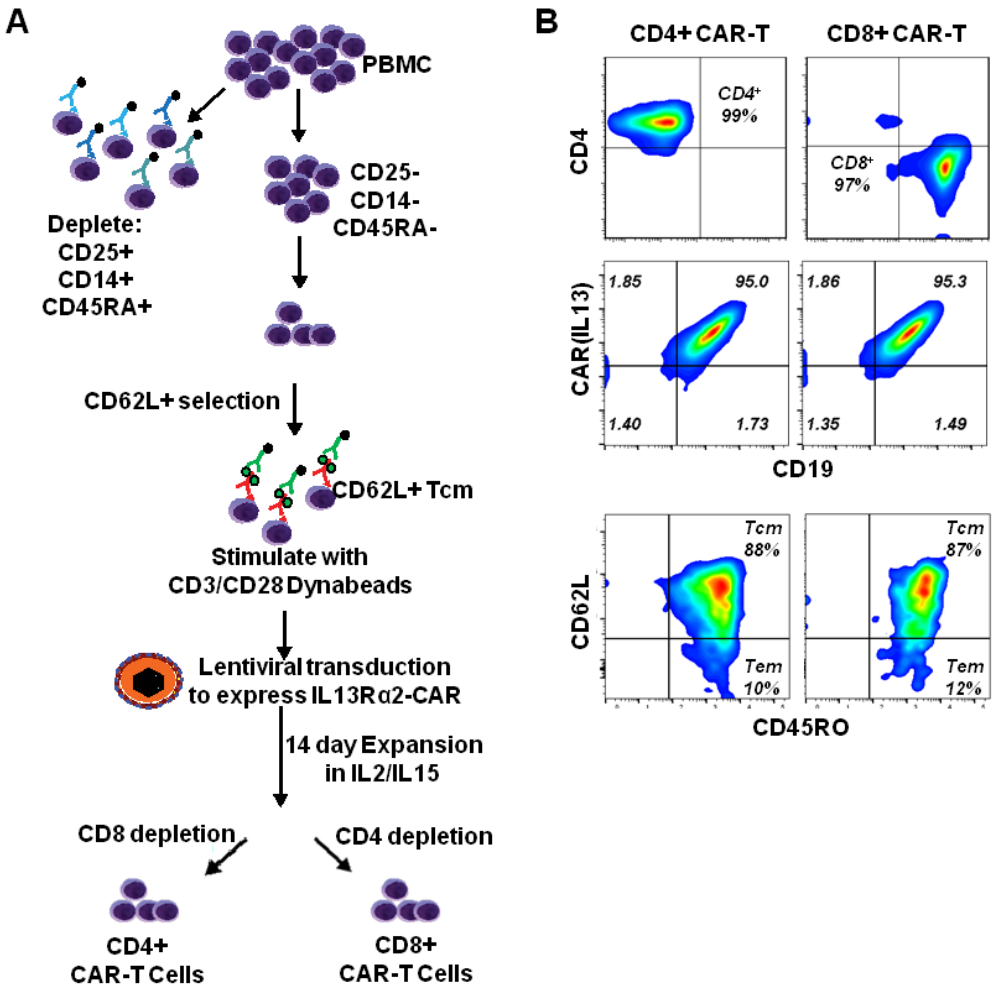
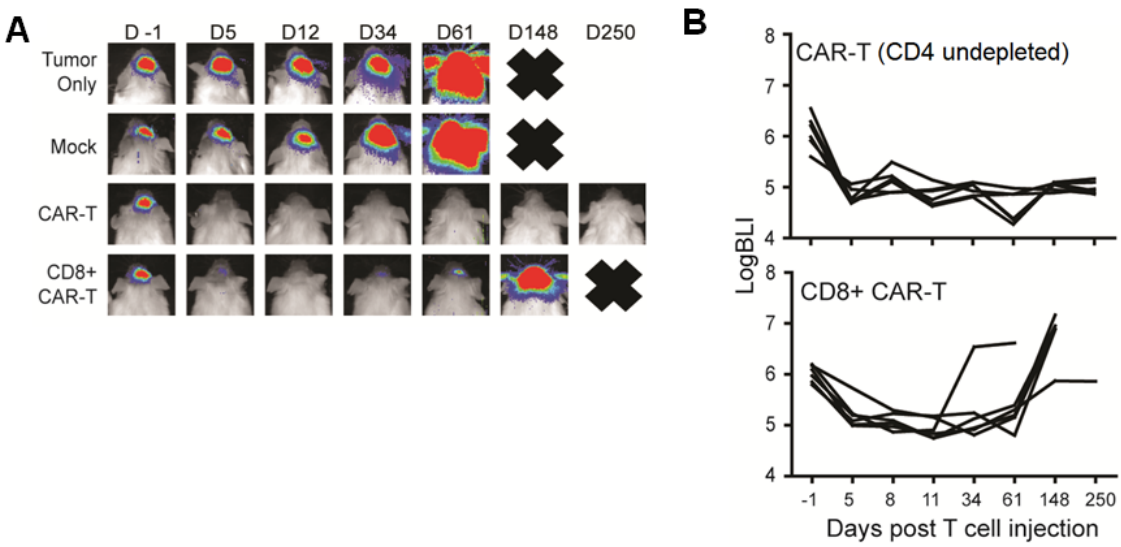
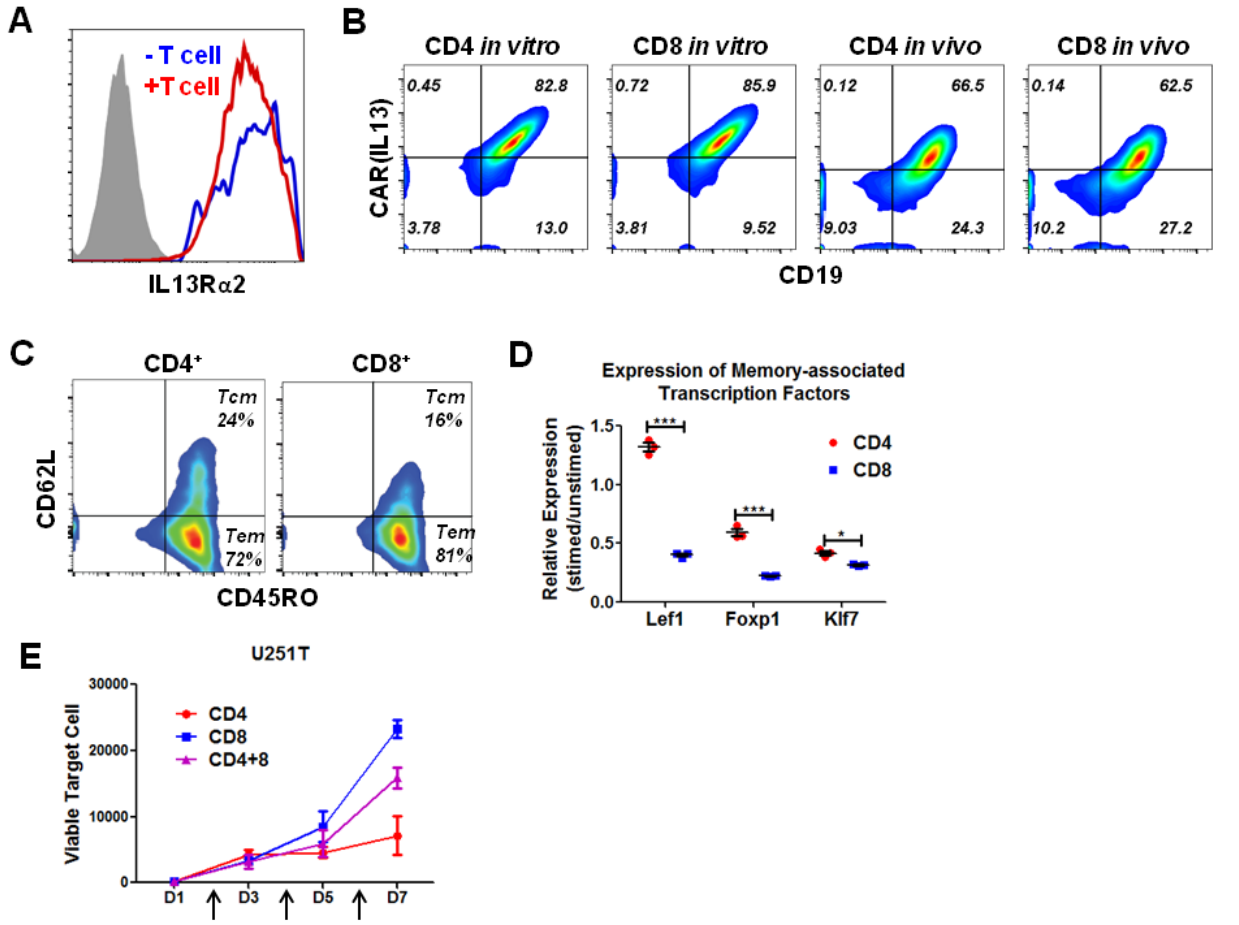


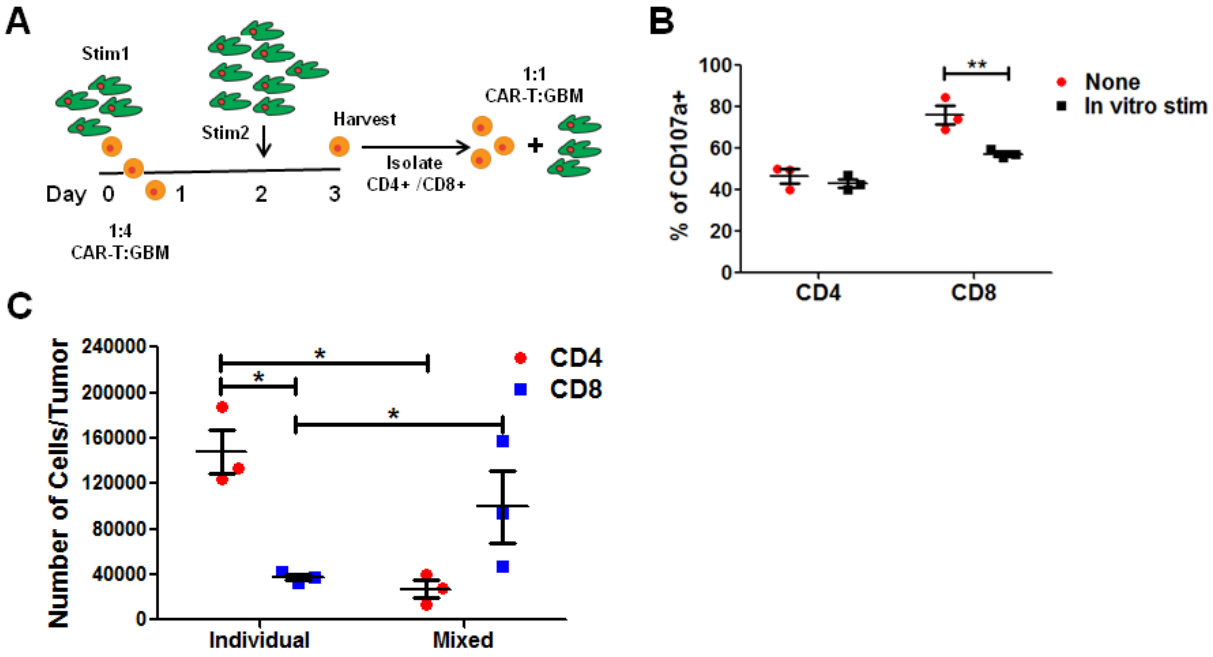
Supplemental Figure 1. CAR transduction and CD4$^+$/CD8$^+$ purification. (A) Schematic description of IL13Ra2-targeted CAR T production, including the enrichment of Tcm from PBMCs, lentiviral transduction and CD4$^+$/CD8$^+$ purification. (B) Flow cytometric analysis of enriched subsets for CD4-CD8 composition, transgene expression and Tcm phenotype.



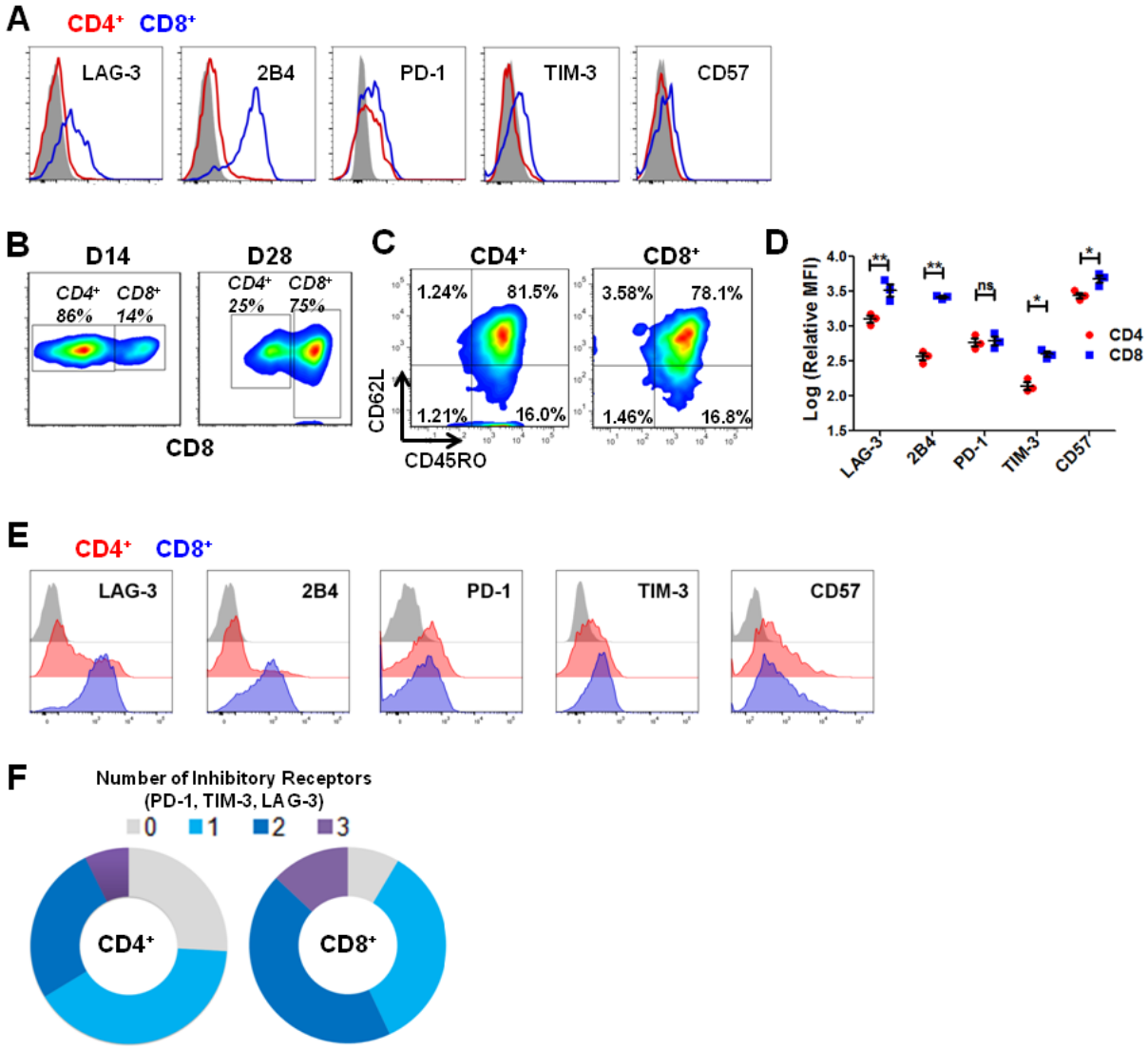
Supplemental Figure 2. CD4$^+$ CAR T cells are required for long-term tumor clearance (A) Biophotonic imaging of representative tumor-bearing mice treated with unselected (CAR T) or CD8-isolated (CD8$^+$ CAR T) cells. X: euthanization due to tumor progression occurred prior to the image collection time point. (B) Bioluminescent Intensity (BLI, Log scale) of each individual mouse over time in the unselected CAR T cell (top) and CD8$^+$ CAR T cell (bottom) treated groups.



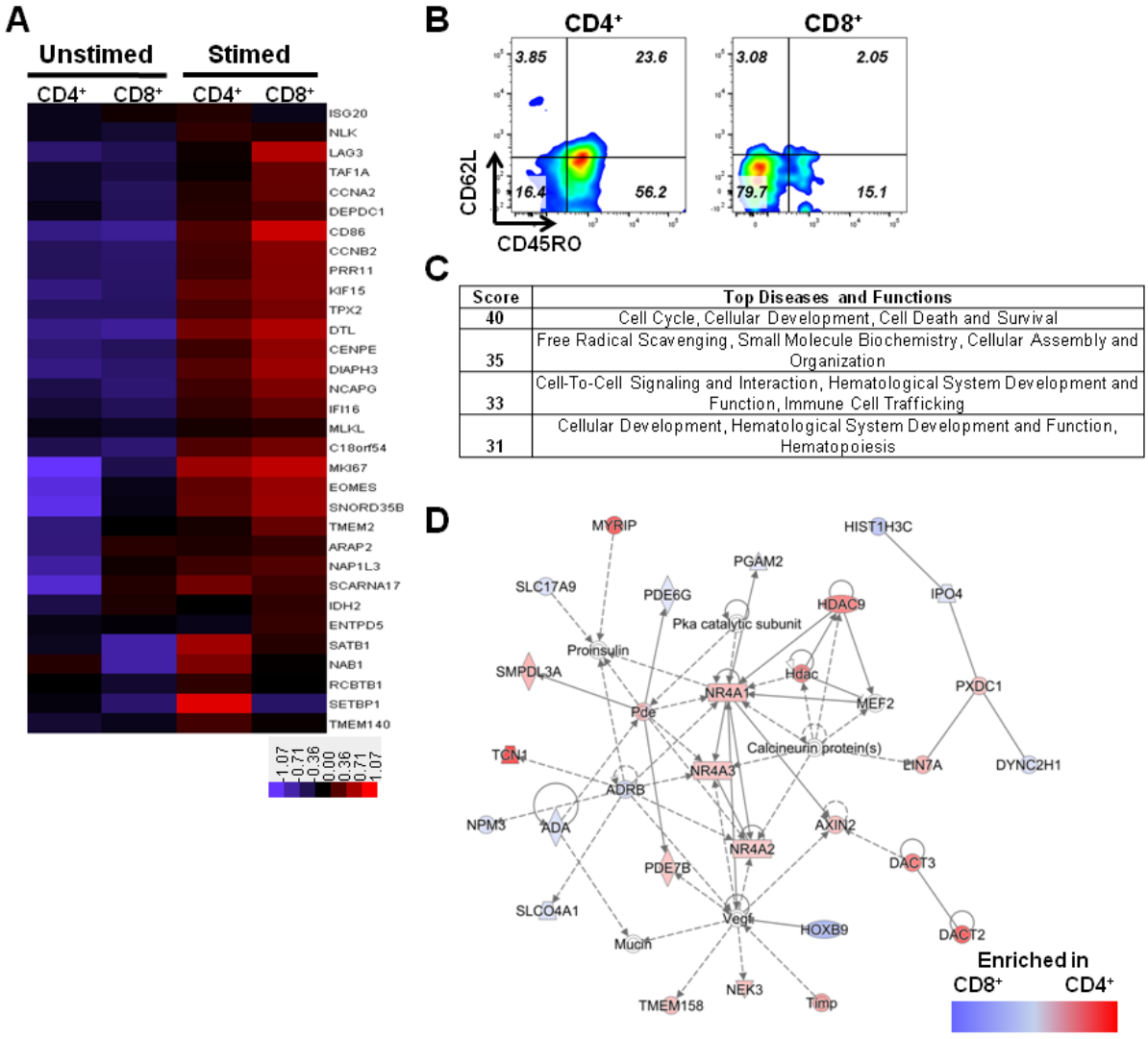
Supplemental Figure 3. Comparison of cytotoxicity between CD4$^+$ and CD8$^+$ CAR T cells. (A) Analysis of antigen expression on remaining PBT030-2 cells at Day 7 of the repetitive tumor challenge assay shown in Figure 2B (using CD8$^+$ CAR T cells). (B) Expression of the CD19 reporter gene and CAR (IL13) on CD4$^+$ and CD8$^+$ CAR T cells after *in vitro* and *in vivo* stimulation. (C) CD62L and CD45RO staining on CD4$^+$ and CD8$^+$ CAR T cells at Day 7 after repetitive tumor challenge. (D) Comparison of memory-associated transcription factor expression between stimulated and unstimulated CD4$^+$ or CD8$^+$ CAR T cells. (E) Antitumor activity of isolated CD4$^+$, CD8$^+$, and mixed (CD4:CD8 at 1:1) CAR T cells during repetitive stimulation with U251T GBM cells (arrows: tumor rechallenge). Remaining viable tumor cell numbers at the indicated time points (n = three replicates) are depicted. *p <0.05, and ***p<0.001 when comparing the CD4$^+$ and CD8$^+$ groups using an unpaired Student's t-test.



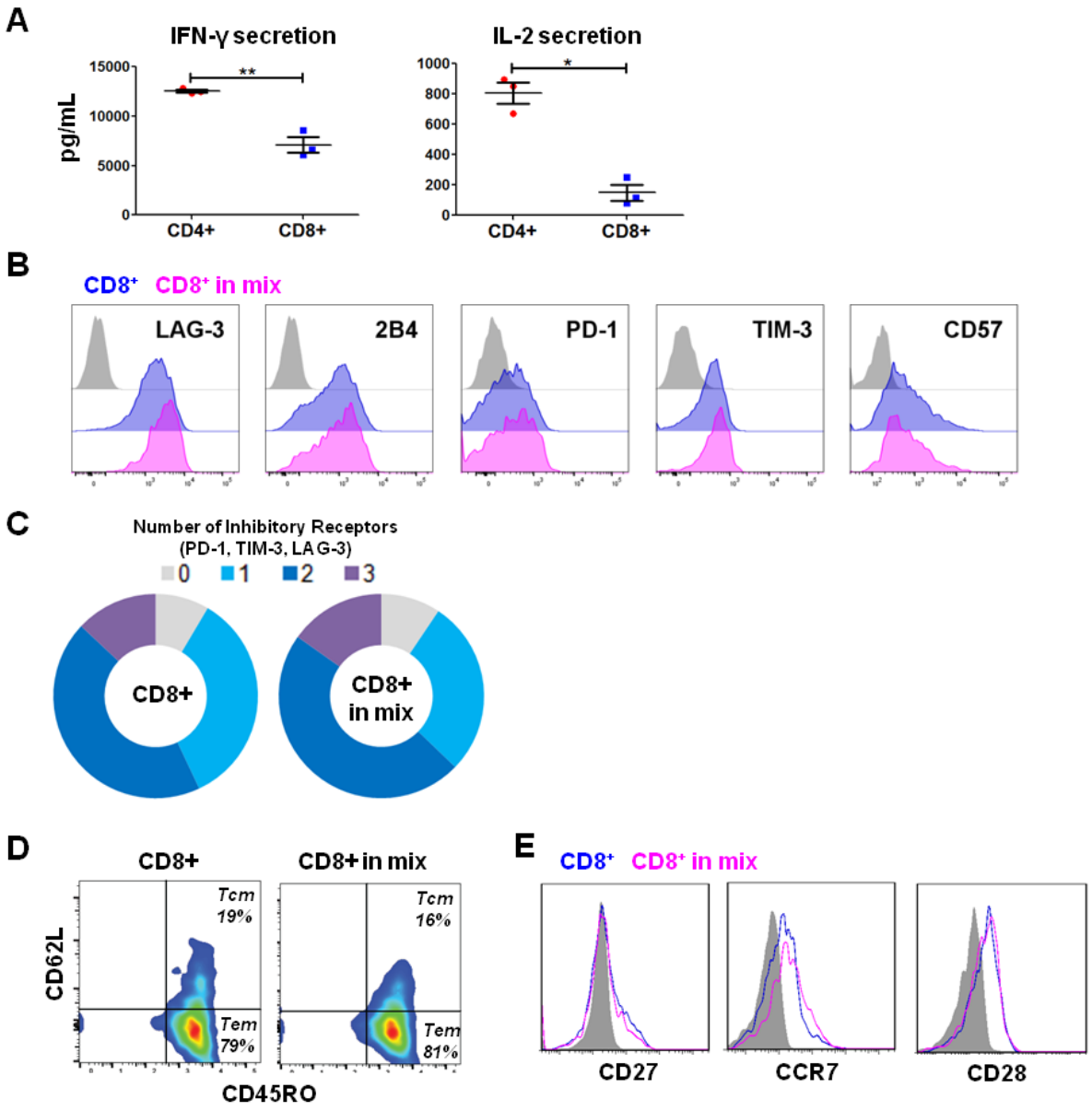
Supplemental Figure 4. Characterization of stimulated CD4$^+$ and CD8$^+$ CAR T cells. (A-B) CD4$^+$ and CD8$^+$ CAR T cells were pre-stimulated with two rounds of PBT030-2 GBM cells, then isolated from the co-culture and re-stimulated to test their activation potential. (A) Schematic of *in vitro* CAR T cell stimulation. (B) Flow cytometric analysis of degranulation was performed on CD19$^+$ gated T cells, and compared to that of CAR T cells without *in vitro* stimulation (none). **p<0.01 when compared to the non- *in vitro* stimulated cells using an unpaired Student's t-test; error bars: ±SEM. (C) T cells (CD4$^+$, CD8$^+$ or a 1:1 mix) underwent *in vivo* stimulation as shown in Figure 2E, and enumerated after harvesting from the tumors. *p<0.05 using an unpaired Student's t-test. Data are representative of CAR T cells from three different donors.



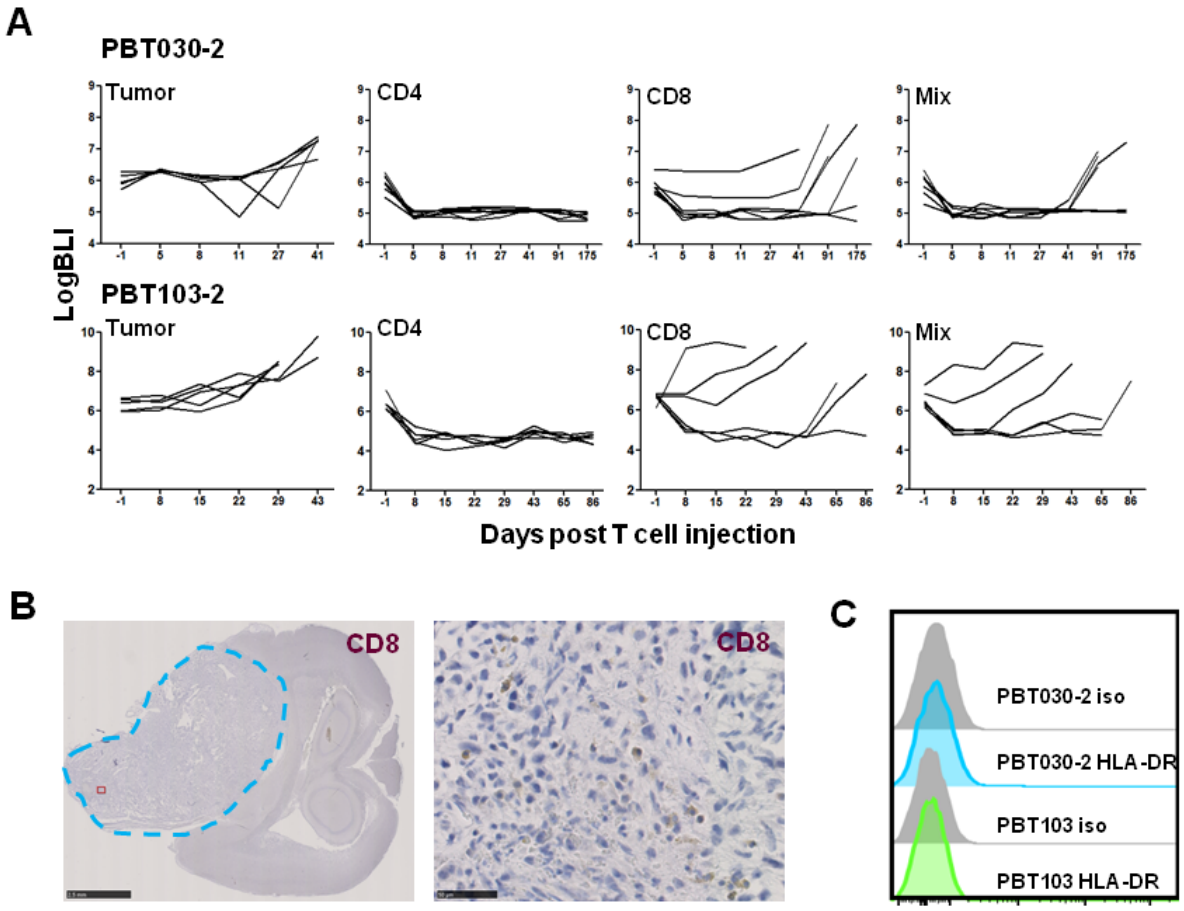
Supplemental Figure 5. CAR T cell exhaustion over extended culture or *in vitro* stimulation. (A) Representative flow cytometric histograms of inhibitory receptor expression (grey peaks: isotype control) on gated $CD4^+$ and $CD8^+$ subsets after extended culture. (B) Representative staining for CD4/CD8 composition of CAR T cells with/without extended culture. (C) CD45RO and CD62L double stain to reveal the memory composition of gated $CD4^+$ and $CD8^+$ subsets after extended culture.(D) Quantification of relative mean fluorescence intensity (MFI, Log scale; with isotype MFI subtracted out) and (E) representative histograms of inhibitory receptor staining on isolated $CD4^+$ and $CD8^+$ CAR T cells after *in vitro* stimulation as depicted in Supplemental Figure 4A (no re-stimulation). *$p<0.05$, and **$p<0.01$ when comparing $CD4^+$ and $CD8^+$ groups using an unpaired Student's t-test; error bars: ±SEM. (F) Graphic representation of the co-expression of PD-1, TIM-3 and LAG-3 on *in vitro* stimulated CAR T cells.



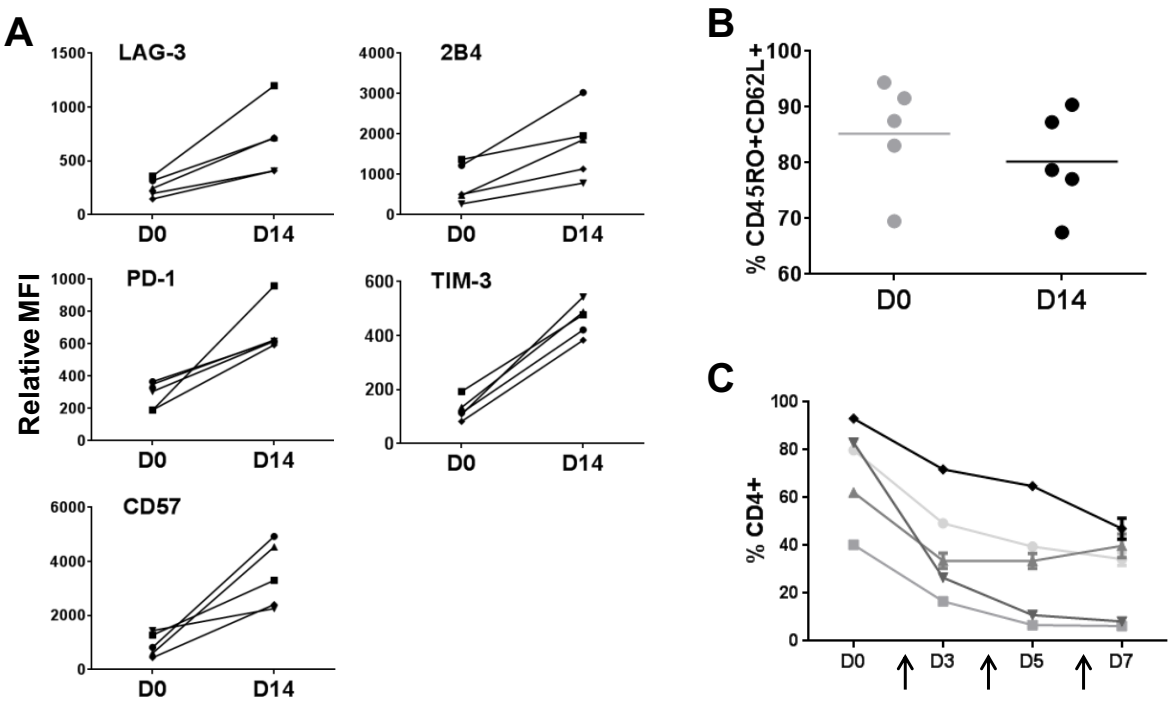
Supplemental Figure 6. Differential transcriptional signature between in vivo stimulated CD4$^+$ and CD8$^+$ cells. (A) RNAseq analysis of exhaustion-contributing genes was performed on CD4$^+$ and CD8$^+$ subsets of unstimulated/stimulated CAR T cells (gene list is identical to Figure 4A but with different orders). (B) CD45RO and CD62L double stain to reveal the memory composition of isolated CD4$^+$ and CD8$^+$ CAR T cells after *in vivo* stimulation. (C) Main networks formed by a total of 407 genes that are the most enriched in stimulated CD4$^+$ and CD8$^+$ cells (204 and 203 respectively in comparison with each other), analyzed by Ingenuity Pathway Analysis. (D) Ingenuity Pathway Analysis of the network with the highest score shown in (C). Gene symbols in red: enriched in CD4$^+$; gene symbols in blue: enriched in CD8$^+$; gene symbols in white: genes within the network but not in the analyzed gene list



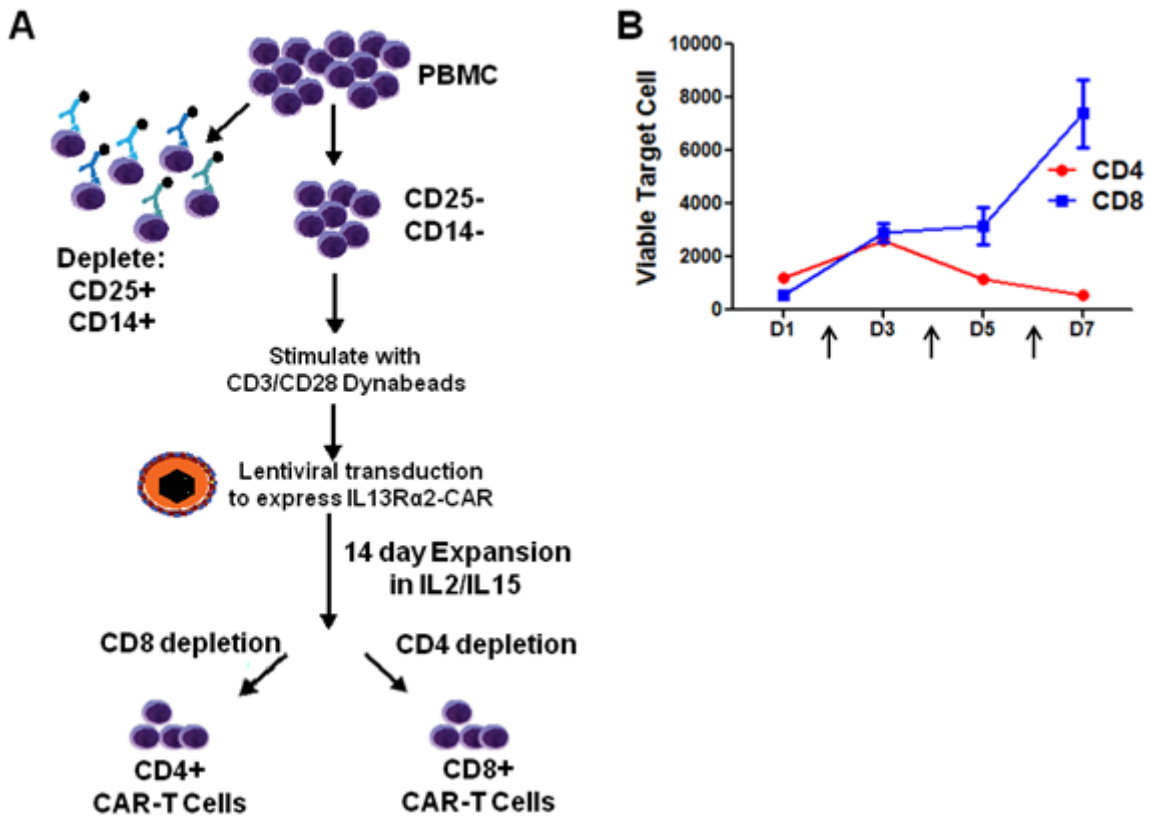
Supplemental Figure 7. Stimulation induced differentiation of CD8$^+$ CAR T cells is not ameliorated by inclusion of CD4$^+$ CAR T cells. (A) Cytokine secretion from CD4$^+$ and CD8$^+$ CAR T cells after co-culturing with PBT030-2 cells (E:T=1:2) for 24 hours. *p<0.05, and **p<0.01 when comparing CD4$^+$ and CD8$^+$ groups using an unpaired Student's t-test; error bars: ±SEM. (B-D) CD8$^+$ CAR T cells were *in vitro* stimulated as shown in Supplemental Figure 4A (no re-stimulation) in the presence/absence of CD4$^+$ CAR T cells. (B) Representative histograms of inhibitory receptor staining on CD8$^+$ CAR T cells post stimulation. (C) Graphic representation of the co-expression of PD-1, TIM-3 and LAG-3 on CD8$^+$ cells after 24 hr stimulation in the presence/absence of co-applied CD4$^+$ cells. (D-E) Flow cytometric staining of CD62L/CD45RO (D) and other T cell memory associated surface proteins (E) on CD8$^+$-gated cells after 24 hr stimulation in the presence/absence of co-applied CD4$^+$ cells.



Supplemental Figure 8. Superior *in vivo* efficacy of CD4$^+$ CAR T cells. (A) ffLuc$^+$ PBT030-2 (top) or PBT103-2(bottom) orthotopic GBM tumors were treated with 0.5x10^6 CD4$^+$ CAR T cells, 0.5x10^6 CD8$^+$ CAR T cells, or a combination of 0.25x10^6 CD4$^+$ and 0.25x10^6 CD8$^+$ CAR T cells. Biophotonic intensity measurements (BLI, Log scale) were plotted of individual tumor-bearing mice before T cell administration and during treatment time. (B) IHC staining for CD8$^+$ cells in the relapsed tumor (after treating with the 1:1 CD4/CD8 mixed CAR T cells). (C) Flow cytometric analysis of HLA-DR expression on the GBM cells used to generate orthotopic tumors.



Supplemental Figure 9. Analysis of CAR T cells derived from GBM patients. CAR T cell therapeutic products engineered from GBM patients (n=5) were either freshly thawed (D0) or underwent extended *in vitro* culture (D14) post transduction and analyzed by flow cytometry for relative MFI of inhibitory receptors (A) as well as the percentage of $CD62L^{+}CD45RO^{+}$ Tcm cells (B). Horizontal lines in (B) indicate mean value. (C) Each of the 5 GBM patient CAR T cell products underwent repetitive tumor challenge (arrows: tumor rechallenge), and were analyzed for the percentage of $CD4^{+}$ cells at the denoted time points.



Supplemental Figure 10. Antitumor efficacy of $CD4^+$ and $CD8^+$ PBMC-derived CAR T cells. (A) Schematic description of IL13Ra2-targeted CAR T cell production from PBMCs (with myeloid and Treg cells depleted). (B) Effector function of $CD4^+$ and $CD8^+$ CAR T cells against repetitively challenged PBT030-2 GBM cells (arrows: tumor rechallenge)

Supplemental Methods

Real-time imaging

For real-time tracing of *in vitro* CAR T cell killing, PBT and CAR T cells were co-cultured and visualized for 72h continuously via an Observer Z1 Live Cell (Zeiss) that contains an incubator with constant 37° C and 5% CO_2. Images were processed and movies generated with Zen (Zeiss).

Detection of cytokine secretion

To measure the quantity of secreted cytokines, T cells were co-cultured with PBT cells (10,000 CAR^+ T cells, 20,000 tumor cells) for 24h. Supernatants were then collected and cytokines were quantified using LEGEND MAX™ Human IL-2 or Human IFN-γ ELISA Kits (BioLegend).

Supplemental Table1. Expression of exhaustion-associated genes shared by $CD4^+$ and $CD8^+$ subsets, analyzed in CAR T cells after *in vivo* stimulation

Supplemental Table2. Expression of mostly enriched genes in $CD4^+$ compared to $CD8^+$ CAR T cells after *in vivo* stimulation

Supplemental Table3. Expression of mostly enriched genes in $CD4^+$ and $CD8^+$ CAR T cells (compared with each other) after *in vivo* stimulation

Supplemental Table4. Primers used in qRT-PCR assays

Supplemental Table5. Antibody information

Supplemental Video1. 72h live imaging (day0-day3) of $CD4^+$ CAR T cells co-cultured with PBT030-2 GBM cells

Supplemental Video2. 72h live imaging (day0-day3) of $CD8^+$ CAR T cells co-cultured with PBT030-2 GBM cells

Supplemental Video3. 72h live imaging (day4-day7) of $CD4^+$ CAR T cells co-cultured with PBT030-2 GBM cells

Supplemental Video4. 72h live imaging (day4-day7) of $CD8^+$ CAR T cells co-cultured with PBT030-2 GBM cells