Cell Reports Medicine, Volume *4*

Supplemental information

CD28-CAR-T cell activation through FYN kinase signaling rather than LCK enhances therapeutic performance

Ling Wu, Joanna Brzostek, Previtha Dawn Sakthi Vale, Qianru Wei, Clara K.T. Koh, June Xu Hui Ong, Liang-zhe Wu, Jia Chi Tan, Yen Leong Chua, Jiawei Yap, Yuan Song, Vivian Jia Yi Tan, Triscilla Y.Y. Tan, Junyun Lai, Paul A. MacAry, and Nicholas R.J. Gascoigne

Table

CRISPR-Cas9 guide RNA (gRNA) sequences for LCK and FYN knock out. Related to STAR method.

gRNA	Sequence
LCK gRNA1	GACCCACTGGTTACCTACGA
LCK gRNA2	GCCGGGAAAAGTGATTCGAG
FYN gRNA1	AGAGTTCACACCTCCAAAGA
FYN gRNA2	ACGGGGACCTTGCGTACGAG
FYN gRNA3	TTGTCCTTTGGAAACCCAAG
FYN gRNA4	GTCCCCCGAATCATTCCTTG
FYN gRNA5	TGGATACTACATTACCACCC

gRNA pool for LCK and FYN knock out. Each of gRNA was selected from http://chopchop.cbu.uib.no/. After screening, LCK gRNA2 and FYN gRNA3 were chosen for LCK and FYN knock out respectively.

Figures

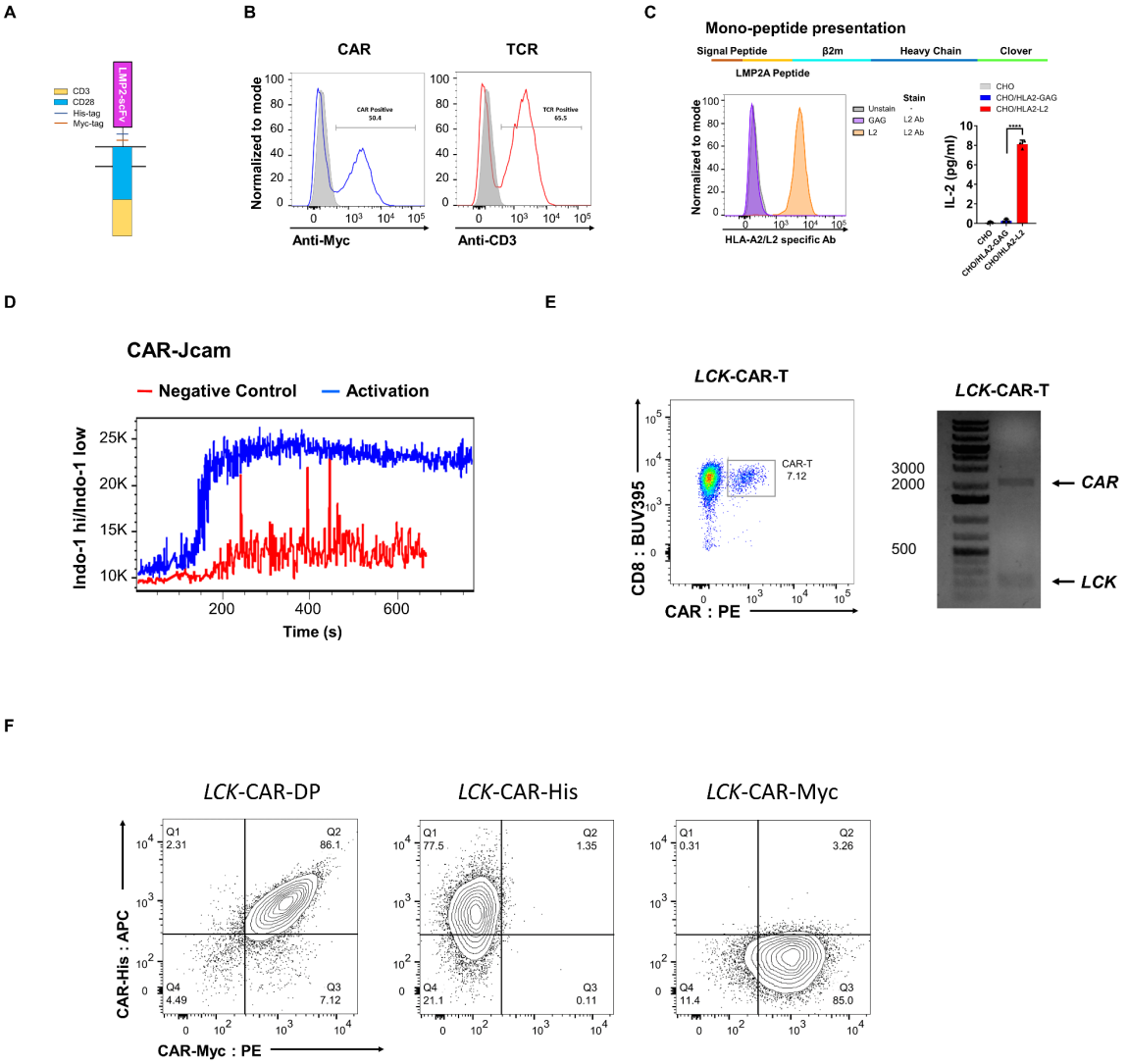


Figure S1. CAR signaling does not require LCK. Related to Figure 1. (**A**) Schematic diagram of CAR construct. Myc-tag was used for the detection of CAR expression. (**B**) Expression of LMP2A peptide (L2)-specific CAR or TCR after lentiviral transduction of Jurkat76. CAR was stained using anti-Myc, TCR with anti-CD3 Abs. (**C**) Schematic diagram of the scHLA construct with linked peptide ("mono-peptide system": upper). Lower left: staining with L2-specific TCR-like Ab on CHO-L2 or CHO-GAG. Lower right: responsiveness of L2-specific CAR-T to CHO-L2 versus CHO-GAG. Mean ±SD of technical triplicates, from 3 experiments. (**D**) Ca^{2+} flux of CAR-Jcam cells. Negative control was PBS, activation by adding specific pMHC tetramer. (**E**) *LCK* locus-

18 targeted CRISPR-Cas9 editing. Left panel shows the percentage of $CD8^+$ CAR^+ CAR-T cells after editing. Right
19 panel is the genotyping of the targeted site in the *LCK* gene. Forward primer: 5'-
20 AGGGAGAGGTGGTGAAACATTA-3', reverse primer: 5'- GAATGGAGTAGGGCATTGAAAG-3'. (**F**)
21 CAR-His and CAR-Myc expression after HDR and cell sort.

22

23

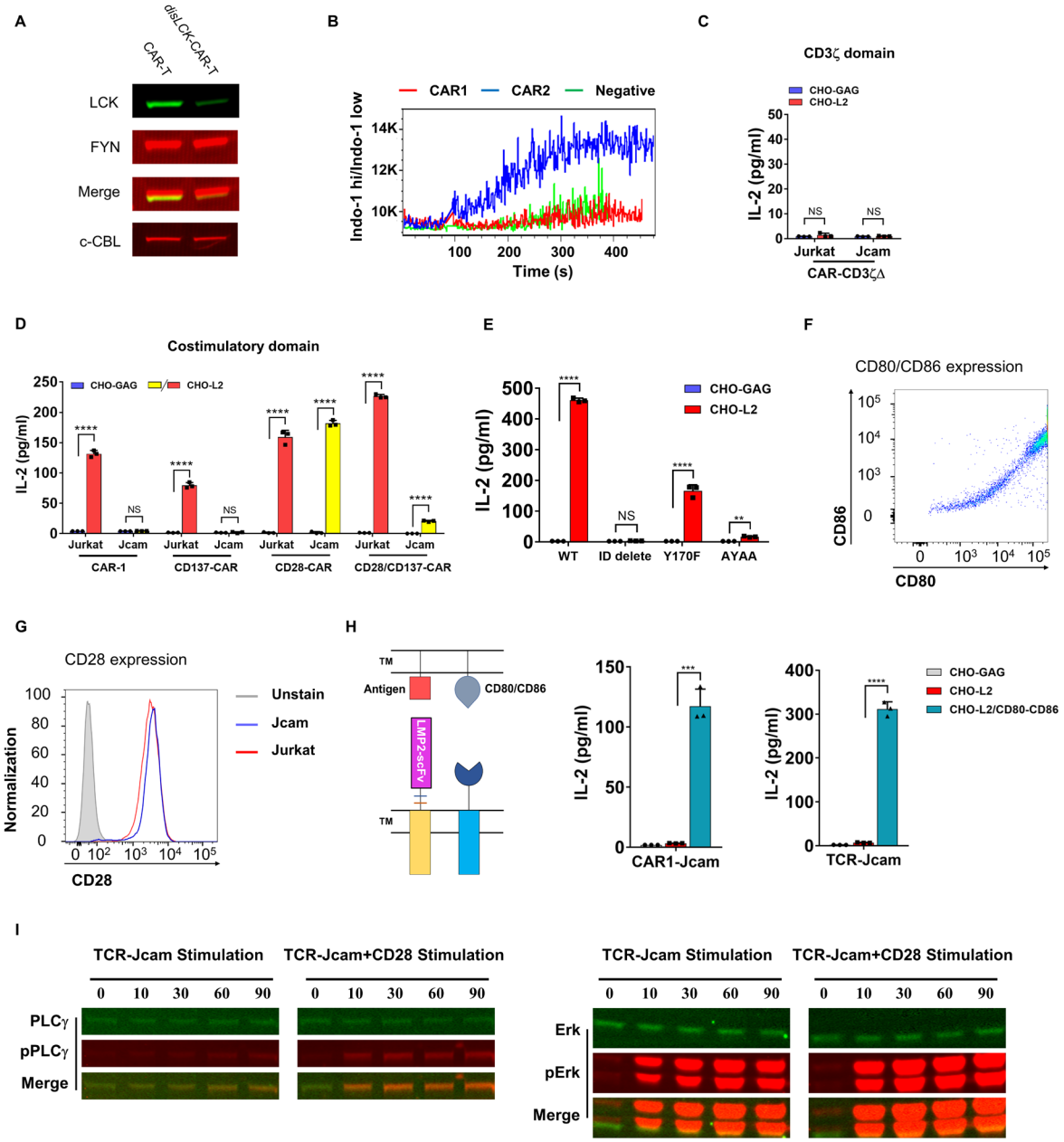


Figure S2. LCK-independent CAR signaling requires CD28 as costimulatory domain. Related to Figure 2.

(**A**) LCK protein expression of sorted *disLCK*-CAR-T cells in comparison with conventional CAR-T. (**B**) Calcium flux of CAR2-Jcam or CAR1-Jcam after specific HLA-A2-L2 tetramer was added into the medium. The second generation CAR with CD28 costimulatory domain in Jcam1.6 cell is labeled as CAR2-Jcam, and the first generation of CAR in Jcam1.6 cell is labeled as CAR1-Jcam. (**C**) IL-2 production of CAR constructs without CD3ζ signaling domain in Jurkat or Jcam cells. (**D**) IL-2 production of CAR constructs with different intracellular domains in Jurkat or Jcam cells. (**E**) IL-2 production of Jcam cells expressing different CD28-CAR mutants. (**F**)

Co-expression of CD80 and CD86 on CHO-L2 APC. The CD80 and CD86 were linked by P2A cleavable linker. CD80-P2A-CD86 construct was on one lentivirus vector. (**G**) Endogenous CD28 expression on Jurkat or Jcam1.6 cell lines. (**H**) Schematic of CD28 costimulation of CAR1 on JCam1.6 T cells and their responsiveness in Jcam1.6 to CHO-L2 expressing co-stimulators CD80 and CD86. (**I**) Phosphorylation of TCR signaling pathway with or without endogenous CD28 costimulation.

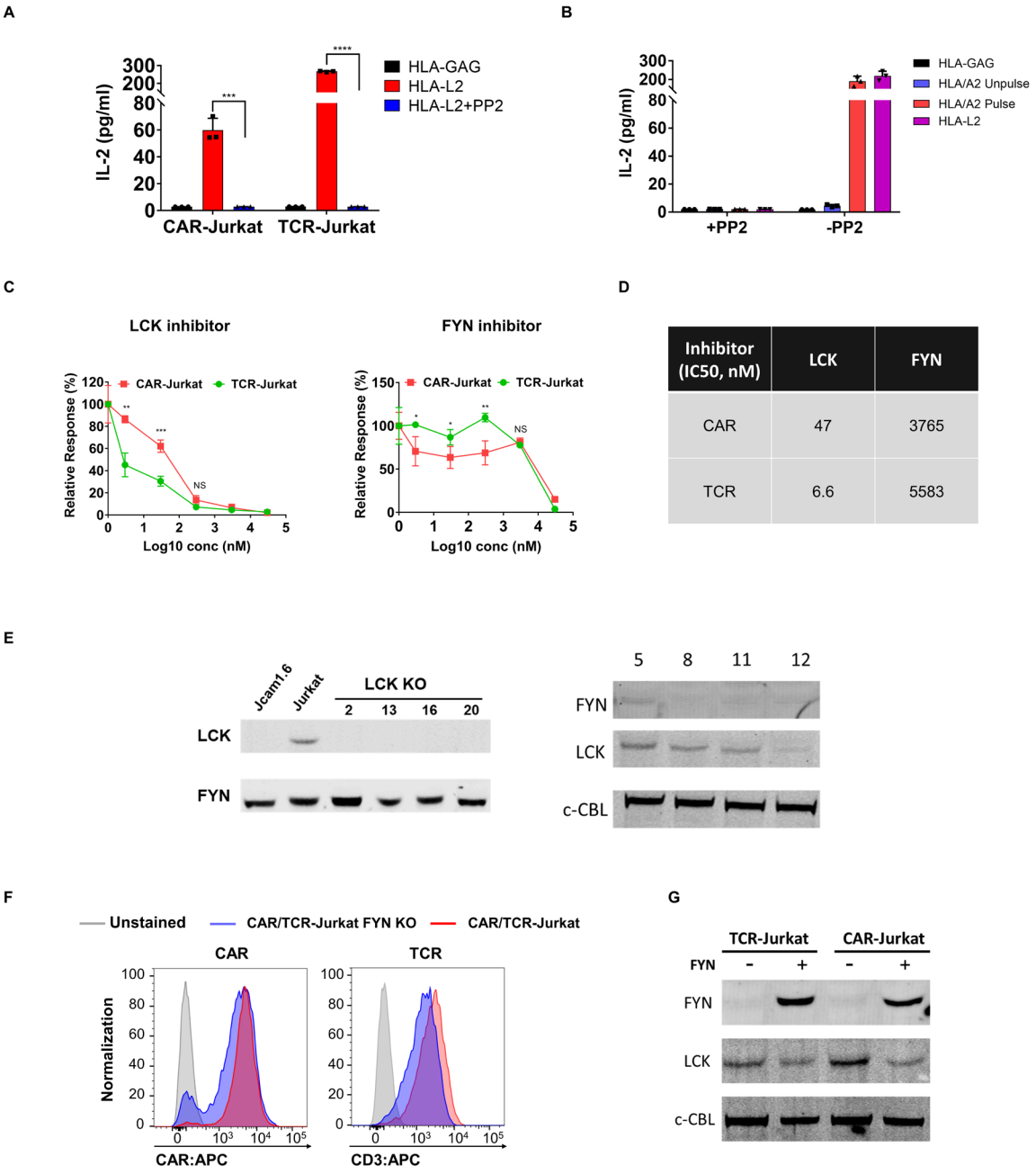


Figure S3. CD28-CAR relies on FYN to transduce downstream signaling. Related to Figure 3. (**A**) Responsiveness of CAR and TCR-Jurkat with SFK inhibitor PP2 (10μM). (**B**) IL-2 production of CAR-Jcam cell with or without SFK PP2. (**C**) Responsiveness of CAR and TCR-Jurkat in the presence of LCK or FYN inhibitors A770041 or SU6656, respectively. IL-2 production was normalized to that without inhibitors as the relative response (%) against log (inhibitor concentration). (**D**) IC50 of LCK- or FYN-specific inhibitors on CAR-Jurkat or TCR Jurkat cell. (**E**) LCK KO and FYN KO single clone selection. Clone 20 in LCK KO was selected as Jurkat LCK KO cell, and Clone 8 in FYN KO was selected as Jurkat FYN KO cell. (**F**) The CAR or TCR expression

47 detection on CAR-Jurkat FYN KO and CAR-Jurkat or on TCR-Jurkat FYN KO and TCR-Jurkat. CD3 was used
48 as an indicator of TCR expression. (**G**) FYN and LCK expression in CAR-Jurkat FYN KO and CAR-Jurkat or on
49 TCR-Jurkat FYN KO and TCR-Jurkat.

50

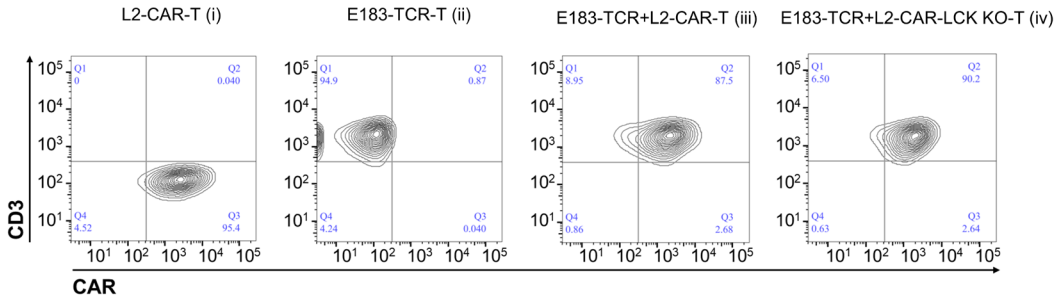


Figure S4. CAR or TCR expression on LCK-sufficient or deficient Jurkat after transduction and cell sort. Related to Figure 4. The TCR is specific for a peptide epitope from HBV antigen, E183. CAR was with the specificity as above, the peptide epitope (L2) from LMP2A protein.

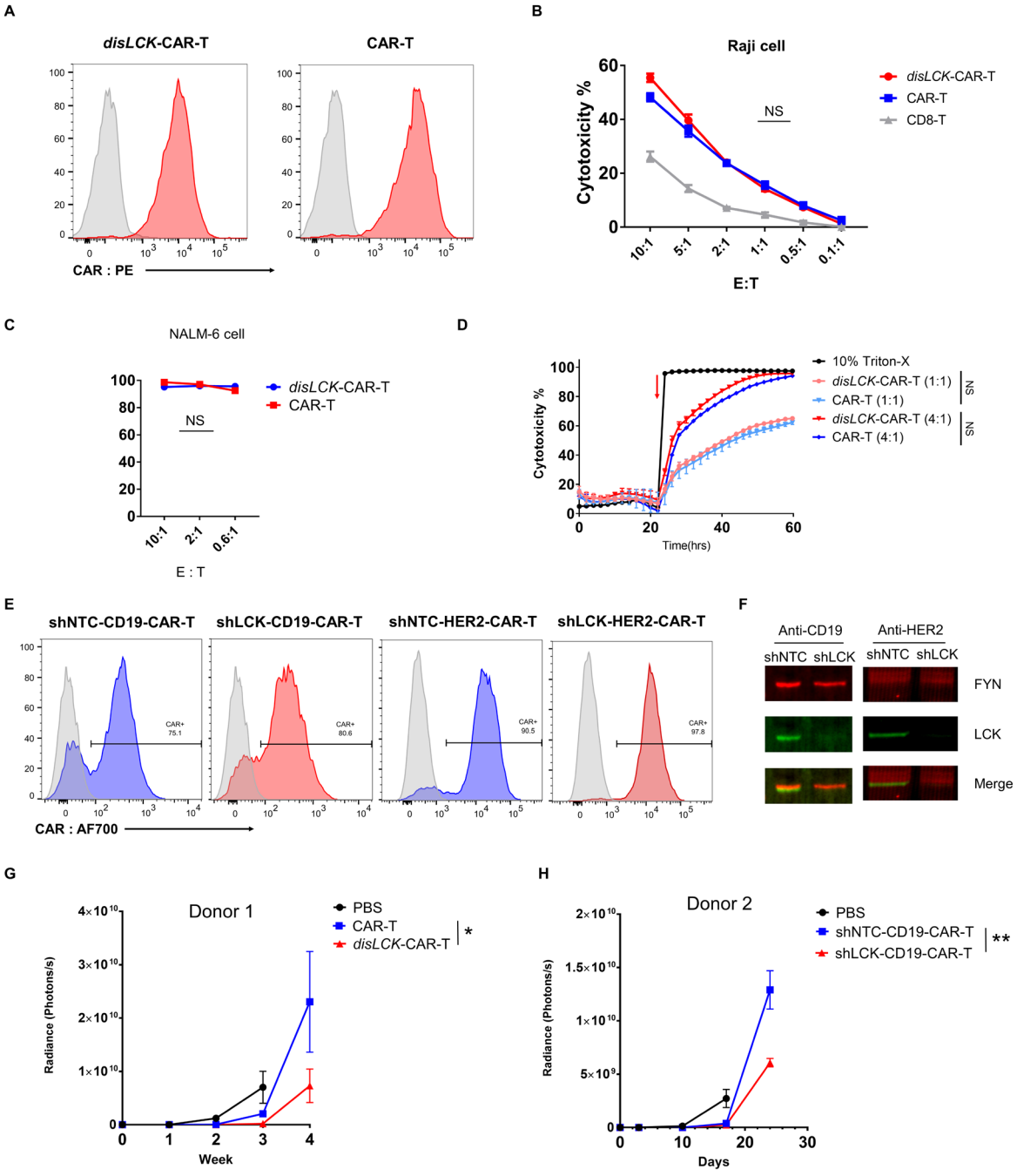
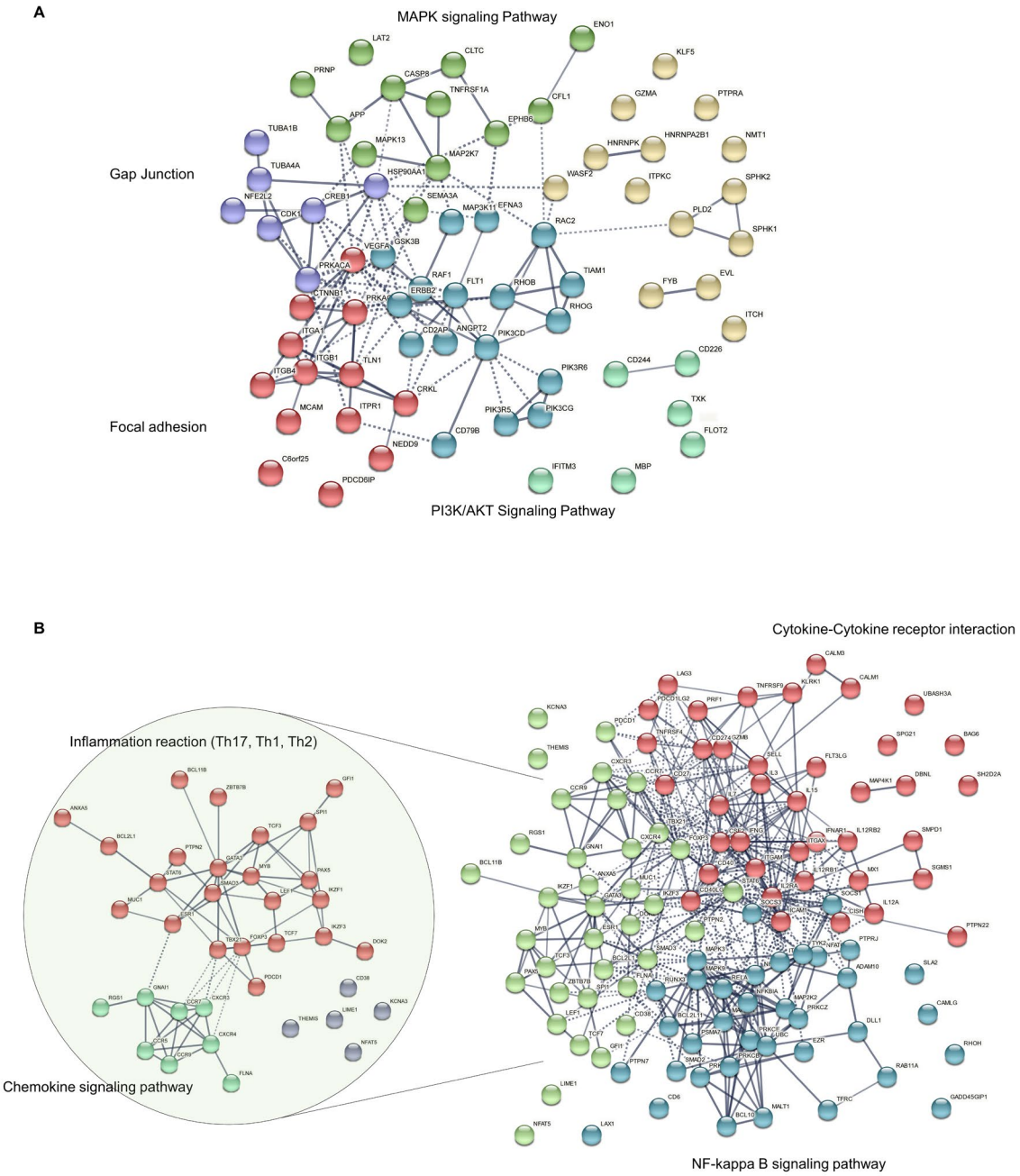


Figure S5. *In vitro and vivo* performance of disLCK-CAR-T cells. Related to Figure 5. (**A**) CAR expression of *disLCK*-CAR-T and conventional CAR-T after sorting and restimulation by feeder cells. Cytotoxicity of conventional CAR-T and *disLCK*-CAR-T to Daudi cells (**B**) and Nalm-6 cells (**C**). (**D**) Real time killing by two conventional CAR-T and *disLCK*-CAR-T cells to CD19-expressing CHO cells. The cytotoxicity was calculated by the formula: 1-cell index(sample)/cell index(media). (**E**) CAR expression of shRNA-CAR-T cells after sorting and restimulation by feeder cells. (**F**) LCK protein expression of sorted shRNA-CAR-T cells. (**G**, **H**) *In vivo*

luciferase signal from Nalm-6 cells at different time points. 2-way ANOVA was used to test the statistical significance.



66

67 **Figure S6. Clustering of molecular interactions within FYN and LCK group. Related to Figure 6.** Cluster
68 of molecular interactions within FYN group (**A**) and LCK group (**B**). The cluster is identified by kmeans=6 (FYN
69 group) and =3 (LCK group). The analysis was done by String webtool analysis. The identification of signal
70 pathway was done by KEGG pathway database.

71

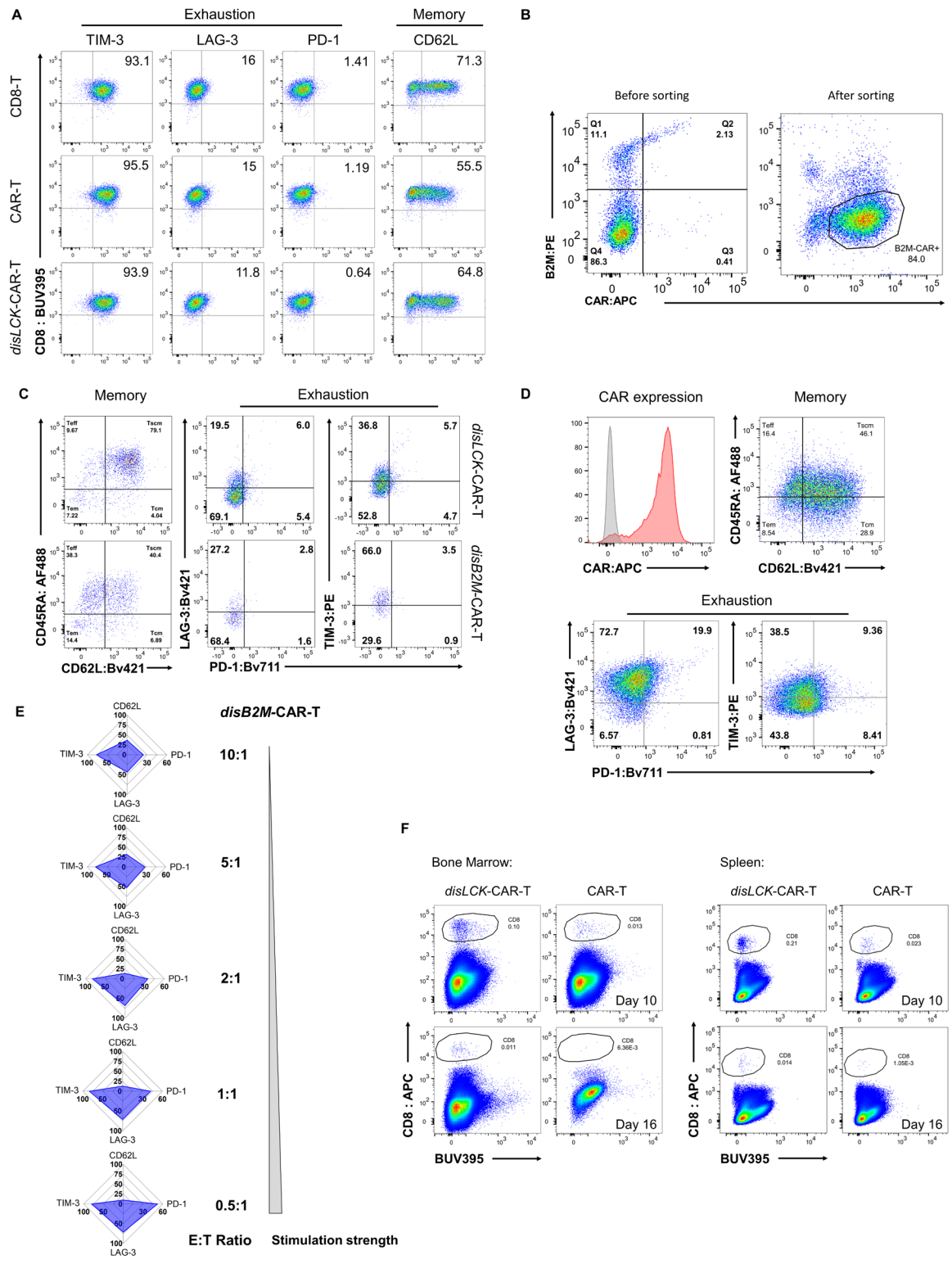


Figure S7. Independence from LCK makes CAR-T cells more specific, memory-like and less exhausted. Related to Figure 6 and Figure 7. (**A**) The memory and exhaustion state of *disLCK*-CAR-T and conventional CAR-T in quiescent state. The data is from another donors' T cells. (**B**) CAR and B2M expression after *B2M* locus-targeted HDR. *disB2M*-CAR-T cells were generated by using gRNA, GGCCGAGAUGUCUCGCUCCG,

through the same process as *disLCK*-CAR-T cells. (**C**) Comparison of the immunotyping of the *disB2M*-CAR-T and *disLCK*-CAR-T cells at resting state. The cells were gated before cell sort. (**D**) *disB2M*-CAR-T cells immunophenotype at day 5 after CAR-T cells sorting and restimulation by feeder cells. (**E**) Radar chart summary of exhaustion and memory marker expression in *disB2M*-CAR-T cells after encountering target cells at different E:T ratios. (**F**) Representative FACS graphs of CAR-T cells in bone marrow and spleen at day 10 and day 16.