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1
Rapid and efficient generation of antigen-specific isogenic T cells from
2
cryopreserved blood samples

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32 ABSTRACT

33 **Objectives** CRISPR/Cas9-mediated gene editing has been leveraged for the modification of human and
34 mouse T cells. However, limited experience is available on the application of CRISPR/Cas9
35 electroporation in cryopreserved T cells collected during e.g. clinical trials.

36 **Methods** PBMCs from healthy donors were used to generate knockout T cell models for interferon-γ
37 (IFNγ), Cbl Proto-Oncogene B (CBLB), Fas cell surface death receptor (Fas) and T cell receptor (TCRαβb)
38 genes. The effect of CRISPR-cas9-mediated gene editing on T cells was evaluated using apoptosis
39 assays, cytokine bead arrays and *ex vivo* and *in vitro* stimulation assays.

40 **Results** Our results demonstrate that CRISPR/Cas9-mediated gene editing of *ex* vivo T cells is efficient
41 and does not overtly affect T cell viability. Cytokine release and T cell proliferation were not affected in
42 gene edited T cells. Interestingly, memory T cells were more susceptible to CRISPR/Cas9 gene editing
43 than naïve T cells. *Ex vivo* and *in vitro* stimulation with antigens resulted in equivalent antigen-specific
44 T cell responses in gene-edited and untouched control cells; making CRISPR/Cas9-mediated gene
45 editing compatible with clinical antigen-specific T cell activation and expansion assays.

46 **Conclusion** Here, we report an optimized protocol for rapid, viable and highly efficient genetic
47 modification in *ex vivo* human antigen specific T cells, for subsequent functional evaluation and/or
48 expansion. Our platform extends CRISPR/Cas9-mediated gene editing for use in gold-standard
49 clinically-used immune-monitoring pipelines and serves as a starting point for development of
50 analogous approaches such as those including transcriptional activators and or epigenetic modifiers.

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54 INTRODUCTION

55 CRISPR/Cas9-mediated gene targeting has been used to significantly improve our understanding of T
56 cell biology and discover regulators of e.g. T cell proliferation and differentiation (1–3). To date, most of
57 this work has been performed using cultured peripheral blood mononuclear cells (PBMCs) from healthy
58 donors, or using patient T cells after an initial *ex vivo* expansion (3). In this setting, cell numbers are not
59 limited and there is generally no need to maintain the clonal repertoire of T cells. By contrast, samples
60 obtained within clinical trials in immune oncology are generally limited and antigen-specificity
61 paramount in understanding the underlying immune biology (4–6). Expanding the CRISPR/Cas9 toolbox
62 to antigen-specific T cells from clinical samples would therefore enable further dissection of anti-cancer
63 immune responses and discovery of novel targets for combination immunotherapy (1).

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

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

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79 RESULTS

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

81 We set out to optimize a CRISPR/Cas9-mediated gene editing protocol compatible with PBMC samples
82 analogous to those routinely procured during clinical trials. PBMCs were processed according to best
83 practice operating protocols for PBMC isolation, cryopreservation and cell density used in storage. Using
84 these standardized PBMC aliquots, we first optimized RNP complex electroporation using efficiency and
85 viability as main parameters. As proof-of-concept, we targeted IFNγ as it is not expressed *ex vivo* in T
86 cells, readily induced upon activation and easily assessable by ELISA or intracellular flow cytometry.
87 Electroporation of Cas9/IFNγ-gRNA RNP complexes into T cells resulted in almost complete abrogation
88 of IFNγ expression in both CD8 and CD4 T cells (figure 1a, c). Electroporation, inclusion of Cas9/gRNA
89 complexes and/or crRNA alone, had no effect on T cell viability as measured using amine-reactive dye
90 staining (figure 1b, e). By varying experimental parameters, we observed that while Cas9 protein
91 concentration did not affect T cell viability, the size of the electroporation cuvette was critically
92 important, resulting in a reduced relative viability when using 20 µL cuvettes compared to 100 µL
93 cuvettes (figure 1d). IFNγ knockout efficiency was similar for all experimental conditions and in both
94 CD4 and CD8 T cells (figure 1d). As lower doses of Cas9 protein were equally effective, we chose 100
95 µg/mL in 100 µL cuvettes as optimal concentration in all subsequent experiments. Analysis of additional
96 parameters of cell viability: 7-AAD, Phosphatidyl Serine (PS) exposure and caspase activation confirmed
97 the minimal loss of cell viability under these conditions (figure 1e). Non-T cells in the PBMC fraction
98 were more sensitive to the T cell optimized protocol, as evident from increased cell death in the total
99 PBMC fraction (figure 1f).

100

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

102 Next, we tested whether our optimized protocol affected T cell function by evaluating cytokine
103 production and proliferation of gene edited T cells. While release of IFNγ into the extracellular space
104 was markedly reduced in IFNγ knockout T cells, no differences were observed in the release of other
105 cytokines, such as TNFα and IL-2 (figure 2a, b, supplementary figure 1). Atypical induction of IL-4, IL-5
106 or IL-10 were also not observed under these conditions (figure 2a, b). We also analyzed whether *ex vivo*
107 gene engineered T cells required recovery following electroporation by activating T cells using anti-
108 CD3/CD28 beads at 0, 30, 60, 120 and 240 minutes after RNP nucleofection, followed by 2 day culture
109 and restimulation using PMA/ionomycin. Under all conditions, production of IFNγ, but not TNFα or IL-2,
110 was specifically abrogated (figure 2c, 2d). Accordingly, anti-CD3/CD28 bead- or PHA-based expansion of
111 T cells directly following electroporation did not impair expansion capacity over a period of 14 days
112 (figure 2e). Furthermore, gene-edited T cells that expanded over this period remained IFNγ-negative,
113 suggesting the optimized protocol did not negatively affect T cell fitness when compared with unedited

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114 T cells (figure 2f and supplemental figure 1). We reasoned that the direct *ex vivo* gene editing, followed
115 by immediate T cell activation, could even be leveraged to knockout genes critical for anti-CD3/CD28
116 bead-based activation, as the steady-state protein levels in the absence of transcription are likely
117 sufficient to drive signaling during the initial 24-48 hours of culture. To test this hypothesis, we disrupted
118 expression of the T cell receptor (TCRαβ) complex. T cells were activated using anti-CD3/CD28 beads
119 directly after RNP electroporation. Again, CRISPR Cas9/TCRαβ-gRNA RNP complex electroporation
120 resulted in high TCRαβ knockout efficiency in both CD8 and CD4 T cells at 48h (figure 2g). As anticipated,
121 TCRαβ knockout T cells proliferated comparably to non-edited T cells over a period of 14 days (figure
122 2h). As for IFNγ, no detrimal effects of gene editing were observed and near-complete loss of TCRαβ
123 was maintained at day 14 (figure 2i).
124
125 <u>*CRISPR/Cas9-mediated gene editing of ex vivo T cells preferentially targets memory T cells*</u>
126 We next determined whether memory or naïve T cells were more amenable to CRISPR/Cas9-mediated
127 gene editing in the *ex vivo* setting. PBMCs were subjected to TCRαβ-targeting RNP electroporation,
128 followed by 8-day culture with cytokines IL-2, IL-15 and/or IL-7 to support memory and naïve T cells,
129 respectively. Cell viability was similar across all conditions (figure 3a). Percentages of memory CD8 T
130 cells were similar between TCRαβ knockout and TCRαβ-expressing cells regardless of the cytokine
131 cocktail used (figure 3b,c). Notably, there was a slight increase in the percentage of memory CD4 T cells
132 after TCRαβ knockout compared to the TCRαβ-proficient cells, independent of cytokine stimulation
133 (figure 3c).
134 To further explore this observation, we investigated the knockout efficiency within the memory and
135 naïve T cell populations (figure 3d-i). The knockout efficiency of memory T cells varied between 60-
136 85%, in $CD8^+$ T cells and between 70-95% in $CD4^+$ T cells (figure 3d,g). By contrast, the knockout
137 efficiency for naïve T cells ranged between 35-80% in CD8 T cells and between 30-80% in CD4 T cells
138 (figure 3e,h). In addition, the percentage of naïve TCRαβ knockout CD8 T cells was higher when
139 compared to naïve TCRαβ knockout CD4 T cells (figure 3e,h). These findings collectively suggest that
140 memory T cells, especially memory CD4 T cells, are slightly more susceptible to CRISPR/Cas9 gene
141 editing than naïve T cells.
142
143 <u>*CRISPR/Cas9-mediated gene editing is compatible with clinical antigen-specific T cell activation and*</u>
144 <u>*expansion assays*</u>
145 Finally, having established the high viability and efficacy of our approach, we evaluated whether our
146 optimized protocol was compatible with best-practice assays for monitoring immune responses in
147 clinical trial settings. We analyzed *ex vivo* peptide stimulation, as well as *in vitro* stimulation (IVS)
148 assays. Using CEF peptides as model antigen, and T cell activation markers CD69 and CD137 as a

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149 readout, we were able to demonstrate equivalent CEF-induced responses in gene-edited and
150 untouched control cells for both the *ex vivo* assay (figure 4a, c) and following IVS (figure 4b, d). These
151 responses were observed for IFNγ knockouts, as well as for Cbl Proto-Oncogene B (CBLB) and Fas cell
152 surface death receptor (Fas) gRNAs previously reported by others (figure 4a-d).
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155 DISCUSSION

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

161 CRISPR/Cas9-mediated gene editing has become an attractive approach for modifying T cells,
162 due to its simplicity, operability, low costs and capability of multiplex genome editing (9,11). However,
163 to date, limited experience on the application of CRISPR/Cas9 electroporation in immune monitoring
164 assays in clinical studies is available. The prerequisites for manipulating T cells in immunoassays are
165 maintaining viability, antigen-specificity and function (4,7). In this study, we demonstrate for the first
166 time that these prerequisites are not affected by CRISPR/Cas9 gene editing, thereby providing a proof-
167 of-principle for the application of CRISPR/Cas9 in immune monitoring assays. In both CD8 and CD4 T
168 cells, a highly efficient gene knock-out up to 90% was reached after a single transfection. Previously
169 published literature, using CRISPR/cas9 RNP electroporation, reported efficiency rates of 20-90% with
170 low efficiency rates in resting primary T cells (9,12–16). Moreover, we demonstrated that CRISPR/Cas9-
171 edited cells could be maintained without stimulation in culture for up to 7 days, or supplemented with
172 a diverse range of cytokines without comprising gene knockout efficacy. Activation of T cells directly, or
173 at a later time-point of choosing did not negatively affect T cell function, expansion, nor did
174 cryopreservation and subsequent restimulation. Finally, while the current work focused on knockout of
175 genes, recent advances in non-homologous end joining (NHEJ)-mediated template incorporating using
176 CRISPR/cas9 should be amenable to the current platform, allowing more complex *ex vivo* T cell
177 engineering (12,14,17).

178 Advances in descriptive immune monitoring have expanded our understanding of anti-cancer
179 immune responses. High-parameter flow, mass and spectral cytometry have allowed the simultaneous
180 assessment of most immune cell populations *ex vivo* (18,19). Imaging using e.g. PET/SPECT allows non-
181 invasive assessment of specific immune cells or immune checkpoints throughout the body, and
182 transcriptomic approaches provide ever deeper dimensional assessment of gene expression, including
183 spatial organization (20–22). By contrast, *ex vivo* functional assays have remained largely unchanged
184 over the years, with peptide stimulation and intracellular flow cytometry, ELISPOT or ELISA used for
185 functional readout on one or two hallmark activation markers (4,23). The platform we present here
186 significantly expands the potential of these assays. By seamlessly integrating CRISPR/Cas9-mediated
187 gene editing into *ex vivo* immune monitoring pipelines, it is possible to assess how T cells from clinically-

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188 treated patients differentially depend on genes-of-interest, but also screen *ex vivo* for novel
189 combination targets to further augment T cell activation (14,15). As demonstrated here, these targets
190 need not be limited to cell surface targets accessible by antibody-based therapeutics (14,15). One
191 interesting observation is that naïve CD4, but not CD8, T cells appear less sensitive to CRISPR/Cas9-
192 mediated gene editing than their memory counterparts. Whether this is the result of the optimized
193 electroporation protocol used, or an intrinsic feature of naïve CD4 T cells is currently unknown.
194 Nevertheless, targeted genes of interest could be knocked out in ~60% of CD4 T cells, establishing our
195 platform as an effective approach for studying both recall and *de novo* immune responses. An
196 interesting approach would be to further combine our platform with recent advances in CRISPR/Cas9-
197 mediated gene editing in monocyte-derived dendritic cells. This could allow reciprocal knockout of
198 ligand-receptor interactions of particular interest for complex ligand-receptor pairs, such as CTLA-
199 4/CD28 and CD80/CD86.

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

203

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205 MATRIALS AND METHODS

206 Peripheral Blood Mononuclear Cells

207 Peripheral blood mononuclear cells (PBMCs) were isolated from blood of healthy volunteers after
208 written informed consent was obtained (Sanquin). For isolation, buffy coats were mixed at a 1:2 ratio
209 with RPMI supplemented with 2,5% FCS, layered on a Ficoll-Paque gradient at a 1:2 volume ratio and
210 centrifuged at 900 g for 20 minutes without brake. PBMC on the interface of the Ficoll-Paque and plasma
211 layers were isolated by pipette (5-10 mL), supplemented with ice-cold PBS to a final volume of 50 mL
212 and centrifuged at 560 g for 8 minutes without brake. PBMC pellets were pooled, supplemented with
213 ice-cold PBS to a final volume of 50 mL and centrifuged at 350 g for 8 minutes without brake. After
214 PBMCs were resuspended in 50 mL of ice-cold PBS, PBMCs were counted using the Bürker counting
215 chamber and centrifuged at 350 g at 8 minutes without brake. Finally, PBMCs were resuspended in 1
216 mL of freezing medium, consisting of 90% FCS and 10% DMSO, at a concentration of $10\text{-}100^6$ cells per
217 cryovial.

218

219 Cas9, tracRNA and crRNA

220 Cas9 (Alt-R Cas 9 Nuclease V3), tracRNA (Alt-R CRISPR-Cas9 tracRNA) and the crRNAs (Alt-R CRISPR-Cas9
221 crRNA) were synthesized by Integrated DNA Technologies (Coralville, USA). Each crRNA was
222 reconstituted in nuclease free duplex buffer (Lonza) to a final concentration of 100 µM. See Table 1 for
223 gRNAs and sequences. For TCRαß: equal volumes of TRAC1 and TRBC were mixed before adding the
224 RNP complex to the PBMCs, see RNP electroporation.

225

226 RNP electroporation

227 PBMCs were counted and resuspended in X-VIVO 15™ at densities as indicated. After optimization as
228 indicated, the final protocol to prepare Cas9/gRNA RNP complexes was as follows: 150 pmol annealed
229 gRNA per nucleofection condition was prepared by combining 1.5 µL tracRNA with 1.5 µL crRNA. The
230 mix was annealed by heating at 95°C for 5 minutes and slowly cooled down to room temperature for 10
231 minutes. Next, 12 µL duplex buffer (Lonza, Basel, Switzerland) was added to get a final concentration
232 150 pmol gRNA in 15 µl. To prepare a Cas9/gRNA RNP complex, 10 µg Cas9 (10 µg/ml) was added to
233 the gRNA complex. This mixture was incubated for 10 minutes at room temperature. Finally, duplex
234 buffer was added to the RNP complex to obtain a final volume of 20 µL.

235 For electroporation, PBMCs were centrifuged and washed once with PBS. Cells were
236 resuspended in 100 µL nucleofection solution at densities as indicated. Nucleofection solution consisted
237 of 82 µL P2 Primary Cell Nucleofector™ Solution (Lonza) and 18 µL Supplement 1 (Lonza, Basel,
238 Switzerland) for 100 µL cuvettes and 16.4 µL P2 Primary Cell Nucleofector™ Solution and 3.6 µL
239 Supplement 1 for 20 µL cuvettes. RNP complex was added to the PBMCs and incubated for 2 minutes

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240 at room temperature. The PBMC/RNP mixture was transferred to a nucleofection cuvette, followed by
241 electroporation of cells using a 4D-Nucleofector machine (Amaxa, program P2, pulse code EH100).
242 PBMCs that did not undergo electroporation served as control and were centrifuged and washed with
243 PBS.
244

245 Intracellular cytokine staining

246 16 hours after electroporation, PBMCs were stimulated for 4 hours with PMA/Ionomycin (eBioscience
247 stimulation cocktail) at 37° degrees. For intracellular staining of IFNγ, tumor necrosis factor alpha
248 (TNFα), and interleukin-2 (IL-2), secretion of cytokines was blocked using golgiplug (protein transport
249 inhibitor; BD 555029). After 4 hours, cells were stained with Zombie Aqua viability dye according to
250 manufacturer's protocol and washed with PBS supplemented with 5% FCS. Next, cells were fixed and
251 permeabilized (CEL FIXATIE & PERMEABILISATIE KIT, GAS-002, Nordic-Mubio) and stained with surface
252 markers CD3, CD8, CD4, and intracellular markers IFNγ and IL-2 for flow cytometer analysis. For the
253 TCRαβ knockout experiments, only cell surface staining with TCRαβ, CD3, CD8 and CD4 antibodies was
254 performed. See supplementary table 2 for characteristics of the antibodies. For fixation and
255 permeabilization cells were incubated with 100 µL/well of reagent A (fix) for 15 minutes at room
256 temperature. Next, cells were washed with PBS and incubated with a mixture of reagent B (perm) and
257 antibodies for 15 minutes at room temperature. In total 100 µL of the mixture reagent B plus antibodies
258 was added per well. After incubation, cells were washed twice, centrifuged and resuspended in flow
259 cytometry buffer (PBS + 2% FCS).

260 Intracellular staining of IFNγ, TNFα and IL-2 was also performed on engineered T cells activated
261 with anti-CD3/CD28 beads (Thermofisher, Cat. 11131D) at 0, 30, 60, 120 and 240 minutes after RNP
262 nucleofection, followed by 2 day culture and restimulation using PMA/ionomycin.
263

264 Apoptosis assay

265 Apoptosis was assessed 24 hours after CRISPR/Cas9-mediated gene editing. PBMCs were counted,
266 centrifuged and resuspended in X-VIVO 15™ at a density of 1-2x10^6 cells/mL. Subsequently, 1 µL Violet
267 live Caspase probe per 0.5 mL of cell suspension was added and cells were incubated for 45 minutes at
268 37°C. After washing, cells were resuspended in fresh medium and incubated for an additional 30
269 minutes at 37°C while protected from light. Afterwards, CD8 and CD3 antibodies were added and cells
270 were incubated for 30 minutes at room temperature. Cells were washed twice with cold PBS and
271 resuspended in 1x Annexin V Binding Buffer at a final concentration of 1x10^6 cells/mL. 1x10^5 cells were
272 incubated with 5 µL of Annexin V and 5 µL of 7-Aminoactinomycin D Invitrogen™ (7-AAD) for 15 minutes
273 at room temperature in the dark. Cells were analyzed within 1 hour using the BD FACSVerse flow
274 cytometer (BD Biosciences).

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275 Cytokine bead array

276 One day after electroporation (10×10^6 cells in 2 mL, 1 mL per well), gene-edited and control PBMCs
277 were incubated with PMA/ionomycin (eBioscience stimulation cocktail) for 4 hours at 37C°.
278 Subsequently, supernatant was collected, and stored at -80°C.
279 The human Th1/Th2 Cytokine Assay (BD™ Cytometric Bead Array (CBA) Human Th1/Th2 Cytokine Kit II)
280 was used to quantitatively measure cytokine production according to the manufacturer's protocol. In
281 brief, supernatant was thawed and 50 µL was used for the cytokine bead array. Samples and standards
282 were incubated with the capture beads for 3 hours at room temperature, while protected from light
283 (Supplementary data, supplementary table 2). After incubation, assay tubes were washed, centrifuged
284 and resuspended before flow cytometer analysis.
285

286 T cell expansion assay

287 PBMCs were plated at a density of 5×10^6/ml in a 24 wells plate. Expansion of gene-edited PBMCs was
288 tested by stimulation with CD3/CD28 beads (20 µL per well, 5×10^6 cells per well) or phytohemagglutinin
289 (L1668 Sigma)(PHA, 10 µg/mL). IL-2 (50 IU/mL) was added to both stimulating conditions. Stimulation
290 was done directly after electroporation. Plates were incubated at 37°C and cells were counted and at
291 day 7, 10 and 14. Cell density was maintained around 1x10^6 cells/mL and medium, IL-2 and/or PHA was
292 refreshed at day 7, and 10.
293

294 *Ex vivo* T cell stimulation

295 PBMCs were resuspended in 500 µL culture medium (X-VIVO 15™ with 10% human serum) and plated
296 in 100 µL/well of 96-well round bottom plates (Thermofisher) at a density of 1x10^6/ml. Cells were *ex*
297 *vivo* stimulated either with viral peptides consisting of a pool of 23 different peptides originating from
298 Cytomegalovirus, Epstein-Barr virus and influenza virus peptides (CEF) or with interleukin 7 (IL-7) and
299 interleukin 15 (IL-15). CEF was synthesized by JPT Peptide Technology (Berlin, Germany). Recombinant
300 human IL-7 (Asp26-His177, size 10 µg) and recombinant human IL-15 (amino acids Asn49-Ser162, size
301 10 µg) were purchased from Biolegend.
302 For *ex vivo* stimulation with CEF, cells were activated one day after electroporation by
303 incubation with CEF peptides at a concentration of 1 µg/ml at 37°C. For *ex vivo* stimulation with IL-7 and
304 IL-15, cells were activated by incubation with IL-7 and IL-15 at a concentration of 25 ng/ml at 37°C at
305 day 8. After 16-26 hours of incubation with CEF or IL-7 and IL-15, cells were stained for T cell phenotype
306 with CD3, CD8 and CD14 and for T cell activation with CD69 and CD137, see supplementary table 2.
307

308 *In vitro* T cell stimulation (IVS)

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309 PBMCs were plated in 2 mL culture medium per well per CRISPR condition of 24-wells plates at a density
310 of $5x10^6$/ml. Culture medium composed of X-VIVO 15™ enriched with 10% human serum, 1%
311 penicillin/streptomycin (P/S), 1% L-glutamine (L-glu) and IL-2 (20 IU/ml). PBMCs were stimulated with
312 CEF for 13 days. A concentration of 1 µg/mL CEF was used. Medium was changed at day 4, 6, 8 and 11,
313 according to the harmonization protocol of short-term *in vitro* culture for expansion of antigen-specific
314 CD8+ T cells (4). At day 13, cells were re-stimulated with CEF at a concentration of 1 µg/mL overnight at
315 37°C and 5% CO_2, while medium served as a negative control and PHA (L1668 Sigma) at 1 µg/mL as
316 positive control. After 16-26 hours cells were stained for phenotype with CD3 and CD8 and for activation
317 with CD69 and CD137, see supplementary table 2 for characteristics of the antibodies.
318
319 *Memory T cells assay*
320 PBMCs were resuspended in 800 µL culture medium (X-VIVO 15™ with 10% human serum) and plated
321 in 100 µL/well of 96-well round bottom plates (Thermofisher) at a density of $0{,}625x10^6$/mL. PBMCs were
322 stimulated with IL-2, IL-7 and/or IL-15 at a concentration of 25 ng/mL or left untreated as indicated.
323 Cells were cultured for 7 days. At day 8, cells were stained for T cell memory phenotype with CD4, CD8,
324 CD45 and TCRα/ß, see supplementary table 2 for characteristics of the antibodies.
325

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326 Declaration of Interest statements:

327 **FF** Outside the submitted work, dr. Foijer reports grants from the Dutch Cancer Society (KWF)

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338 grants and non-financial support from Aduro Biotech, in part relating to a patent for Antibodies targeting
339 CD103 (de Bruyn et al. No. 62/704,258).

340 There are no conflicts of interest to disclose for the remaining authors.

341

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407 SUPPLEMENTARY TABLES

408

409 Supplementary table 1

gRNA		Sequence
IFNγ		AAA GAG TGT GGA GAC CAT CA
CBLB		TGCACAGAACTATCGTACCA
Fas		GAGGGTCCAGATGCCCAGCA
TCRα		TGTGCTAGACATGAGGTCTA
TCRβ		GGAGAATGACGAGTGGACCC

410

411

412 Supplementary table 2

413

Antibody	Color	Cat No	Supplier	Experiment
CD3	APC-eFluor 780	47-0038-42	eBioscience™, ThermoFisher	*Ex vivo* and *in vitro* T cell stimulation
CD3	PE	12-0038-41/42	eBioscience™, ThermoFisher	Apoptosis assay
CD3	FITC	555332	BD	Intracellular cytokine staining
CD4	PE	12-0048-42	eBioscience™, ThermoFisher	Intracellular cytokine staining Memory T cell assay
CD8	BV421	562428	eBioscience™, ThermoFisher	Intracellular cytokine staining *Ex vivo* and *in vitro* T cell stimulation Memory T cell assay
CD8	APC-eFluor 780	47-0088-42	eBioscience™, ThermoFisher	Apoptosis assay Intracellular cytokine staining
CD45RA	Pe-Cy7	25-0458-41	eBioscience™, ThermoFisher	Memory T cell assay
CD69	FITC	11-0699-41	eBioscience™, ThermoFisher	*Ex vivo* and *in vitro* T cell stimulation
CD137	PE	12-1379-42	eBioscience™, ThermoFisher	*Ex vivo* and *in vitro* T cell stimulation
TCRα/ß	APC	17-9986-41	eBioscience™, ThermoFisher	Memory T cell assay Intracellular cytokine staining
IFNγ	PerCP-Cy5.5	45-7319-42	eBioscience™, ThermoFisher	Intracellular cytokine staining
TNFa	APC	17-7349-82	eBioscience™, ThermoFisher	Intracellular cytokine staining
IL-2	PE-Cy7	25-7029-42	eBioscience™, ThermoFisher	Intracellular cytokine staining

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417 Supplementary table 3

418

Tube label	Concentration (pg/mL)	Cytokine Standard dilution
1	0 (negative control)	No standard dilution (Assay Diluent only)
2	20	1:256
3	40	1:128
4	80	1:64
5	156	1:32
6	312,5	1:16
7	625	1:8
8	1250	1:4
9	2500	1:2
10	5000	Top Standard

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421 FIGURE DESCRIPTIONS

422 **Figure 1. CRISPR/Cas9 electroporation *ex vivo* T cells does not affect T cell viability**

423 Electroporation of PBMCs with Cas9/IFNγ gRNA RNP complexes results in almost complete abrogation
424 of IFNγ expression in both CD8 and CD4 T cells. 16 hours after electroporation, PBMCs were
425 stimulated for 4 hours with PMA/Ionomycin. (A, C) Representative flow cytometry contour plot
426 showing the expression of IFNγ production in CD8 T cells. (B) Scatterplots demonstrating the viability
427 of PBMCs after electroporation alone, with Cas9/gRNA complexes and/or crRNA alone. Viability was
428 measured using amine reactive-dye. (D) Effect of different cuvette sizes (100 and 20µL) and different
429 Cas9 protein concentrations (0, 100, 250 en 500 µg/ml) on cell viability. (E) Analysis of additional
430 parameters of cell viability was performed using 7-AAD, Phosphatidyl Serine (PS) exposure and
431 caspase activation in CD8 and CD4 T cells(E) and in the total PBMC fraction(F).
432 Blue dots represent unstimulated PBMCs; orange dots represent PBMCs stimulated with
433 PMA/ionomycin for 4 hours; green dots represent all PBMCs; in e, middle scatterpot, blue dots
434 represent CD8 T cells and orange dots CD4 T cells.
435

436 **Figure 2. CRISPR/Cas9-mediated gene editing of *ex vivo* T cells does not affect T cell function**

437 IFNγ, TNFα, IL-2, IL-4, IL-5 and IL-10 release was evaluated using a cytokine bead array kit. (A,B)
438 Electroporation with Cas9/IFNγ gRNA RNP complexes results in reduced IFNγ release whereas no
439 differences are observed in the release of TNFα and IL-2, 2 days after electroporation. Atypical
440 induction of IL-4, IL-5 or IL-10 are also not observed. (C) t-SNE plots showing the reduced IFNγ release
441 after stimulation with beads at t=0 and t=4 hours after electroporation. (D) IFNγ, TNFα and IL-2
442 cytokine-producing CD8 T cells after activation with anti-CD3/CD28 beads at 0, 30, 60, 120 and 240
443 minutes after RNP nucleofection, followed by 2 day culture and restimulation using PMA/ionomycin.
444 (E) Scatterplot showing the anti-CD3/CD28 bead- or PHA-based expansion of T cells directly following
445 electroporation over 14 days. (F) IFNγ and IL-2 production of 14 days expanded gene-edited CD8 T
446 cells. (G) Flow cytometry contour plot showing the TCRαβ knockout in CD8 and CD4 T cells 48 hours
447 after electroporation (G). (H) Expansion capacity of anti-CD3/CD28 activated TCRαβ knockout cells
448 over 14 days. (I) Flow cytometry contour plot left) and scatterplot (right) showing the TCRαβ knockout
449 in CD8 and CD4 T cells after 14 days.

450

451 **Figure 3. CRISPR/Cas9 gene editing preferentially targets memory T cells.**

452 Gene editing of TCRαβ, IFNγ, CBLB or FAS in PBMCs. (A) Viability of CD8 versus CD4 TCRαβ knockout T
453 cells after *in vitro* stimulation with IL-2, IL-15 and/or IL-7 for eight days. (B) Percentages of CD8 and (C)
454 CD4 memory T cells within TCRαβ knockout T cells compared to TCRαβ-proficient cells after co-

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455 culturing with IL-2, IL-15 and/or IL-7 for eight days. (D) Percentages of CD8 memory and (E) CD8 naïve
456 T cells expressing TCRαβ eight days after gene editing. (F) Gene editing efficiency of CD8 memory T
457 cells compared to CD8 naïve T cells. (G) Percentages of CD4 memory and (H) CD4 naïve T cells
458 expressing TCRαβ eight days after gene editing of the TCRαβ receptor. (I) Gene editing efficiency of
459 CD4 memory T cells compared to CD4 naïve T cells. Culture conditions as indicated.
460

461 **Figure 4. CRISPR/Cas9 editing does not influence the antigen-specificity of T cells.**
462 Gene editing of IFNγ, CBLB or FAS in PBMCs. (A) Representative flow cytometry contour plots showing
463 the expression of T cell activation markers CD69 and CD137 after *ex vivo* and (B) post-IVS stimulation
464 with CEF. (C) Scatterplot representing the percentage of CD8 T cells expressing both activation
465 markers CD137 and CD69 within variable CRISPR gene editing conditions and untouched control cells
466 after *ex vivo* stimulation at day 2 and (D) post-IVS stimulation with and without CEF peptides at day 13.
467 Blue dots represent PBMCs not stimulated with CEF peptides and orange dots PBMCs stimulated with
468 CEF.

469
470 **Supplementary figure 1. CRISPR/Cas9-mediated gene editing of *ex vivo* T cells does not affect T cell**
471 **function.**
472 Scatterplots representing the IFNγ-, IL-2, and TNFα producing CD8 and CD4 T cells after activation with
473 anti-CD3/CD28 beads, or PHA, 14 days after expansion (relates to figure 2f).
474

475 **Supplementary figure 2. Gating strategy.**
476 Scatterplots representative for the gating strategy used throughout the manuscript. The depicted
477 scatterplots show gene edited PBMCs after CBLB knockout and IVS stimulation with CEF. Cell
478 populations are depicted above the FACS images and the gated population are depicted below.
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480
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Figure 1. CRISPR/Cas9 electroporation ex vivo T cells does not affect T cell viability

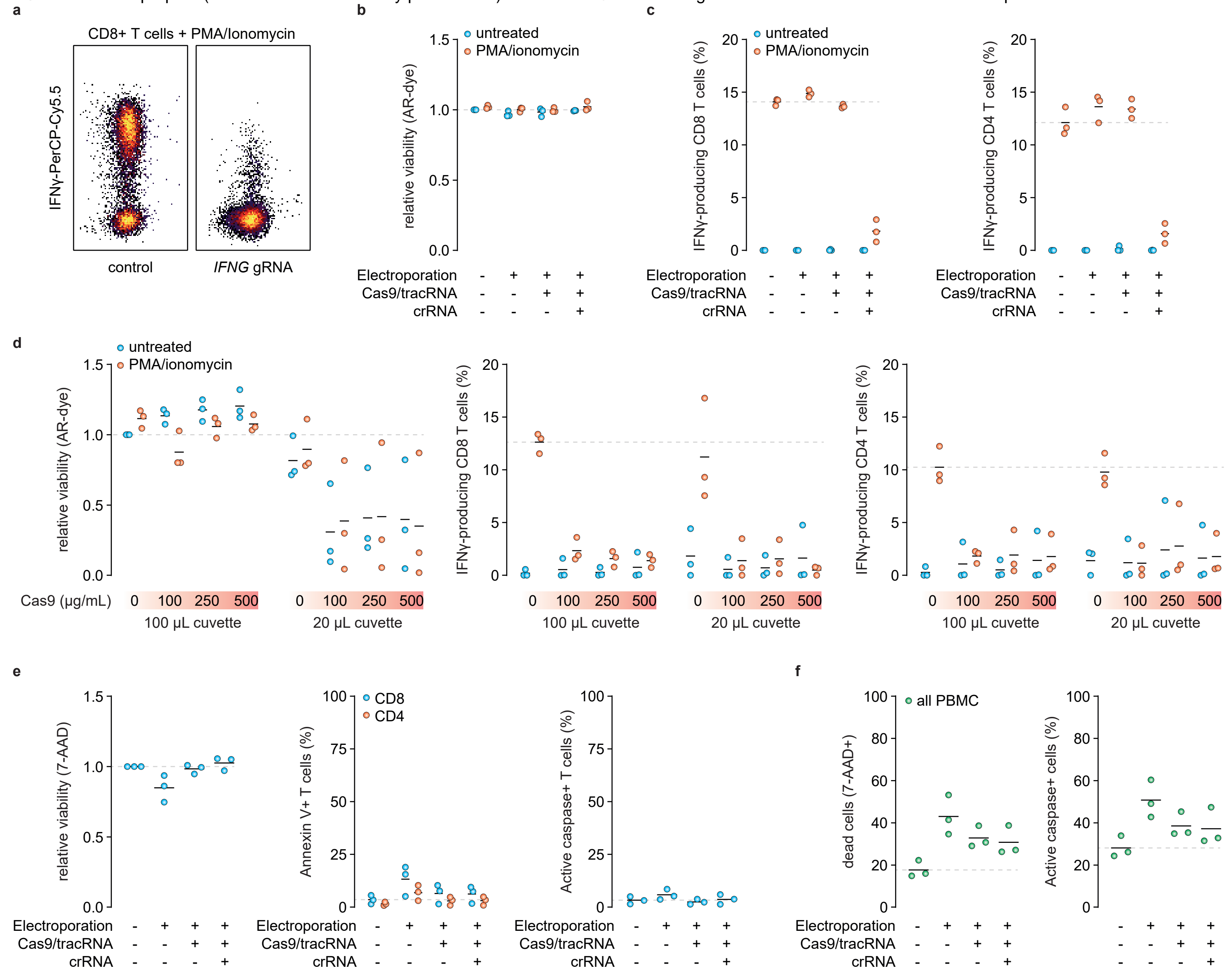

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Figure 2. CRISPR/Cas9-mediated gene editing of ex vivo T cells does not affect T cell function

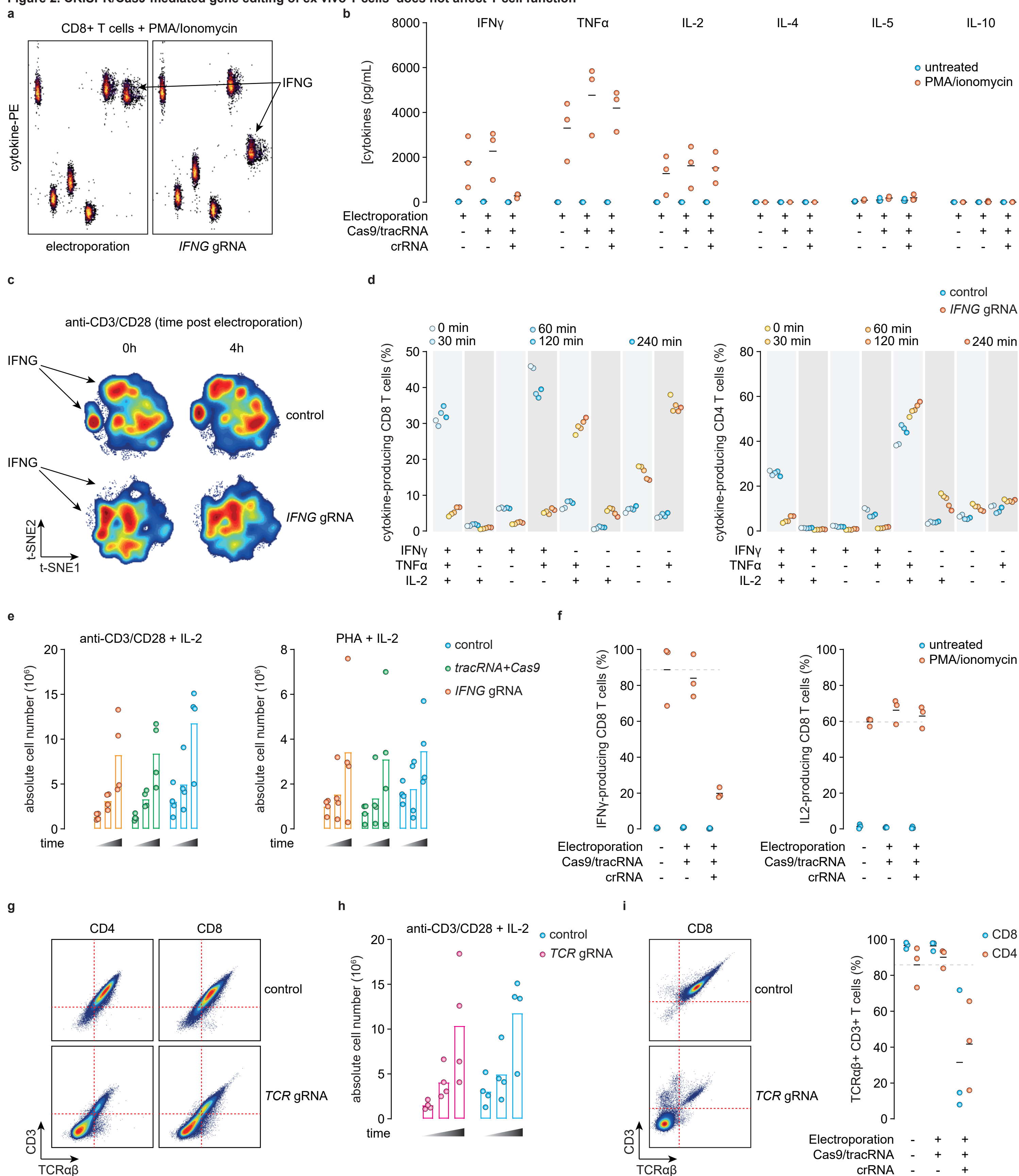

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Figure 3. CRISPR/Cas9 gene editing preferentially targets memory T cells.

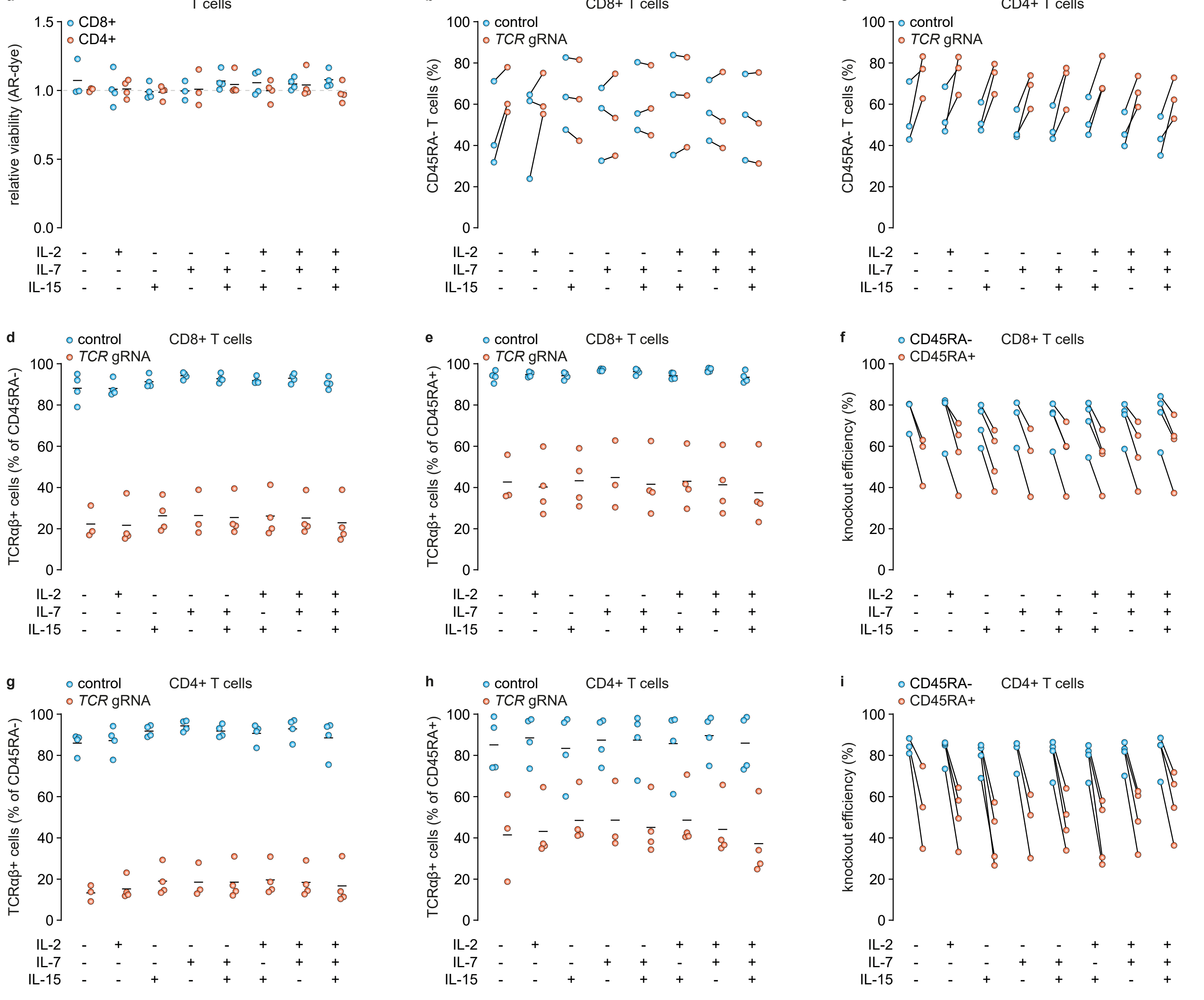

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Figure 4. CRISPR/Cas9 editing does not influence the antigen-specificity of T cells