

SUPPLEMENTAL MATERIAL

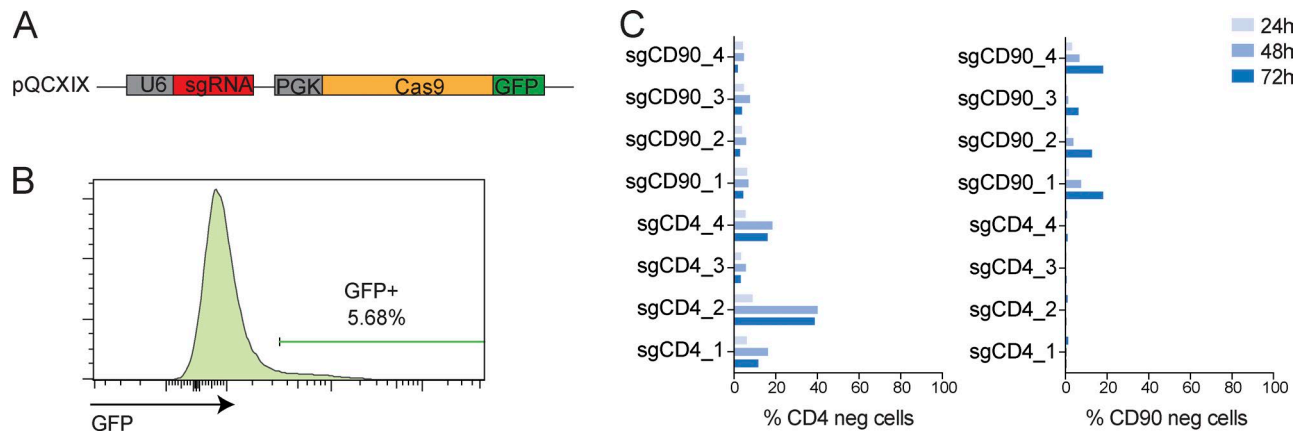
Seki and Rutz, <https://doi.org/10.1084/jem.20171626>

Figure S1. **Low efficiency of retroviral transduction of sgRNA/Cas9 all-in-one construct in primary CD4⁺ T cells.** (A) Schematic depiction of all-in-one retroviral construct expressing a sgRNA from a U6 polymerase III promoter and a Cas9/GFP fusion protein from a PGK promoter. (B) Expression of the retroviral construct as measured by GFP expression 48 h after retroviral transduction in CD4⁺ mouse T cells (gated on live cells). (C) Percentage CD4 or CD90 negative (KO) T cells 24, 48, and 72 h after retroviral transduction with sgRNA/Cas9 constructs targeting either CD4 or CD90 in CD4⁺ T cells (gated on live GFP⁺ cells). Data are representative of two independent experiments.

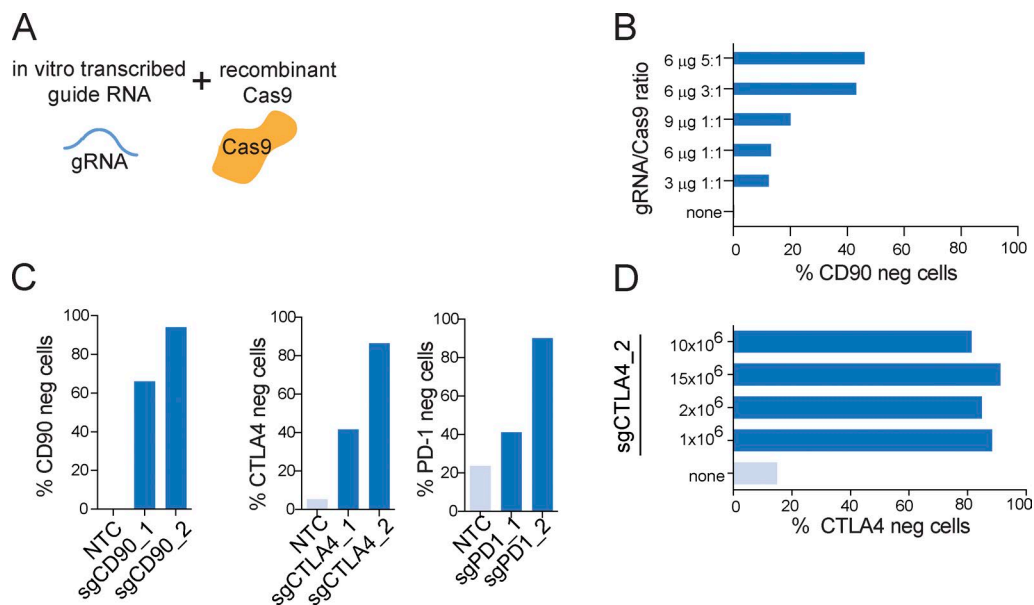


Figure S2. **Electroporation of in vitro transcribed sgRNA and recombinant Cas9 protein leads to highly efficient gene KO in primary T cells.** (A) Schematic depiction of RNP components, in vitro-transcribed sgRNA, and recombinant Cas9 protein. (B) Titration of amount and ratio of sgRNA/Cas9 targeting CD90 in activated mouse CD8⁺ T cells. Data are representative of two independent experiments. (C) KO efficiency as measured by flow cytometry 3 d after RNP transfection targeting CD90, CTLA4, and PD1 in activated mouse CD8⁺ T cells. Data are representative of two independent experiments. (D) Titration of number of activated mouse CD8⁺ T cells transfected with sgRNA targeting CD90. Data are representative of two independent experiments.

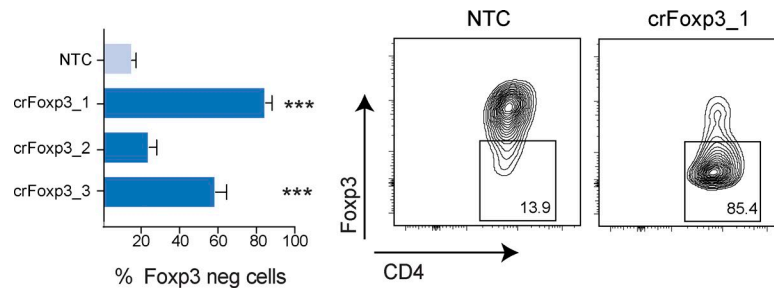


Figure S3. **KO of Foxp3 in expanded human regulatory T cells.** KO efficiency of targeting FoxP3 in human regulatory T cells expanded in vitro for 7 d was analyzed by flow cytometry 72 h after RNP transfection. Data are presented as mean \pm SD ($n = 2$) and representative of two independent experiments. ***, $P < 0.001$ by one-way ANOVA.

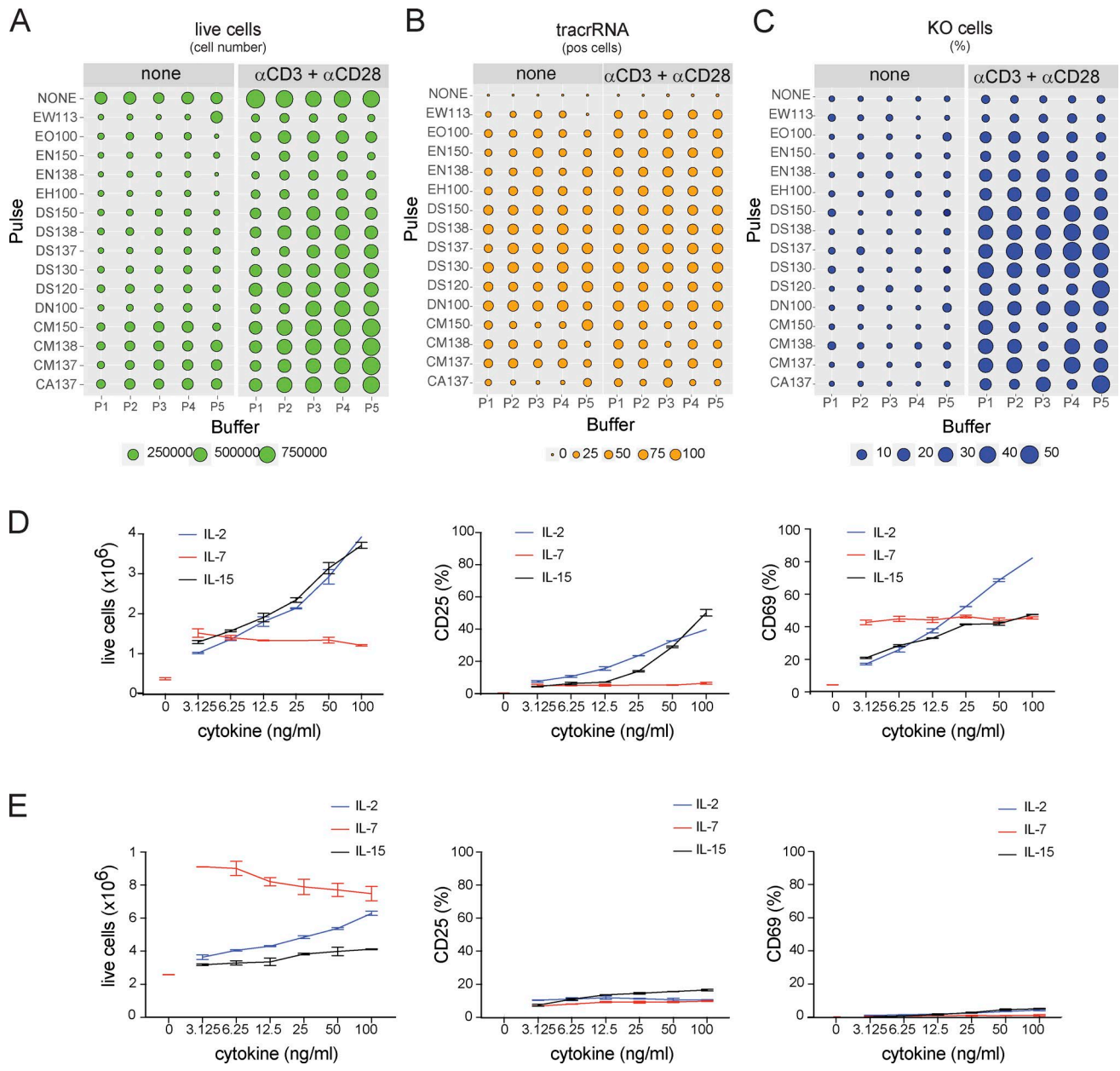


Figure S4. **Optimization of RNP transfection conditions in nonactivated mouse T cells.** (A–C) Systematic optimization of nucleofection parameters for RNP transfection of nonactivated mouse CD8⁺ T cells. Cell viability (A), transfection efficiency as determined by tracrRNA positive cells (B), and KO efficiency (%; C) of mouse CD8⁺ T cells transfected with RNP targeting CD90 and cultured for 2 d either without any stimulation or with anti-CD3/anti-CD28 stimulation. Data are from one experiment. (D and E) Analysis by flow cytometry of viable cells and CD25 and CD69 expression after 3 d of culture and titration of IL-2, IL-7, or IL-15 in CD8⁺ (D) or CD4⁺ mouse T cells (E). Data are presented as mean \pm SD ($n = 2$) and representative of two independent experiments.

Table S1. **gRNA sequences**

Name	Sequence (5'-3')	Name	Sequence (5'-3')
sgRNA (plasmid constructs)			
mCd4_1	ACTCCTAGCTGTCACTCAA	mCd90_1	ATGGCGGCAGTCCAGGCCGA
mCd4_2	AAGGGAAGACGCTGGTGCT	mCd90_2	CTTGGTGTATTCTCATGG
mCd4_3	TAAGTTTATTGATGATGAG	mCd90_3	TGCTGGATGGAGTTATCCT
mCd4_4	AGAGTTGCTATCCAAGGTC	mCd90_4	CTTCTCTTCTCTCGGGTC
sgRNA (IVT)			
mCd90_1	GGCGAAGGTTTTGGTTCACC	mCtla4_1	GACCCAACCTTCAGTGGTGT
mCd90_2	AAAGTAGTCGCCCTCATCCT	mCtla4_2	ATGGAAAGCTGGCGACACCA
mPd1_1	CGGAGGATCTTATGCTGAAC		
mPd1_2	GACACACGGCGCAATGACAG		
crRNA			
mPd1_1	ACAGCCCAAGTGAATGACCA	hCXCR4_1	GAAGCGTGATGACAAAGAGG
mPd1_2	TGAATGACCAGGTACCTGC	hCXCR4_2	AGGGAAGCGTGATGACAAAG
mPd1_3	AGTTGAGCTGGCAATCAGGG	hCXCR4_3	ACGGCATCAACTGCCAGAA
mCtla4_1	AGGTCCGGGTGACTGTGCTG	hCCR7_1	TACCTACCTGCTCAACCTGG
mCtla4_2	GACAAATGACCAAATGACTG	hCCR7_2	CATCAGCATTGACCGCTACG
mCtla4_3	ATGGAAAGCTGGCGACACCA	hCCR7_3	AAAGTGGACACCGAAGACCC
mCd90_1	CCGCCATGAGAATAACACCA	hCD127_1	TCAGGCACCTTACCTCCACG
mCd90_2	CCTTGGTGTATTCTCATGG	hCD127_2	CAGGCACCTTACCTCCACGA
mCd90_3	GAGCAGGAGAGCGACGCTGA	hCD127_3	CAAGTCGTTTCTGGAGAAAG
mCd8a_1	ATCCCAACAAGATAACGT	hIFNG_1	CCAGAGCATCCAAAAGAGTG
mCd8a_2	TGAAGCCATATAGACAACGA	hIFNG_2	AAAGAGTGTGGAGACCATCA
mCd8a_3	TGGGTGAGTCGATTATCCTG	hIFNG_3	TGCAGGTCATTGAGATGTAG
mFoxp3_1	TGGACGCACTTGGAGCACAG	hFOXP3_1	CTTGAGGGAGAAGACCCAG
mFoxp3_2	TCTACCCACAGGATCAATG	hFOXP3_2	GAGGGAGAAGACCCAGTGG
mFoxp3_3	CTTGAGGGAGAAGACCCAG	hFOXP3_3	GGGTGCACCTGCAGCACAG
NTC	Negative control crRNA #1 (IDT 1072544)	hPD1_1	CTGCAGCTTCTCCAACACAT
		hPD1_2	CGTGTACACAACTGCCCAA
		hPD1_3	GCCCTGGCCAGTCGTCTGGG
		hCTLA4_1	CCAGGTGACTGAAGTCTGTG
		hCTLA4_2	CACTGTACCCCGACCTCAG
		hCTLA4_3	GCACGTGGCCAGCCTGCTG
		hTIGIT_1	CAGGCACAATAGAAACAACG
		hTIGIT_2	GACCTGGGTCACTTGTGCCG
		hTIGIT_3	CAGGCATGCTTCTGCCAG