\alpha\beta$ -targeting RNP electroporation, followed by an 8-day culture with cytokines IL-2, IL-15 and/or IL-7 to support memory and naïve T cells, respectively. Cell viability was similar across all conditions (Figure 3a). Percentages of memory CD8 T cells were similar between TCR $\alpha\beta$ knockout and TCR $\alpha\beta$ -expressing cells regardless of the cytokine cocktail used (Figure 3b, c). Notably, there was a slight increase in the percentage of memory CD4 T cells after TCR $\alpha\beta$ knockout compared with the TCR $\alpha\beta$ -proficient cells, independent of cytokine stimulation (Figure 3c).

To further explore this observation, we investigated the knockout efficiency within the memory and naïve T-cell populations (Figure 3d–i). The knockout efficiency of memory T cells varied between 60% and 85% in CD8⁺ T cells and between 70% and 95% in CD4⁺ T cells (Figure 3d, g). By contrast, the knockout efficiency for naïve T cells ranged between 35% and 80% in CD8 T cells and between 30% and 80% in CD4 T cells (Figure 3e, h). In addition, the percentage of naïve TCR $\alpha\beta$ knockout CD8 T cells was higher when compared with naïve TCR $\alpha\beta$ knockout CD4 T cells (Figure 3e, h). These findings collectively suggest that memory T cells, especially memory CD4 T cells, are slightly more susceptible to CRISPR/Cas9 gene editing than naïve T cells.

CRISPR/Cas9-mediated gene editing is compatible with clinical antigen-specific T-cell activation and expansion assays

Finally, having established the high viability and efficacy of our approach, we evaluated whether our optimized protocol was compatible with best-practice assays for monitoring immune responses in clinical trial settings. We analyzed *ex vivo* peptide stimulation as well as *in vitro* stimulation assays. Using cytomegalovirus, Epstein–Barr virus and influenza virus (CEF) peptides as model antigen, and T-cell activation markers CD69 and CD137 as a readout, we were able to demonstrate equivalent CEF peptide-induced responses in gene-edited and untouched control cells for both the *ex vivo* assay (Figure 4a, c) and following 13-day *in vitro* stimulation (Figure 4b, d). These responses were observed for IFN γ knockouts, as well as for Cbl proto-oncogene B (CBLB) and Fas cell surface death receptor (Fas) gRNAs previously reported by others (Figure 4a–d).

DISCUSSION

We describe a rapid, robust and flexible platform for CRISPR/Cas9-mediated gene editing in primary *ex vivo* human T cells. We have optimized efficiency, viability as well as culture conditions to allow rapid and direct *ex vivo* modification of T cells for subsequent functional evaluation and/or expansion. Our platform is amenable to integration into clinically used pipelines for immune response evaluation using antigens of choice.

CRISPR/Cas9-mediated gene editing has become an attractive approach for modifying T cells, owing to its simplicity, operability, low costs and capability of multiplex genome editing.^{9,11} However, limited experience on the application of CRISPR/Cas9 electroporation in immune-monitoring assays in clinical studies is available. The prerequisites for manipulating T cells in immunoassays are maintaining viability, antigen-specificity and function.^{4,7} In this study, we demonstrate for the first time that these prerequisites are not affected by CRISPR/Cas9 gene editing, thereby providing a proof of principle for the application of CRISPR/Cas9 in immune-monitoring assays. In both CD8 and CD4 T cells, a highly efficient gene knockout up to 90% was reached after a single transfection. Previously published literature, using CRISPR/Cas9 RNP electroporation, reported efficiency rates of 20–90% with low efficiency rates in resting primary T cells.^{9,12–16} Moreover, we demonstrated that CRISPR/Cas9-edited cells could be maintained without stimulation in culture for up to 7 days, or supplemented with a diverse range of cytokines without comprising gene knockout efficacy. Activation of T cells directly, or at a later timepoint of choosing, did not negatively affect T-cell function or expansion, nor did cryopreservation and subsequent restimulation. Finally, while the current work focused on knockout of genes, recent advances in nonhomologous end joining-mediated template incorporation using CRISPR/Cas9 should be amenable to the current platform, allowing more complex *ex vivo* T-cell engineering.^{12,14,17}

Advances in descriptive immune monitoring have expanded our understanding of anticancer immune responses. High parameter flow, mass and spectral cytometry have allowed the simultaneous assessment of most immune cell populations *ex vivo*.^{18,19} Imaging using, for example, positron emission tomography/single photon emission computed tomography allows noninvasive assessment of specific immune cells or immune checkpoints throughout the body, and transcriptomic approaches provide ever deeper dimensional assessment of gene expression, including spatial organization.^{20–22} By contrast, *ex vivo* functional assays have remained largely unchanged over the years,

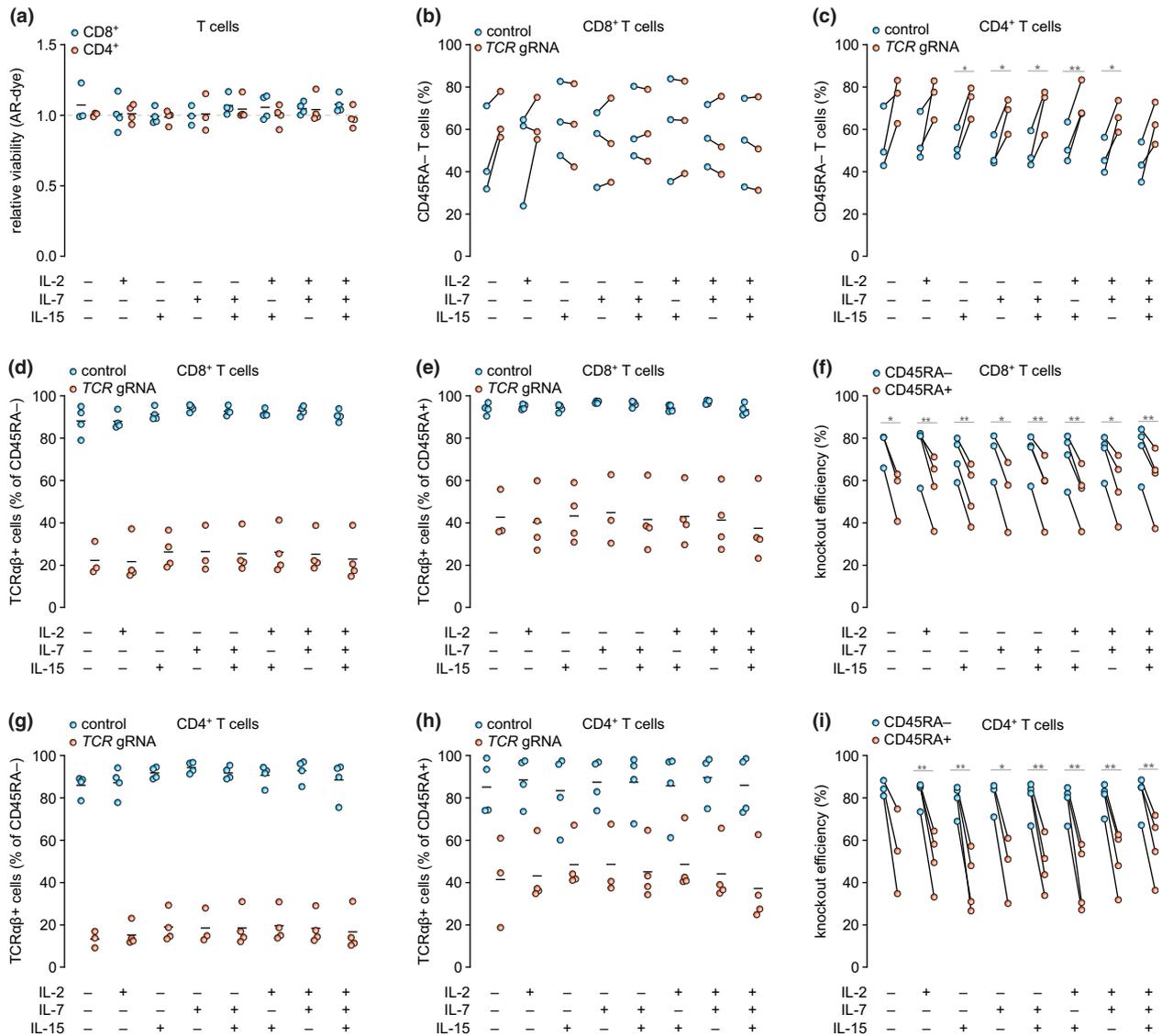


Figure 3. CRISPR/Cas9 gene editing preferentially targets memory T cells. Gene editing of TCRαβ in PBMCs. **(a)** Viability of CD8 versus CD4 TCRαβ knockout T cells after *in vitro* stimulation with IL-2, IL-15 and/or IL-7 for 8 days. **(b)** Percentages of CD8 and **(c)** CD4 memory T cells within TCRαβ knockout T cells compared with TCRαβ-proficient cells after coculturing with IL-2, IL-15 and/or IL-7 for 8 days. **(d)** Percentages of CD8 memory and **(e)** CD8 naive T cells expressing TCRαβ 8 days after gene editing. **(f)** Gene editing efficiency of CD8 memory T cells compared with CD8 naive T cells. **(g)** Percentages of CD4 memory and **(h)** CD4 naive T cells expressing TCRαβ 8 days after gene editing of the TCRαβ receptor. **(i)** Gene editing efficiency of CD4 memory T cells compared with CD4 naive T cells. Culture conditions as indicated. Experiments were performed in triplicate or quadruplicate. Paired *t*-tests were performed where appropriate. **P* ≤ 0.05 and ***P* ≤ 0.01. AR, amine reactive; CRISPR/Cas9, clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9; gRNA, guide RNA; IL, interleukin; PBMC, peripheral blood mononuclear cells; TCR, T-cell receptor.

with peptide stimulation and intracellular flow cytometry, ELISPOT or ELISA used for functional readout on one or two hallmark activation markers.^{4,23} The platform we present here significantly expands the potential of these assays. By seamlessly integrating CRISPR/Cas9-mediated gene editing into *ex vivo* immune-monitoring pipelines, it is possible to not only assess how T cells from clinically treated patients differentially depend on genes of interest,

but also screen *ex vivo* for novel combination targets to further augment T-cell activation.^{14,15} As demonstrated here, these targets need not be limited to cell surface targets accessible by antibody-based therapeutics.^{14,15} One interesting observation is that naïve CD4, but not CD8, T cells appear less sensitive to CRISPR/Cas9-mediated gene editing than their memory counterparts. Whether this is the result of the optimized electroporation protocol used

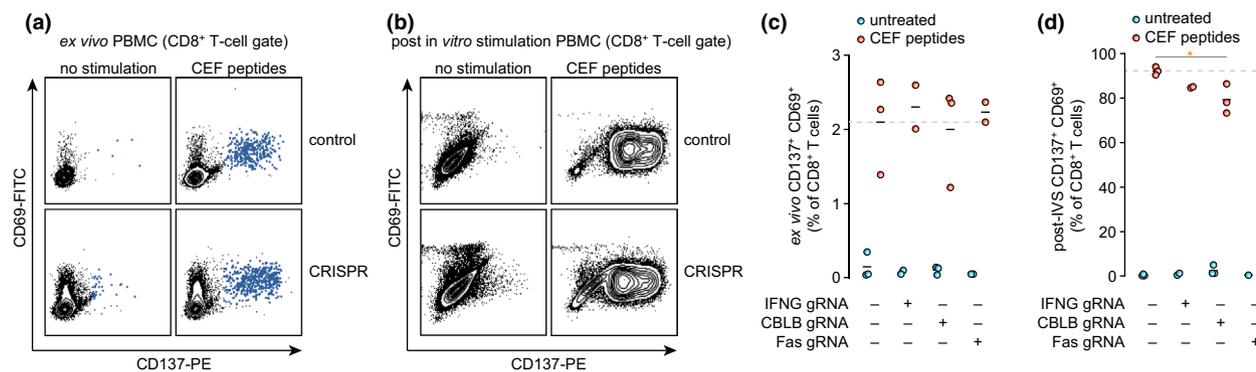


Figure 4. CRISPR/Cas9 editing does not influence the antigen specificity of T cells. Gene editing of IFN γ , CBLB or FAS in PBMCs. **(a)** Representative flow cytometry contour plots showing the expression of T-cell activation markers CD69 and CD137 after *ex vivo* and **(b)** after IVS for 13 days with CEF peptides. **(c)** Scatterplot representing the percentage of CD8 T cells expressing both activation markers CD137 and CD69 within variable CRISPR gene editing conditions and untouched control cells after *ex vivo* stimulation at day 2 and **(d)** after IVS with and without CEF peptides at day 13. Blue dots represent PBMCs not stimulated with CEF peptides and orange dots PBMCs stimulated with CEF peptides. Experiments were performed in duplicate or triplicate. One-way ANOVA was performed where appropriate. $*P \leq 0.05$. CBLB, Cbl proto-oncogene B; CEF, cytomegalovirus, Epstein–Barr virus and influenza virus; CRISPR/Cas9, clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9; FAS, fas cell surface death receptor; FITC, fluorescein isothiocyanate; IFN, interferon; IVS, *in vitro* stimulation; PBMC, peripheral blood mononuclear cells; PE, phycoerythrin.

or an intrinsic feature of naïve CD4 T cells remains currently unknown. Nevertheless, targeted genes of interest could be knocked out in around 60% of CD4 T cells, establishing our platform as an effective approach for studying both recall and *de novo* immune responses. An interesting approach would be to further combine our platform with recent advances in CRISPR/Cas9-mediated gene editing in monocyte-derived dendritic cells. This could allow for reciprocal knockout of ligand–receptor interactions of particular interest for complex ligand–receptor pairs, such as CTLA-4/CD28 and CD80/CD86.

Taken together, our platform represents an advance on previous reports on CRISPR/Cas9-mediated gene editing and extends this approach for use in gold-standard clinically used immune-monitoring pipelines.

METHODS

Peripheral blood mononuclear cells

PBMCs were isolated from buffy coats of healthy blood donors. The procedure was approved by the Ethical Advisory Council located in Amsterdam, The Netherlands. All experiments using human PBMCs were in compliance with the Helsinki declaration and written informed consent was obtained from all donors. For isolation, buffy coats were first diluted with Roswell Park Memorial Institute medium supplemented with 2.5% fetal calf serum (FCS, Bodinco BV, Alkmaar, Netherlands) at a 1:2 ratio. Next, the diluted PBMCs were layered on a Ficoll-Paque (Cytiva, Uppsala, Sweden) gradient at a 1:2 volume ratio and centrifuged at 900g for 20 min without brake. PBMCs on the interface of the Ficoll-

Paque and plasma layers were isolated by pipette (5–10 mL) and washed. Hereto, tubes were supplemented with ice-cold phosphate-buffered saline (PBS, Thermo Fisher Scientific, Waltham, USA) to a final volume of 50 mL and centrifuged at 560g for 8 min without brake. PBMC pellets were pooled and washed with PBS. Hereto, PBMCs were supplemented with ice-cold PBS to a final volume of 50 mL and centrifuged at 350g for 8 min without brake. After PBMCs were resuspended in 50 mL of ice-cold PBS, PBMCs were counted using the Bürker counting chamber and centrifuged at 350g at 8 min without brake. Finally, PBMCs were resuspended in 1 mL of freezing medium, consisting of 90% FCS and 10% dimethyl sulfoxide (DMSO, Merck, Darmstadt, Germany), at a concentration of $10\text{--}100 \times 10^6$ cells per cryovial.

Cas9, tracrRNA and crRNA

Cas9 (Alt-R Cas 9 Nuclease V3), tracrRNA (Alt-R CRISPR/Cas9 tracrRNA) and the CRISPR RNAs (crRNA; Alt-R CRISPR/Cas9 crRNA) were synthesized by Integrated DNA Technologies (Coralville, IA, USA). Both tracrRNA and the crRNAs were each reconstituted in nuclease-free duplex buffer (Lonza, Basel, Switzerland) to a final concentration of 100 μM . Hereto, 2 nM of tracrRNA or crRNA was dissolved in 20 μL nuclease free buffer. See Supplementary table 1 for gRNAs and sequences. For TCR $\alpha\beta$ equal volumes of T cell receptor alpha1+2 (TRAC1+2) and beta (TRBC) were mixed before adding the RNP complex to the PBMCs (see the “RNP electroporation” section).

RNP electroporation

Cryopreserved PBMCs were thawed, counted and resuspended in X-VIVO 15 (Lonza, Basel, Switzerland) at a concentration

of 100×10^6 cells mL^{-1} . After optimization of our protocol as indicated in Figure 1d and main text, a standard concentration of crRNA of 1.5 μL , tracrRNA of 1.5 μL and Cas9 of 10 μg was used in a 100- μL cuvette and prepared as follows. First, a gRNA complex was assembled. For each RNP electroporation condition, a concentration of 150 pmol gRNA complex is needed. Hereto, 1.5 μL tracrRNA was combined with 1.5 μL crRNA. This mixture was annealed by heating at 95°C for 5 min, followed by slowly cooling down to room temperature for 10 min. Next, 12 μL duplex buffer (Lonza, Basel, Switzerland) was added to get a final concentration 150 pmol gRNA complex in 15 μL of total volume. To prepare a Cas9-gRNA RNP complex, 10 μg Cas9 (5 μL) was added to the gRNA complex. This mixture was incubated for 10 min at room temperature. Finally, duplex buffer was added to the RNP complex to obtain a final volume of 20 μL . For the experiments performed with the 20- μL cuvette size all final concentrations were maintained equal.

For the preparation of PBMCs, PBMCs were centrifuged and washed once with PBS (Thermo Fisher Scientific, Waltham, USA). Cells were resuspended in 100 μL for 100- μL -cuvette size or 20 μL for 20- μL -cuvette size nucleofection solution at a density of 100×10^6 cells mL^{-1} . Therefore, 10×10^6 cells and 2×10^6 cells were used for 100- and 20- μL cuvettes, respectively. Nucleofection solution consisted of 82 μL P2 Primary Cell Nucleofector Solution (Lonza, Basel, Switzerland) and 18 μL Supplement 1 (Lonza, Basel, Switzerland) for 100- μL cuvettes and 16.4 μL P2 Primary Cell Nucleofector Solution and 3.6 μL Supplement 1 for 20- μL cuvettes. The Cas9-gRNA RNP complex was added to the PBMCs and incubated for 2 min at room temperature. For nucleofection the PBMC/Cas9/gRNA RNP mixture was transferred to a nucleofection cuvette and electroporated by using a 4D-Nucleofector machine (Amaxa, program P2, pulse code EH100). The Amaxa 4D-Nucleofector machine (Lonza, Basel, Switzerland) was used for all CRISPR experiments. After electroporation cells were resuspended in prewarmed culture medium at a concentration of 5×10^6 cells mL^{-1} . PBMCs that did not undergo electroporation served as control and were only centrifuged and washed with PBS.

Intracellular cytokine staining

After 16 h of electroporation, PBMCs were stimulated for 4 h with PMA/ionomycin (eBioscience stimulation cocktail, Waltham, USA) at 37°C. A volume of 1 μL PMA/ionomycin was used per 500 μL sample. For intracellular staining of IFN γ , TNF α and IL-2, secretion of cytokines was blocked using 1 μL GolgiPlug (protein transport inhibitor; BD Biosciences, San Jose, CA, USA) per 1 mL sample. After 4 h of incubation, cells were washed, stained with amine reactive viability dye (Zombie Aqua; BioLegend, CA, USA) according to manufacturer's protocol for 15 min and washed with PBS (Thermo Fisher Scientific, Waltham, USA) supplemented with 5% FCS (Bodinco BV, Alkmaar, Netherlands). Next, cells were fixed and permeabilized (FIX&PERM® Cell Fixation and Permeabilization Kit, GAS-002, Nordic-Mubio, Susteren, the Netherlands) and stained with surface markers CD3, CD8 and

CD4, and intracellular markers IFN γ and IL-2 for flow cytometry analysis. For the TCR $\alpha\beta$ knockout experiments, only cell surface staining with TCR $\alpha\beta$, CD3, CD8 and CD4 antibodies was performed. See Supplementary table 3 for characteristics of the antibodies. For fixation and permeabilization cells were incubated with 100 μL /well of reagent A (fix) for 15 min at room temperature. Next, cells were washed with PBS and incubated with a mixture of reagent B (perm) and appropriate antibodies for 15 min at room temperature. In total, 100 μL of the mixture reagent B plus antibodies was added per well. After incubation, cells were washed twice, centrifuged and resuspended in flow cytometry buffer (PBS + 2% FCS).

Intracellular staining of IFN γ , TNF α and IL-2 was also performed on engineered T cells activated with anti-CD3/CD28 beads (Thermo Fisher Scientific, Waltham, USA; catalog number 11131D) at 0, 30, 60, 120 and 240 min after RNP nucleofection, followed by a 2-day culture and restimulation using PMA/ionomycin.

Apoptosis assay

After RNP electroporation, PBMCs were resuspended in X-VIVO 15 (Lonza, Basel, Switzerland), plated at a density of 5×10^6 mL^{-1} in a 24-well plate and left untreated. Apoptosis assay was performed 24 h after CRISPR/Cas9-mediated gene editing. For the apoptosis assay, PBMCs were counted, centrifuged and resuspended in X-VIVO 15 (Lonza, Basel, Switzerland) at a density of $1-2 \times 10^6$ cells mL^{-1} . Subsequently, 1 μL of Violet Live Caspase (BD Biosciences, San Jose, CA, USA) probe per 0.5 mL of cell suspension was added and cells were incubated for 45 min at 37°C. After washing, cells were resuspended in fresh medium and incubated for an additional 30 min at 37°C while protected from light. Afterward, CD8 and CD3 antibodies were added, and cells were incubated for 30 min at room temperature. Cells were washed twice with cold PBS (Thermo Fisher Scientific, Waltham, USA) and resuspended in 1 \times Annexin V Binding Buffer (BD Biosciences, San Jose, CA, USA) at a final concentration of 1×10^6 cells mL^{-1} ; 1×10^5 cells were incubated with 5 μL of Annexin V and 5 μL of 7-aminoactinomycin D (BD Biosciences, San Jose, CA, USA) for 15 min at room temperature in the dark. Cells were analyzed within 1 h using the BD FACSVerser flow cytometer (BD Biosciences, San Jose, CA, USA).

Cytokine bead array

One day after electroporation (10×10^6 cells in 2 mL, 1 mL per well), gene-edited (cas9/IFN γ gRNA RNP and Cas9/tracrRNA) and control (electroporation only) PBMCs were incubated with PMA/ionomycin (eBioscience stimulation cocktail, Waltham, USA) for 4 h at 37°C. After stimulation, the supernatant was collected for the cytokine bead array analysis and stored at -80°C .

The human Th1/Th2 Cytokine Assay (BD Cytometric Bead Array Human Th1/Th2 Cytokine Kit II, San Jose, CA, USA) was used to quantitatively measure cytokine production. The

kit uses bead array technology to simultaneously detect multiple cytokines (IL-2, IL-4, IL-6, IL-10, TNF and IFN γ). Hereto, six bead populations with different fluorescent intensities have been coated with specific capture antibodies. According to the manufacturer's protocol, supernatant of the stimulated PBMCs samples was thawed and 50 μ L was used for the cytokine bead array. Samples and standards were incubated with the capture beads for 3 h at room temperature, while protected from light; see Supplementary table 4 for an overview of the dilutions used. After incubation, assay tubes were washed, centrifuged and resuspended before flow cytometry analysis. Cells were analyzed within 1 h using the BD FACSVerser flow cytometer (BD Biosciences, San Jose, CA, USA).

T-cell expansion assay

PBMCs were plated at a density of 5×10^6 mL $^{-1}$ in a 24-well plate. Expansion of gene-edited PBMCs (Cas9/IFN γ gRNA and Cas9/TCR $\alpha\beta$ -gRNA) was tested by stimulation with CD3/CD28 beads (20 μ L per well, 5×10^6 cells per well) or phytohemagglutinin (L1668 Sigma; 10 μ g mL $^{-1}$, Mannheim, Germany) and compared with control PBMCs (no nucleofection). IL-2 (50 IU mL $^{-1}$) was added to both stimulating conditions. Stimulation was performed directly after electroporation. Plates were incubated at 37°C and cells were manually counted with trypan blue (Sigma-Aldrich, Steinheim, Germany) for viability, using Bürker Chamber (Marienfeld, Germany) at days 7, 10 and 14. Cell density was maintained at about 1×10^6 cells mL $^{-1}$ and medium, IL-2 (Novartis, Arnhem, Netherlands) and/or phytohemagglutinin was refreshed at days 7 and 10.

Memory T cells assay

After RNP electroporation PBMCs were resuspended in 800 μ L culture medium, comprising X-VIVO 15 (Lonza, Basel, Switzerland) with 10% human serum (Sigma-Aldrich, USA). PBMCs were plated in 100 μ L/well of 96-well round-bottomed plates (Thermo Fisher Scientific, Waltham, USA) at a density of $5\text{--}10 \times 10^6$ cells mL $^{-1}$ ($0.5\text{--}1 \times 10^6$ cells/well) for both electroporated and non-electroporated (control) PBMCs. Afterward, PBMCs were stimulated with IL-2, IL-7 and/or IL-15 or left untreated as indicated. For IL-2 a concentration of 20 IU mL $^{-1}$ was used and for IL-15 and IL-7 a concentration of 25 ng mL $^{-1}$ was used. PBMCs were stimulated for 7 days. Medium was not changed during these 7 days. At day 8, cells were stained for T-cell memory phenotype with CD4, CD8, CD45 and TCR α/β antibodies according to manufacturer's protocol (see Supplementary table 3 for characteristics of the antibodies). In brief, cells were washed with PBS (Thermo Fisher Scientific, Waltham, USA) and incubated with Zombie Aqua for 15 min on room temperature. Subsequently, cells were stained with appropriate antibodies for 30 min on ice, followed by washing twice. Finally, cells were resuspended in FACS buffer (PBS enriched with 2% FCS) and analyzed using BD FACSVerser flow cytometer (BD Biosciences, San Jose, CA, USA).

Ex vivo T-cell stimulation

After RNP electroporation, both non-electroporated PBMCs (control) and electroporated PBMCs were resuspended in 500 μ L of culture medium (X-VIVO 15 with 10% human serum) and plated in 100 μ L/well of 96-well round-bottomed plates (Thermo Fisher Scientific, Waltham, USA) at a density of 10×10^6 cells mL $^{-1}$ per well (1×10^6 cells/well) and 20×10^6 cells mL $^{-1}$ per well (2×10^6 cells/well), respectively. Cells were *ex vivo* stimulated either with viral peptides consisting of a pool of 23 different peptides originating from CEF peptides or with IL-7 and IL-15. CEF peptides were synthesized by JPT Peptide Technology (Berlin, Germany). Recombinant human IL-7 (Asp26–His177, size 10 μ g) and recombinant human IL-15 (amino acids Asn49–Ser162, size 10 μ g) were purchased from BioLegend, San Diego, CA, USA. IL-2 was purchased from Clinigen, Yardley, USA.

For *ex vivo* stimulation with CEF peptides, cells were stimulated overnight 1 day after electroporation by incubation with CEF peptides at a concentration of 1 μ g mL $^{-1}$ at 37°C.

For *ex vivo* stimulation with IL-7 and IL-15, cells were first left untreated and activated 8 days after electroporation by overnight incubation with IL-7 and IL-15 at a concentration of 25 ng mL $^{-1}$ at 37°C. After 16–26 h of incubation with CEF peptides or IL-7 and IL-15, cells were stained for T-cell phenotype with CD3 and CD8 and for T-cell activation with CD69 and CD137 according to manufacturer's protocol (see Supplementary table 3 for characteristics of the antibodies). In brief, cells were washed with PBS (Thermo Fisher Scientific, Waltham, USA) and incubated with Zombie Aqua for 15 min at room temperature. Subsequently, cells were washed and stained with CD3, CD8, CD69 and CD137 antibodies for 30 min on ice. Afterward, cells were washed twice, resuspended in FACS buffer (PBS enriched with 2% FCS) and analyzed using BD FACSVerser flow cytometer (BD Biosciences).

In vitro T-cell stimulation

Directly after RNP electroporation, electroporated PBMCs were plated in 2 mL culture medium per well per CRISPR condition of 24-well plates at a density of 5×10^6 mL $^{-1}$ (10×10^6 cells/well). Non-electroporated cells (control) were plated in 2 mL of culture medium per well of a 24-well plate at a density of 2.5×10^6 cells mL $^{-1}$ (5×10^6 cells/well). For both control and knockout PBMCs, culture medium comprised X-VIVO 15 (Lonza, Basel, Switzerland) enriched with 10% human serum (Sigma-Aldrich, USA), 1% penicillin/streptomycin (Thermo Fisher Scientific, Grand Island, USA), 1% L-glutamine (Thermo Fisher Scientific, Paisley, Scotland) and IL-2 (Clinigen, Yardley, USA) (20 IU mL $^{-1}$). After control and knockout PBMCs were plated in culture medium both were stimulated with CEF peptides for 13 days. A concentration of 1 μ g mL $^{-1}$ CEF peptides was used. Medium was changed at days 4, 6, 8 and 11, according to the harmonization protocol of short-term *in vitro* culture for expansion of antigen-specific CD8 $^+$ T cells.⁴ At day 13, cells were restimulated with CEF peptides at a concentration of

1 $\mu\text{g mL}^{-1}$ overnight at 37°C and 5% CO₂. The medium served as a negative control and phytohemagglutinin (L1668; Sigma) at 1 $\mu\text{g mL}^{-1}$ as a positive control. After 16–26 h cells were stained for phenotype with CD3 and CD8 and for activation with CD69 and CD137 according to manufacturer's protocol (see Supplementary table 3 for characteristics of the antibodies). In brief, cells were washed with PBS (Thermo Fisher Scientific, Waltham, USA) and incubated with Zombie Aqua for 15 min at room temperature. Next, cells stained with CD3, CD8, CD69 and CD137 antibodies for 30 min on ice. Afterward, cells were washed twice, resuspended in FACS buffer (PBS enriched with 2% FCS) and analyzed using BD FACSVerse flow cytometer (BD Biosciences).

Statistical analysis

Statistical analysis was performed using GraphPad Prism version 8. Differences between groups were evaluated by paired and unpaired two-tailed Student's *t*-tests, or one-way ANOVA. A *P*-value of < 0.05 was considered to be statistically significant (Supplementary table 2).

CONFLICT OF INTEREST

Outside the submitted work, Dr Floris Foijer reports grants from the Dutch Cancer Society (KWF). Outside the submitted work, Professor Hans W Nijman reports grants from the Dutch Cancer Society (KWF), the European Research Council (ERC), Health Holland and Immunicum; nonfinancial support from AIMM Therapeutics, BioNTech and Surflay; grants and shares and nonfinancial support from ViciniVax; in addition, Professor Nijman has grants and nonfinancial support from Aduro Biotech, in part relating to a patent for antibodies targeting CD103 (de Bruyn *et al.* No. 62/704,258). Outside the submitted work, Dr Marco de Bruyn reports grants from the Dutch Cancer Society (KWF), the European Research Council (ERC), Health Holland, Immunicum; nonfinancial support from BioNTech, Surflay and AIMM Therapeutics; grants and nonfinancial support from ViciniVax; In addition, Dr de Bruyn has grants and nonfinancial support from Aduro Biotech, in part relating to a patent for antibodies targeting CD103 (de Bruyn *et al.* No. 62/704,258). There are no conflicts of interest to disclose for the remaining authors.

AUTHOR CONTRIBUTION

Anneke L Eerkens: Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Project administration; Validation; Visualization; Writing—original draft; Writing—review and editing. **Annegé Vledder:** Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Project administration; Validation; Visualization; Writing—original draft; Writing—review and editing. **Nienke van Rooij:** Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Project administration; Writing—review and editing. **Floris Foijer:** Supervision; Writing—review and editing. **Hans W Nijman:** Conceptualization;

Supervision; Writing—original draft; Writing—review and editing. **Marco de Bruyn:** Conceptualization; Methodology; Supervision; Visualization; Writing—original draft; Writing—review and editing.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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