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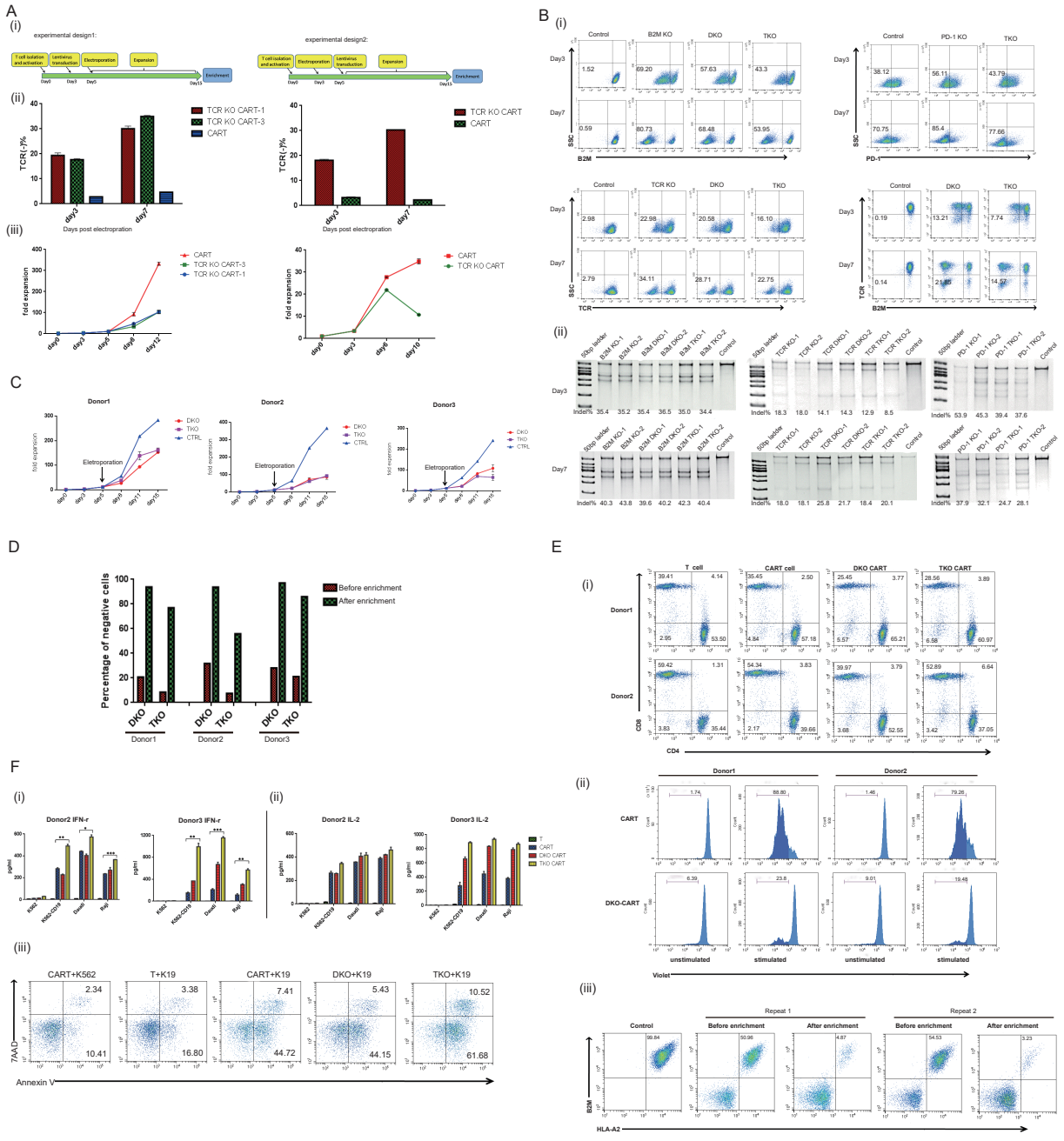

FigureS2. Multiplex gene editing mediated by CRISPR-Cas9 in CAR-T cells.

A. Optimization of the procedure of generating gene edited CAR-T cells. (i) Schematics of two strategies. (ii) Flow cytometric analysis (mean±SEM, n=2) of the frequency of negative cells by different strategies. Left panel is the result from strategy one. TKO-CART-1 is the sample using $1x10^6$ CART cells for electroproration, and TKO-CART-3 used $3x10^6$ cells. Right panel is the result from strategy two, $1x10^6$ CART cells were used. (iii) Cell viability analysis of the two

strategies. Left is the result from strategy one and the right is from strategy two. **B.** Genome editing results in CAR-T cells. $3x10^6$ CAR-T cells were elctroprorated with Cas9 protein and sgRNAs targeting each gene as indicated before. 5 groups were developed: *B2M* KO, TCR KO, *PD-1* KO, DKO and TKO. 3 days and 7 days later, gene editing efficiency was determined by Surveyor assay and surface expression of the target genes was analyzed by FACS. (i) Surface expression of B2M, TCR and PD-1 3 days and 7 days post electroproration. (ii) Quantification of Cas9 RNP mediated indel mutations by Surveyor assay 3 days and 7 days post electroproration. **C.** Proliferation of DKO and TKO CAR-T cells generated using strategy one. The bar figures show cell number count (mean±SEM, n=2) of RNP-treated CAR-T cells and control CAR-T cells from day 0 to day 15. **D.** Quantification of DKO and TKO CAR-T cells before and after enrichment using magnetic beads, using FACS analysis (mean±SEM, n=2). **E.** Characterization of DKO and TKO CAR-T cells. (i) CD4 and CD8 surface expression in DKO and TKO CAR-T cells from two independent donors. (ii) DKO CAR-T cell proliferation after anti-CD3 and anti-CD28 stimulation. (iii) Surface expression of HLA-A and B2M before and after magnetic beads enrichment. **F.** Cytokine production and cytotoxicity analysis of DKO and TKO CAR-T cells. (i) IFN-r and (ii) IL-2 production (mean±SEM, n=2) of 2 independent donors. *p<0.05, **p<0.01, ***p<0.001. (iii) Flow cytometric plot of cytotoxicity analysis of DKO and TKO CAR-T cells at 10:1 E:T ratio.