Supplementary Information

Optimizing CRISPR-Cas9-Mediated TCR Knockout in Primary Mouse CD8$^+$, CD4$^+$, and Regulatory T Cells

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Supplemental Information includes ten supplementary figures.

Supplementary Figure 1: Related to Figure 1: Optimization of *TRAC* gene knockout in the Jurkat cell line.

Supplementary Figure 2: Related to Figure 1: Optimization of *TRAC* gene knockout in the Jurkat cell line.

Supplementary Figure 3: Related to Figure 3: Optimizing the pipeline for TCR knockout in primary mouse T cells and functional validation of edited cells.

Supplementary Figure 4: Related to Figure 3: Optimizing the pipeline for TCR knockout in primary mouse T cells and functional validation of edited cells.

Supplementary Figure 5: Related to Figure 3: Optimizing the pipeline for TCR knockout in primary mouse T cells and functional validation of edited cells.

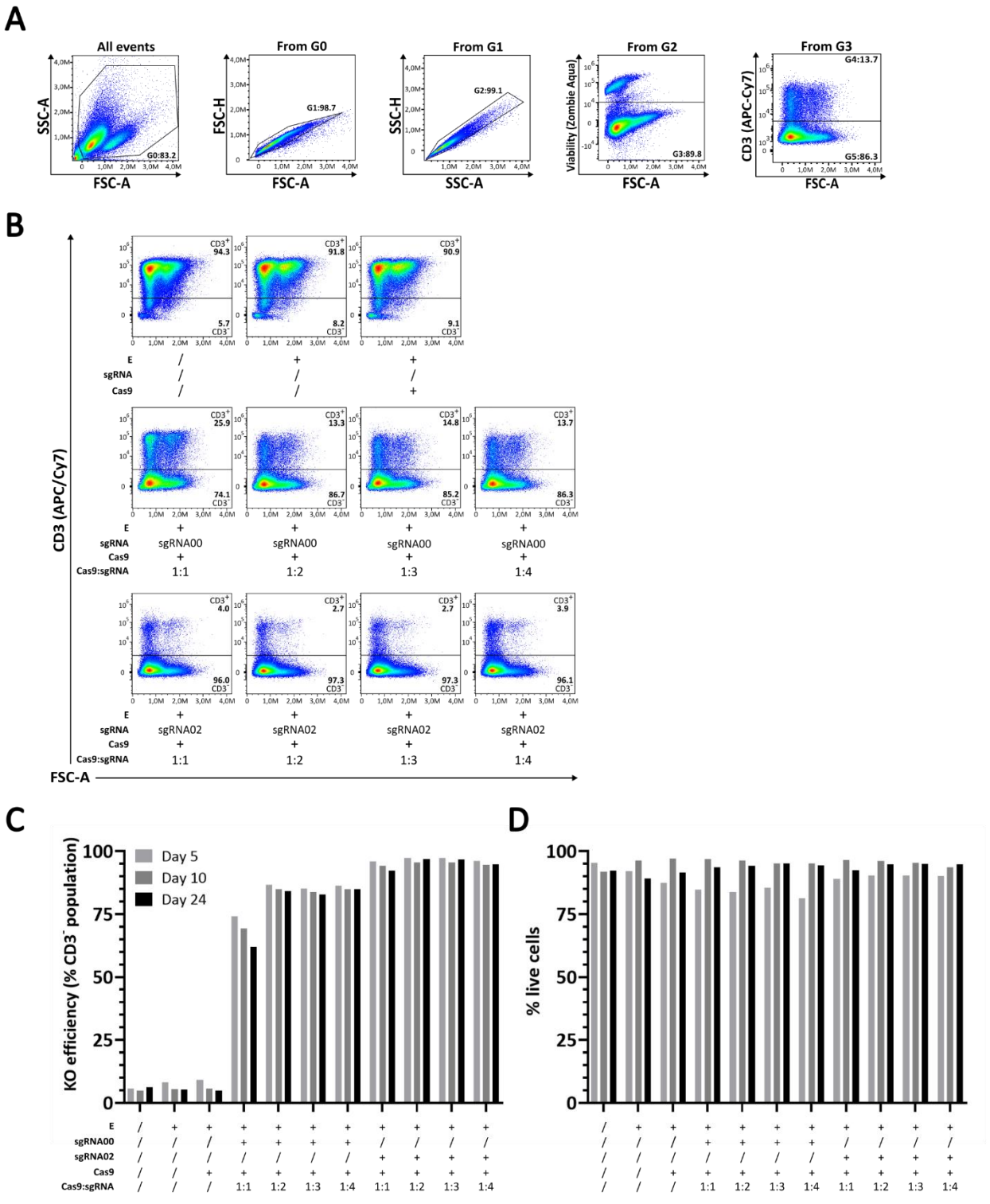
Supplementary Figure 6: Related to Figure 4: Translation of the genome editing pipeline and TCR knockout strategy to primary mouse Treg cells.

Supplementary Figure 7: Related to Figure 4: Translation of the genome editing pipeline and TCR knockout strategy to primary mouse Treg cells.

Supplementary Figure 8: Related to Figure 4: Translation of the genome editing pipeline and TCR knockout strategy to primary mouse Treg cells.

Supplementary Figure 9: Related to Figure 4: Translation of the genome editing pipeline and TCR knockout strategy to primary mouse Treg cells.

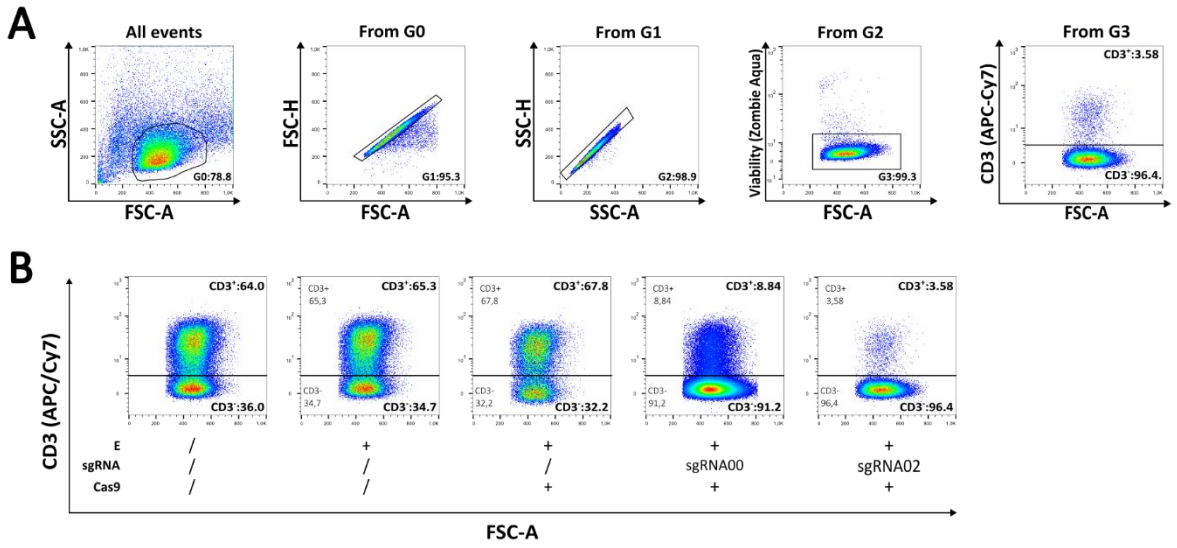
Supplementary Figure 10: Related to Figure 4: Translation of the genome editing pipeline and TCR knockout strategy to primary mouse Treg cells.



Supplementary Figure 1: Related to Figure 1: Optimization of *TRAC* gene knockout in the Jurkat cell line.

(A) Gating strategy for flow cytometric analysis of surface CD3 expression. Main cell population was gated based on FSC-A and SSC-A (gate G0). Doublets were excluded using FSC-H vs. FSC-A (gate G1) and SSC-H vs. SSC-A (gate G2) plots. Dead cells were eliminated based on staining with Zombie

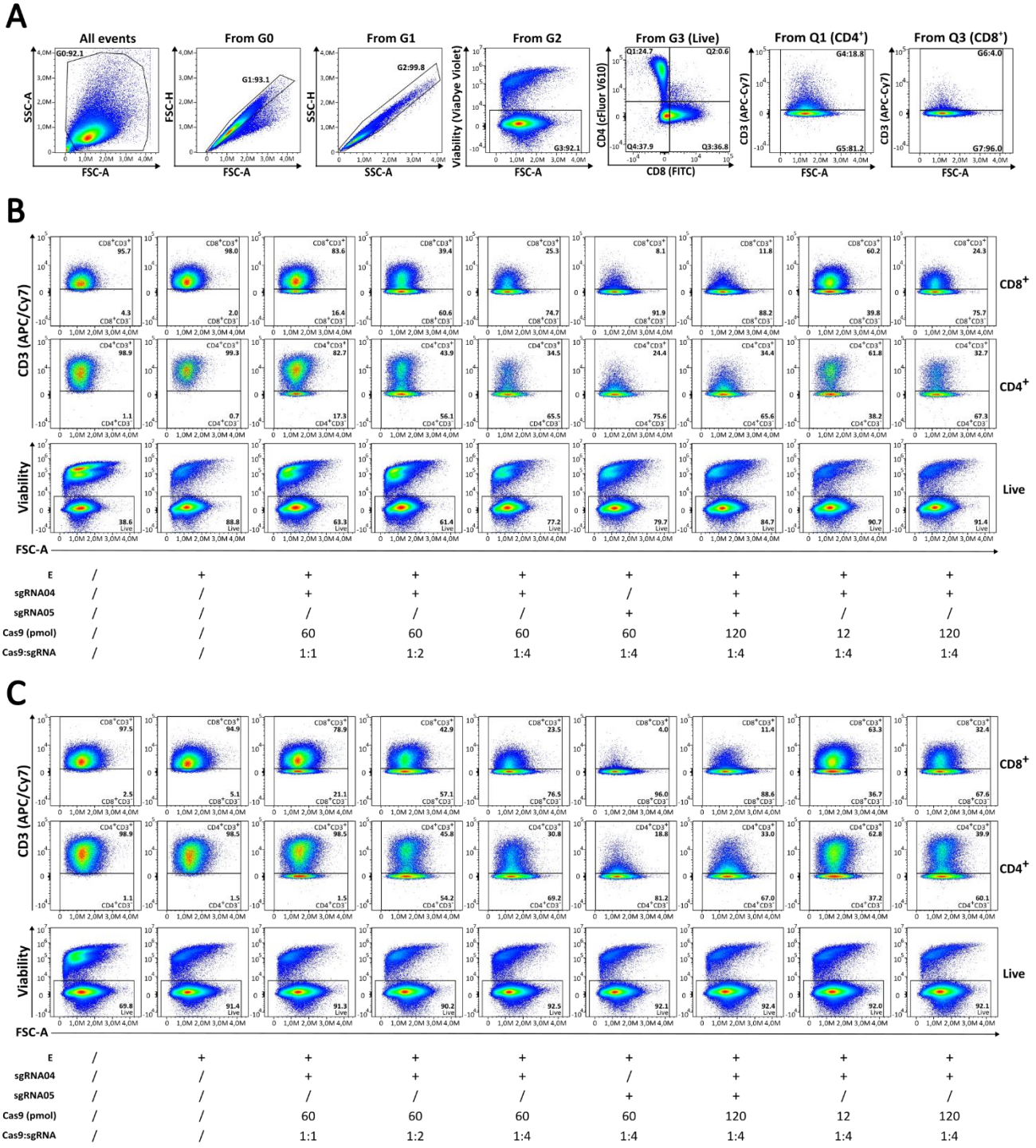
Aqua viability dye (gate G3). Gating to distinguish $CD3^+$ from $CD3^-$ cells was performed on the population of live cells (gates G4, G5). (B) Representative flow cytometric analysis of CD3 surface expression, used as a surrogate marker for surface TCR expression, in non-electroporated and mock-electroporated cells (top), and in cells treated with sgRNA00 (middle) or sgRNA02 (bottom) across varying Cas9:sgRNA ratios. (C and D) Titration of RNP complexes at a fixed 1:2 Cas9:sgRNA molar ratio. (C) KO efficiency and (D) cell viability were evaluated 5 and 10 days post-electroporation. Data in C and D are presented as mean ± SD from three technical replicates (after electroporation, each sample was divided into three wells). ****$p < 0.0001$, *$p < 0.05$, ns – not significant (one-way ANOVA, Tukey's multiple comparisons test, we show statistics calculated for day 5). "E", electroporation; "/", no reagent added or procedure applied.



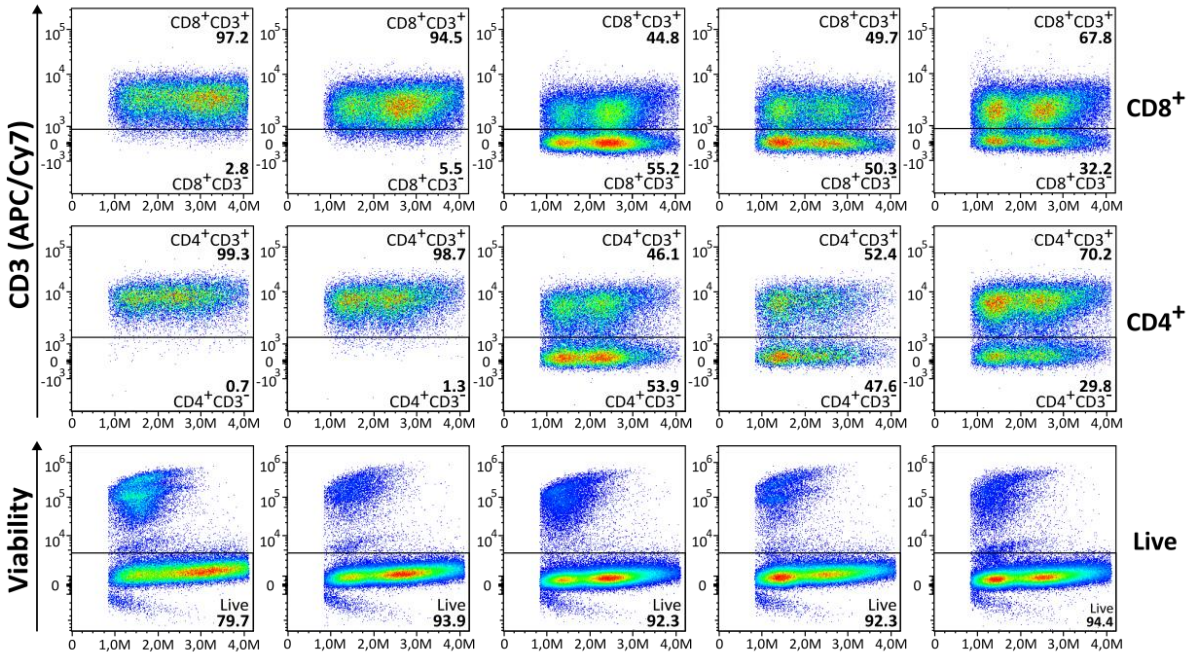
Supplementary Figure 2: Related to Figure 1: Optimization of *TRAC* gene knockout in the Jurkat cell line.

(A) Gating strategy for flow cytometric analysis of surface CD3 expression. The main cell population was gated based on FSC-A and SSC-A (gate G0). Doublets were excluded using FSC-H vs FSC-A (gate G1) and SSC-H vs SSC-A (gate G2) plots. Dead cells were excluded based on staining with Zombie Aqua viability dye (gate G3). Gating to distinguish $CD3^+$ from $CD3^-$ cells was performed on the population of live cells. (B) Flow cytometric analysis of CD3 surface expression, used as a surrogate marker for surface TCR expression, was conducted in non-electroporated cells, mock-electroporated cells, and cells treated with reference sgRNA00 or sgRNA02, which was designed with the web tool CHOPCHOP, using 150 pmol sgRNA and 60 pmol Cas9. Electroporation was performed with the NEPA21 electroporator (NepaGene). Before electroporation, cells were resuspended in OptiMEM to a concentration of 1×10^7 cells/ml. Immediately before electroporation, RNP complexes were prepared by mixing 150 pmol of sgRNA with 60 pmol of Cas9 protein per transfection in RNase-free tubes, followed by a 10-minute incubation at room temperature. Simultaneously, 5 µl of OptiMEM was prepared for each mock-electroporated and non-electroporated controls. 95 µl of cells (0.95×10^6 cells) were added to the RNP complexes or OptiMEM and incubated for 2 minutes. 100 µl of the sample was added to each electroporation cuvette. Before electroporation, the impedance of the samples was measured and adjusted to between 30 and 55 Ω. After electroporation, samples were transferred from the cuvettes into a 12-well plate containing warm R10e medium and resuspended to

disperse cell clumps. All subsequent cell culture was performed in R10e medium. Flow cytometry was performed 9 days after electroporation using a MACSQuant flow cytometer. No biological or technical replicates were included in this preliminary study. “E”, electroporation; “/”, no reagent added or procedure applied.



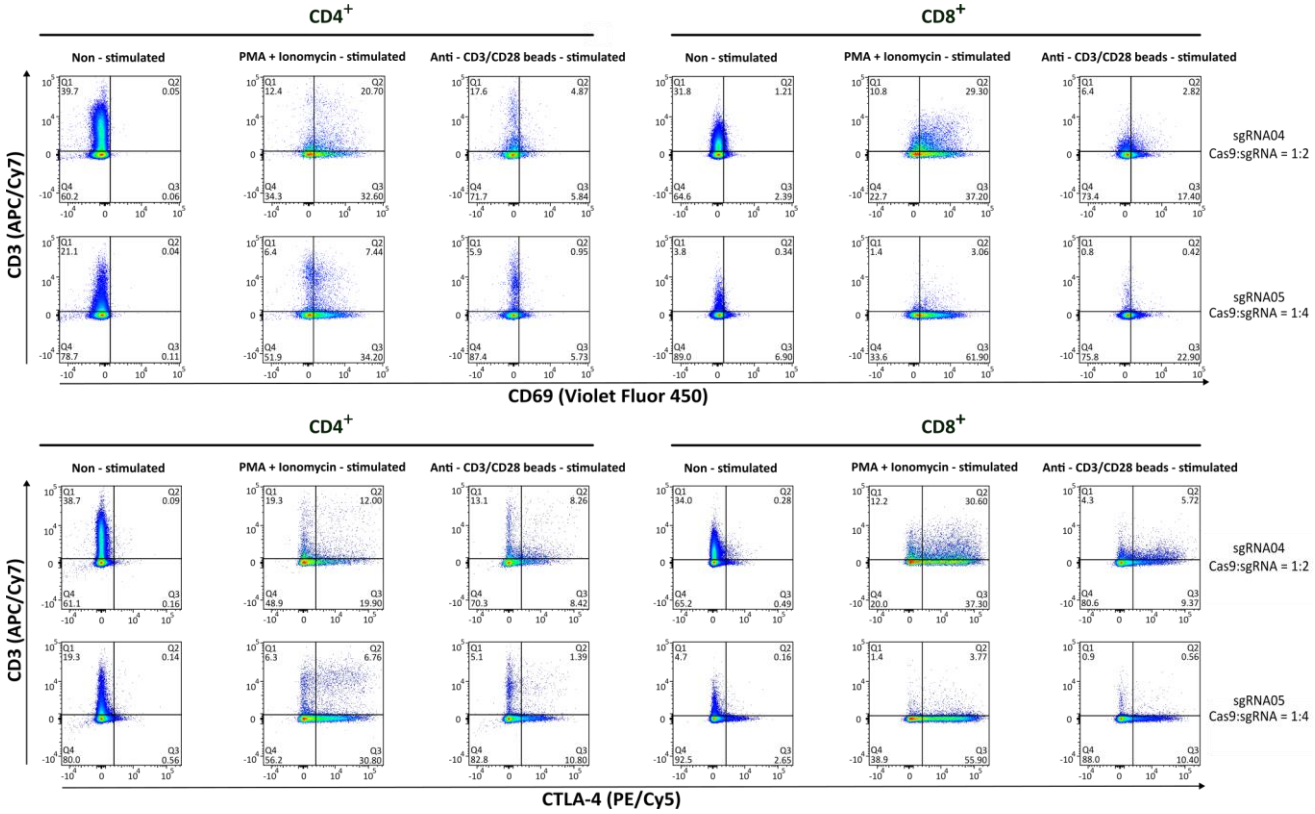
Supplementary Figure 3: Related to Figure 3: Optimizing the pipeline for TCR knockout in primary mouse T cells and functional validation of edited cells.
(A) Gating strategy for flow cytometric analysis of surface CD3 expression on $CD4^+$ and $CD8^+$ cells. Main cell population was gated based on FSC-A and SSC-A (gate G0). Doublets were excluded using FSC-H vs. FSC-A (gate G1) and SSC-H vs. SSC-A (gate G2) plots. Dead cells were eliminated based on staining with ViaDye Violet viability dye (gate G3). From live cells, $CD4^+$ and $CD8^+$ T cell populations were gated using a quadrant gate (from G3, quadrant 1 and quadrant 3, respectively). CD3 expression was then assessed on the gated $CD4^+$ (gates G4 and G5) and $CD8^+$ cell populations (gates G6 and G7). The gating strategy is shown for the biological replicate from Supplementary Figure 2C. (B and C) Related to Figure 3A. Flow cytometric analysis of CD3 surface expression, in $CD8^+$ (top) and $CD4^+$ (middle) T cells, and cell viability (bottom) in two additional biological replicates. Cells were treated with sgRNA04 and/or sgRNA05 targeting the *Trac* locus across varying Cas9:sgRNA ratios and different amounts of RNP complexes. "E", electroporation; "/", no reagent added or procedure applied.



E	/	+	+	+	+
sgRNA04	/	/	+	+	+
sgRNA05	/	/	/	/	/
Cas9 (pmol)	/	/	60	60	60
Cas9:sgRNA	/	/	1:2	1:2	1:2
Cell number (x10^6)	2	2	2	1	0,3

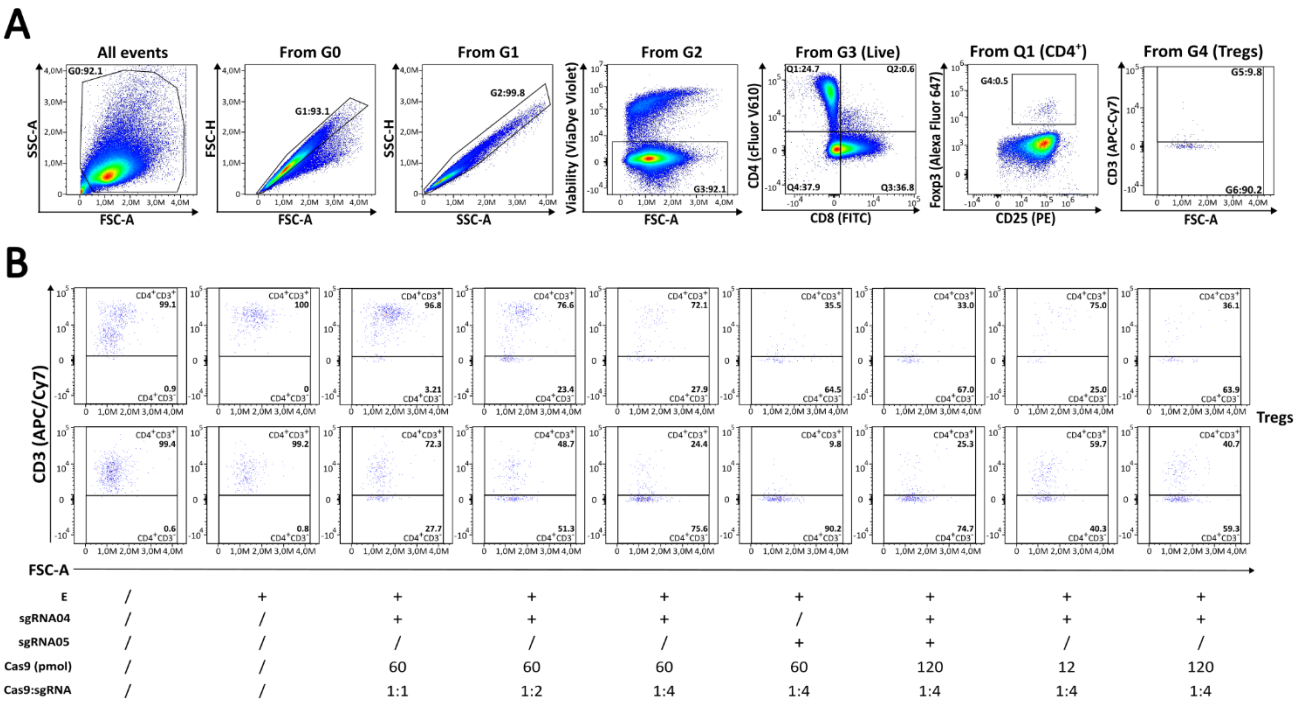
Supplementary Figure 4: Related to Figure 3: Optimizing the pipeline for TCR knockout in primary mouse T cells and functional validation of edited cells.

Flow cytometric analysis of CD3 surface expression in $CD8^+$ (top) and $CD4^+$ (middle) T cells, and cell viability (bottom). Cells were treated with sgRNAs targeting the *Trac* locus at varying cell input numbers, while maintaining constant concentrations and ratio of sgRNA04 and Cas9. "E", electroporation; "/", no reagent added or procedure applied.



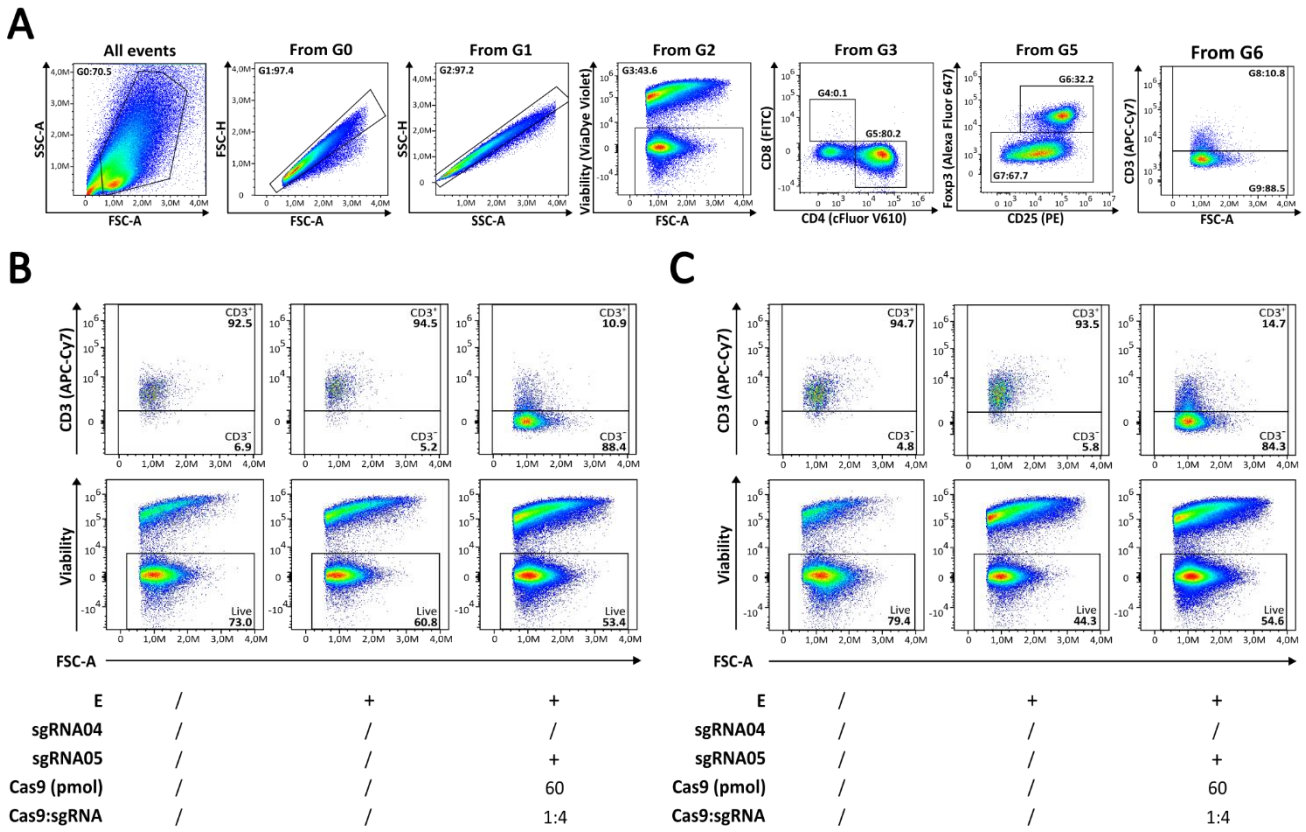
Supplementary Figure 5: Related to Figure 3: Optimizing the pipeline for TCR knockout in primary mouse T cells and functional validation of edited cells.

Gating strategy to analyze expression of CD69 and CTLA-4 activation markers under varying knockout efficiencies and stimulation conditions. Representative gating strategy used to quantify CD69 and CTLA-4 expression within $CD3^+$ and $CD3^-$ fractions of $CD4^+$ and $CD8^+$ T-cell populations. Four days post debeading and electroporation, cells were either left unstimulated or stimulated for 24 hours with PMA and ionomycin or anti-CD3/CD28 activation beads. Two knockout conditions were assessed: intermediate (sgRNA04, Cas9:sgRNA = 1:2) and high (sgRNA05, Cas9:sgRNA = 1:4) knockout efficiency.



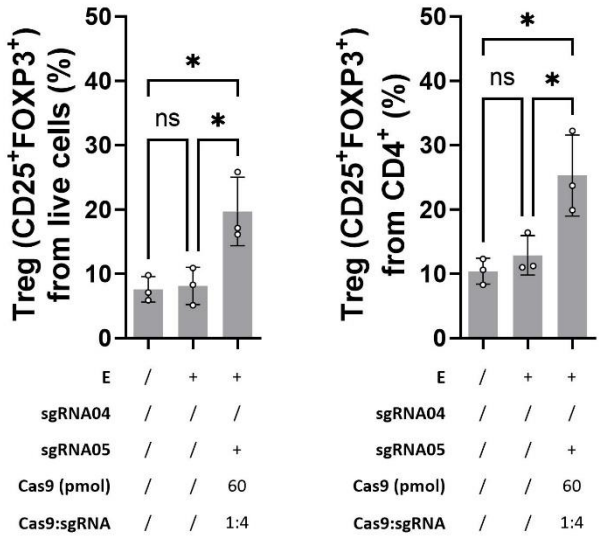
Supplementary Figure 6: Related to Figure 4: Translation of the genome editing pipeline and TCR knockout strategy to primary mouse Treg cells.

(A) Related to Figures 4A and 4B. Gating strategy for flow cytometric analysis of surface CD3 expression on $CD25^{+}FOXP3^{+}$ Treg cells identified within the complex mixture of $CD4^{+}$ and $CD8^{+}$ T cells from the experiment shown in Figure 3. Main cell population was gated based on FSC-A and SSC-A (gate G0). Doublets were excluded using FSC-H vs. FSC-A (gate G1) and SSC-H vs. SSC-A (gate G2) plots. Dead cells were eliminated based on staining with ViaDye Violet viability dye (gate G3). From live cells, $CD4^{+}$ and $CD8^{+}$ T cell populations were gated using a quadrant gate (from G3, quadrant 1 and quadrant 3, respectively). Among the $CD4^{+}$ cells, the Treg cells were selected based on the expression of CD25 and FOXP3 (gate G4). Subsequently, CD3 expression was analyzed in the Treg population (gates G5 and G6). The gating strategy is shown for the biological replicate from Supplementary Figure 5B, bottom panel. (B) Related to Figures 4A and 4B. Flow cytometric analysis of CD3 surface expression was performed on Treg cells identified within a complex mixture of $CD4^{+}$ and $CD8^{+}$ T cells, across two additional biological replicates. Cells were treated with sgRNA04 and/or sgRNA05 targeting the *Trac* locus across varying Cas9:sgRNA ratios and different amounts of RNP complexes. "E", electroporation; "/", no reagent added or procedure applied.

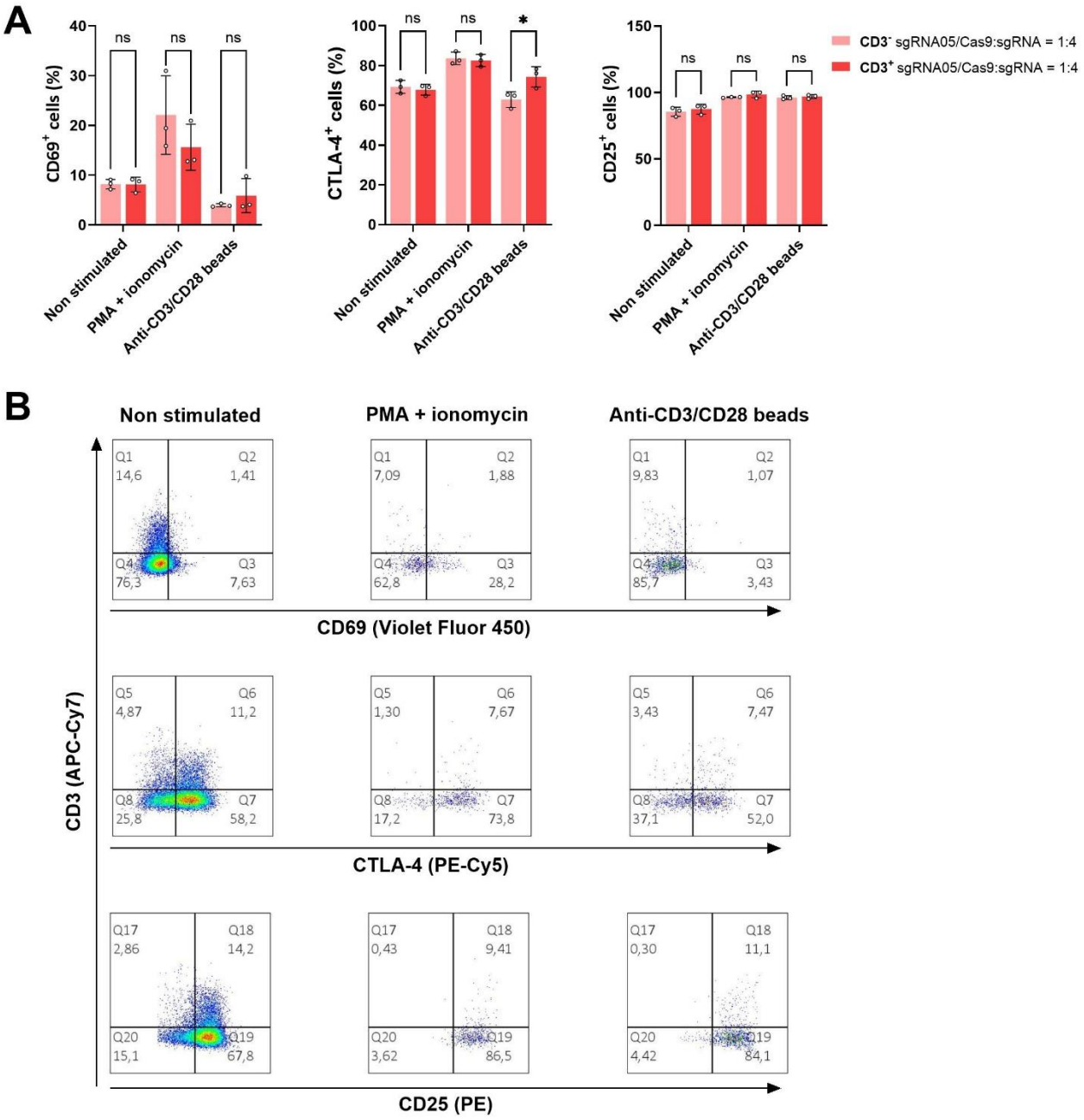


Supplementary Figure 7: Related to Figure 4: Translation of the genome editing pipeline and TCR knockout strategy to primary mouse Treg cells.

(A) Related to Figures 4C-E. Gating strategy for flow cytometric analysis of surface CD3 expression on Treg cells. Main cell population was gated based on FSC-A and SSC-A (gate G0). Doublets were excluded using FSC-H vs. FSC-A (gate G1) and SSC-H vs. SSC-A (gate G2) plots. Dead cells were eliminated based on staining with ViaDye Violet viability dye (gate G3). Among the $CD4^+$ cells (gate G5), the Treg cells were selected based on the expression of CD25 and FOXP3 (gate G6). Subsequently, CD3 expression was analyzed in the Treg population (gates G8 and G9). (B and C) Related to Figures 4C-E. Flow cytometric analysis of CD3 surface expression, in Treg (top) and cell viability (bottom) in two additional biological replicates. "E", electroporation; "/", no reagent added or procedure applied.

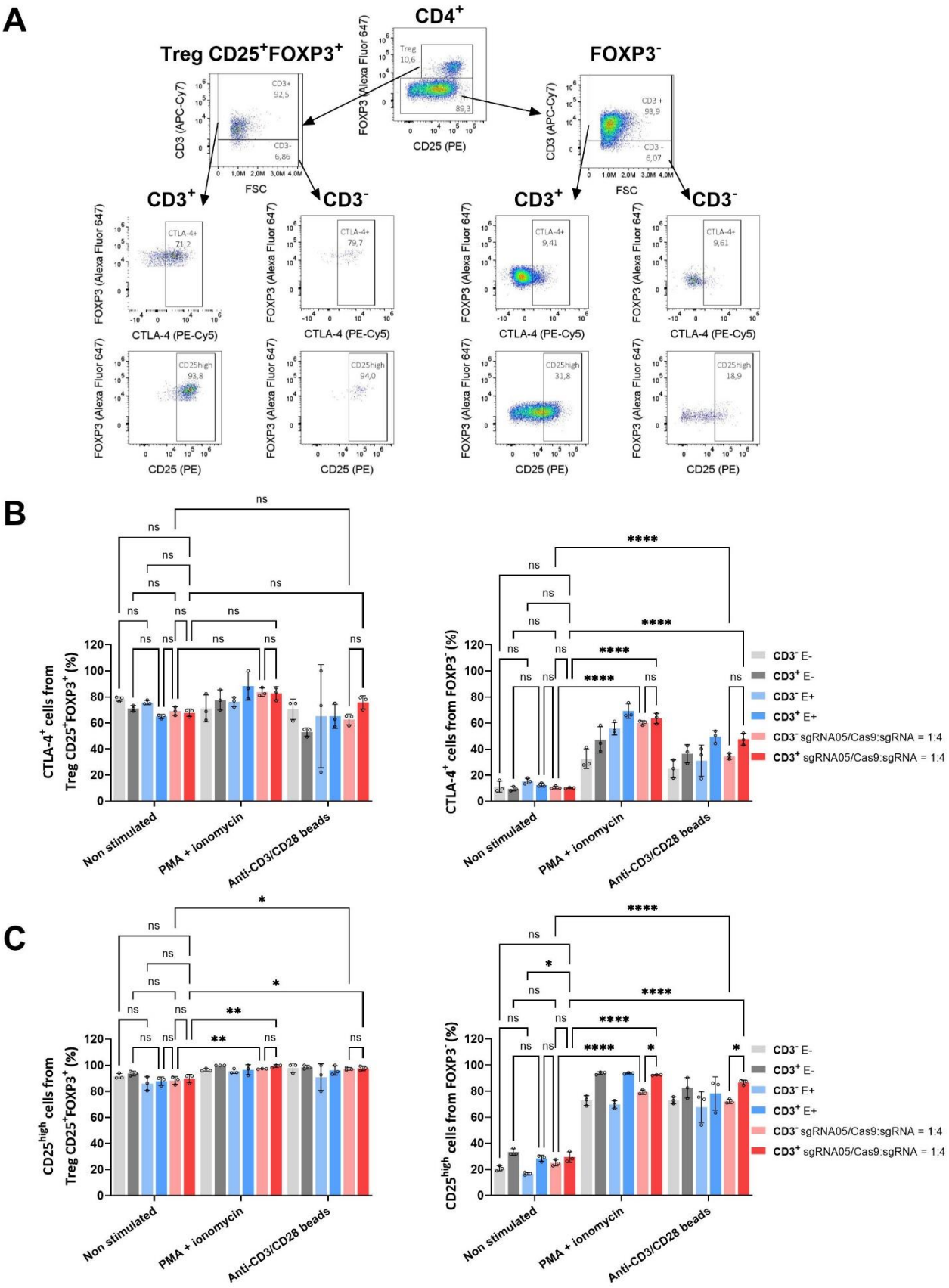


Supplementary Figure 8: Related to Figure 4: Translation of the genome editing pipeline and TCR knockout strategy to primary mouse Treg cells.
Frequency of $FOXP3^{+}CD25^{+}$ Treg cells among total live cells (left) and within the $CD4^{+}$ T-cell compartment (right) following electroporation. Quantification of the Treg phenotype is shown as the percentage of $CD25^{+}FOXP3^{+}$ cells across three biological replicates. Data are presented as mean ± SD and are representative of two independent experiments. *$p < 0.05$, ns – not significant (one-way ANOVA, Tukey's multiple comparisons test). "E", electroporation; "/", no reagent added or procedure applied.



Supplementary Figure 9: Related to Figure 4: Translation of the genome editing pipeline and TCR knockout strategy to primary mouse Treg cells.

Four days post debeading and electroporation, Treg cells were either left unstimulated or stimulated with PMA and ionomycin, or with anti-CD3/CD28 activation beads for 24 h. The percentages of CD69-, CTLA-4-, and CD25-expressing cells within CD3-positive or CD3-negative populations under conditions of high KO efficiency (sgRNA05/Cas9:sgRNA = 1:4) were determined by flow cytometry. (A) Frequencies of $CD69^+$, $CTLA\text{-}4^+$, and $CD25^+$ cells in $CD3^-$ and $CD3^+$ populations. (B) Representative gating strategy for assessing CD69, CTLA-4, and CD25 expression in $CD3^+$ and $CD3^-$ Treg fractions under the indicated stimulation conditions, shown for cultures with high KO efficiency. Data in A are presented as mean ± SD of three biological replicates. *$p < 0.05$, ns – not significant (two-way ANOVA, Tukey's multiple comparisons test).


A
CD4+
Treg CD25+FOXP3+
FOXP3-
CD3+
CD3-
CD3 (APC-Cy7)
FOXP3 (Alexa Fluor 647)
CD25 (PE)
FSC
CTLA-4 (PE-Cy5)
B
CTLA-4+ cells from Treg CD25+FOXP3+ (%)
CTLA-4+ cells from FOXP3- (%)
CD3- E-
CD3+ E-
CD3- E+
CD3+ E+
CD3- sgRNA05/Cas9:sgRNA = 1:4
CD3+ sgRNA05/Cas9:sgRNA = 1:4
Non stimulated
PMA + ionomycin
Anti-CD3/CD28 beads
C
CD25high cells from Treg CD25+FOXP3+ (%)
CD25high cells from FOXP3- (%)

Supplementary Figure 10: Related to Figure 4: Translation of the genome editing pipeline and TCR knockout strategy to primary mouse Treg cells.

Four days post debeading and electroporation, Treg cells were either left unstimulated or stimulated with PMA + ionomycin, or with anti-CD3/CD28 activation beads for 24 h. The percentages of CTLA-4-, and $CD25^{high}$-expressing cells within CD3-positive or CD3-negative populations of $CD25^{+}FOXP3^{+}$Treg and $FOXP3^{-}$ cells under conditions of high KO efficiency (sgRNA05/Cas9:sgRNA = 1:4) were determined by flow cytometry. (A) Representative flow cytometry plots showing the gating strategy used to identify $FOXP3^{+}CD25^{+}$ Treg cells and $FOXP3^{-}$ cells within the $CD4^{+}$ population, followed by separation into $CD3^{+}$ and $CD3^{-}$ fractions in non-electroporated and non-stimulated samples. CTLA-4^{+} and $CD25^{high}$ populations were subsequently analyzed within $CD3^{+}$ and $CD3^{-}$ Treg and FOXP3- subsets. (B) Frequency of CTLA-4^{+} cells within Treg cells (left) and FOXP3- cells (right) under non-stimulated conditions or following stimulation with PMA and ionomycin or anti-CD3/CD28 beads. (C) Frequency of $CD25^{high}$ cells within Treg cells (left) and FOXP3- cells (right) under the same stimulation conditions. Data in B and C are presented as mean ± SD of three biological replicates. "E", electroporation *$p < 0.05$, **$p< 0.01$, ****$p < 0.0001$, ns – not significant (two-way ANOVA, Tukey's multiple comparisons test).