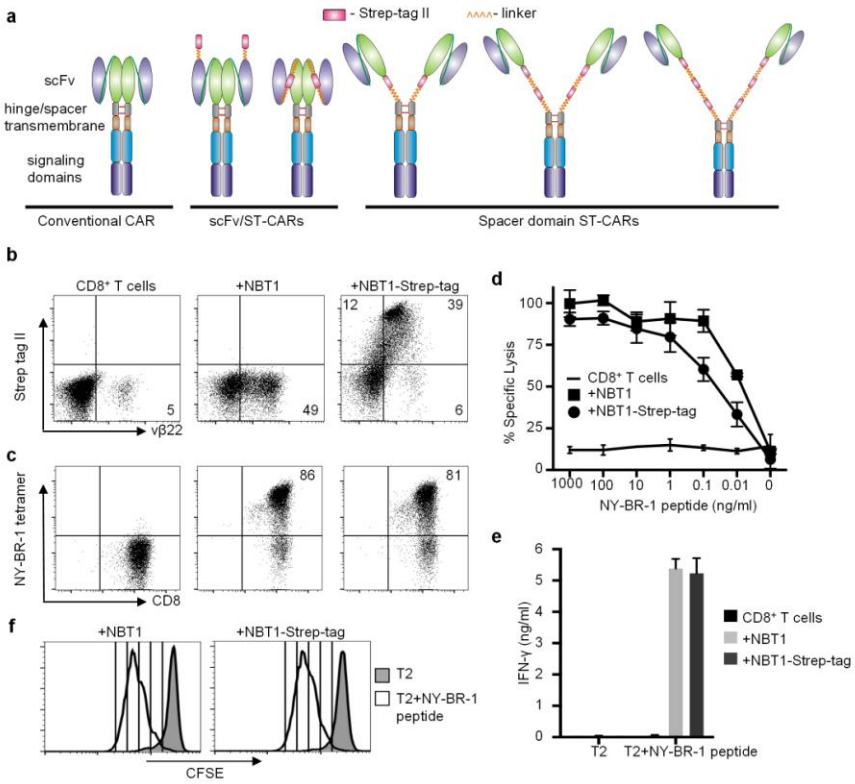


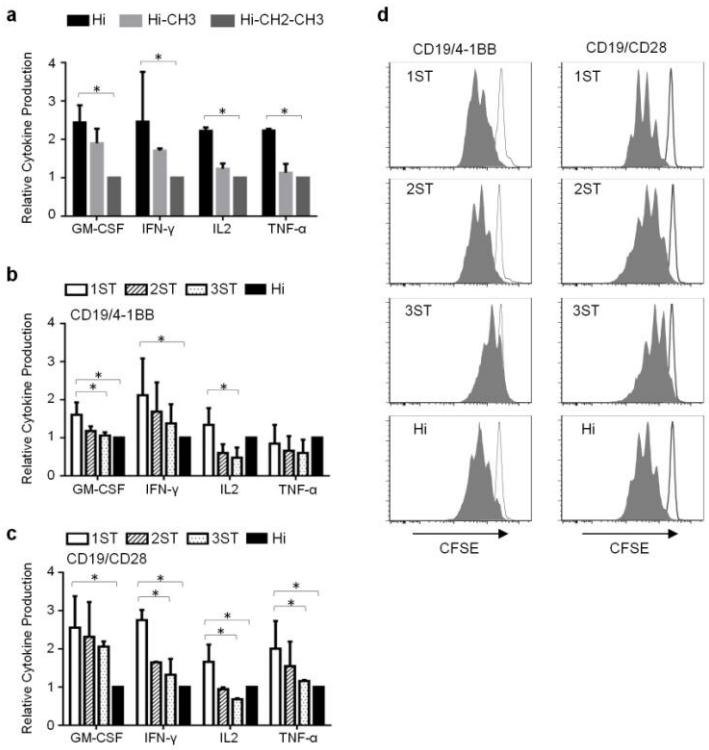
Supplementary Figure 1

Design of functional *Strep*-tag II chimeric antigen and T-cell receptors.

(a) Schematic of conventional and Strep-tag CAR designs. The conventional CD19 CAR encodes the antigen-specific single chain variable fragment, an IgG4 hinge spacer and CD28 transmembrane domain, and an effector domain containing a signaling module comprised of 4-1BB or CD28 fused to CD3ζ. Strep-tag CARs were designed either with a Strep-tag II sequence at the N-terminus of the scFv (ST-scFv), between the VL and VH chains (VL-ST-VH), or with one or more Strep-tag II sequences (scFv-ST) between the scFv and the hinge. Gly/Ser linker sequences were inserted on one or both sides of the Strep-tag II and are indicated by the zigzag line.

(b,c) Incorporating Strep-tag II into a TCR does not impair surface expression or tetramer binding. CD8+ T cells were transduced with either an unmodified NY-BR-1-specific TCR (NBT1) or a Strep-tag II version of NBT1 (NBT1-Strep-tag), and analyzed for surface expression of the TCR Vβ22 chain of NBT1 and Strep-tag II. (c) Transduced T cells were enriched for Vβ22+ cells, expanded, and analyzed for NY-BR-1-multimer binding. Data are representative of two independent experiments.

(d,e,f) Strep-tag II TCRs are functional. T cells transduced with the wild-type NBT1 TCR and with the NBT1 strep-tag TCR were tested for cytotoxicity (d), cytokine production (e), and proliferation against T2 cells alone or loaded with NY-BR-1 peptide. Untransduced CD8+ T cells were used as a control for cytotoxicity and cytokine assays. The dose of NY-BR-1 peptide in (e) and (f) is 0.1 ng/ml. Data are representative of three independent experiments and error bars represent mean ± SD.



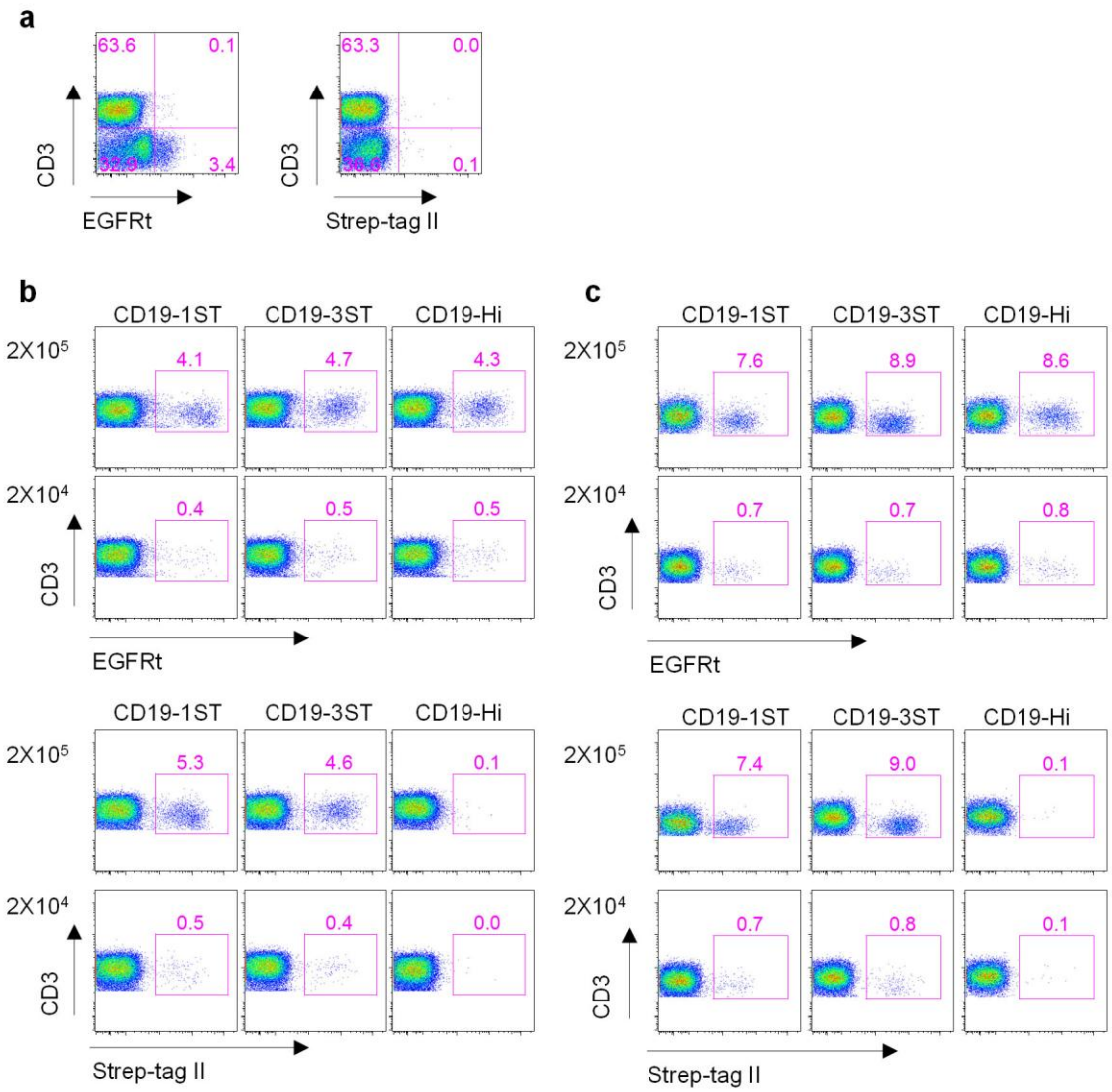
Supplementary Figure 2

Altering spacer length with *Strep*-tag II can optimize CAR function.

(**a**) Comparison of cytokine production by $CD8^+$ T cells expressing CD19 4-1BB/CD3ζ CARs with various IgG4 Fc spacer lengths (Hinge only: Hi; Hinge-CH3: Hi-CH3; Hinge-CH2-CH3: Hi-CH2-CH3). CAR-T cells were co-cultured with $CD19^+$ Raji cells (2:1 ratio) for 24h and supernatants were analyzed using the Luminex Multiplex platform. The data are derived from three independent experiments using T cells from different donors. Data are expressed as means ± SD and normalized such that the mean cytokine release by T cells expressing the CD19-CAR Hi-CH2-CH3 spacer is designated as 1. Statistical analysis was performed using the Student's t test. * $P<0.05$

(**b, c**) Cytokine production by $CD8^+$ T cells expressing CD19 4-1BB/CD3ζ (b) or CD28/CD3ζ CARs (c) encoding 1, 2, or 3 Strep-tag II sequences in the spacer domain compared to T cells expressing the identical CD19 CARs with an IgG4 hinge (Hi) only spacer domain. The assays were performed as described in (a). Data from three independent experiments are expressed as mean ± SD normalized to the cytokine release by T cells expressing the CD19-Hi CAR. Statistical analysis was performed using the Student's t test. * $P<0.05$

(**d**) Proliferation of T cells expressing CD19Hi-4-1BB/CD3ζ, CD19 Hi-CD28/CD3ζ or CD19 Strep-tag CARs with 4-1BB/CD3ζ and CD28/CD3ζ intracellular signaling domains. T cells were labeled with CFSE, stimulated with $CD19^+$ Raji tumor cells (solid grey) or medium only (white), and analyzed for CFSE dilution 5 days after stimulation. Data are representative of three independent experiments



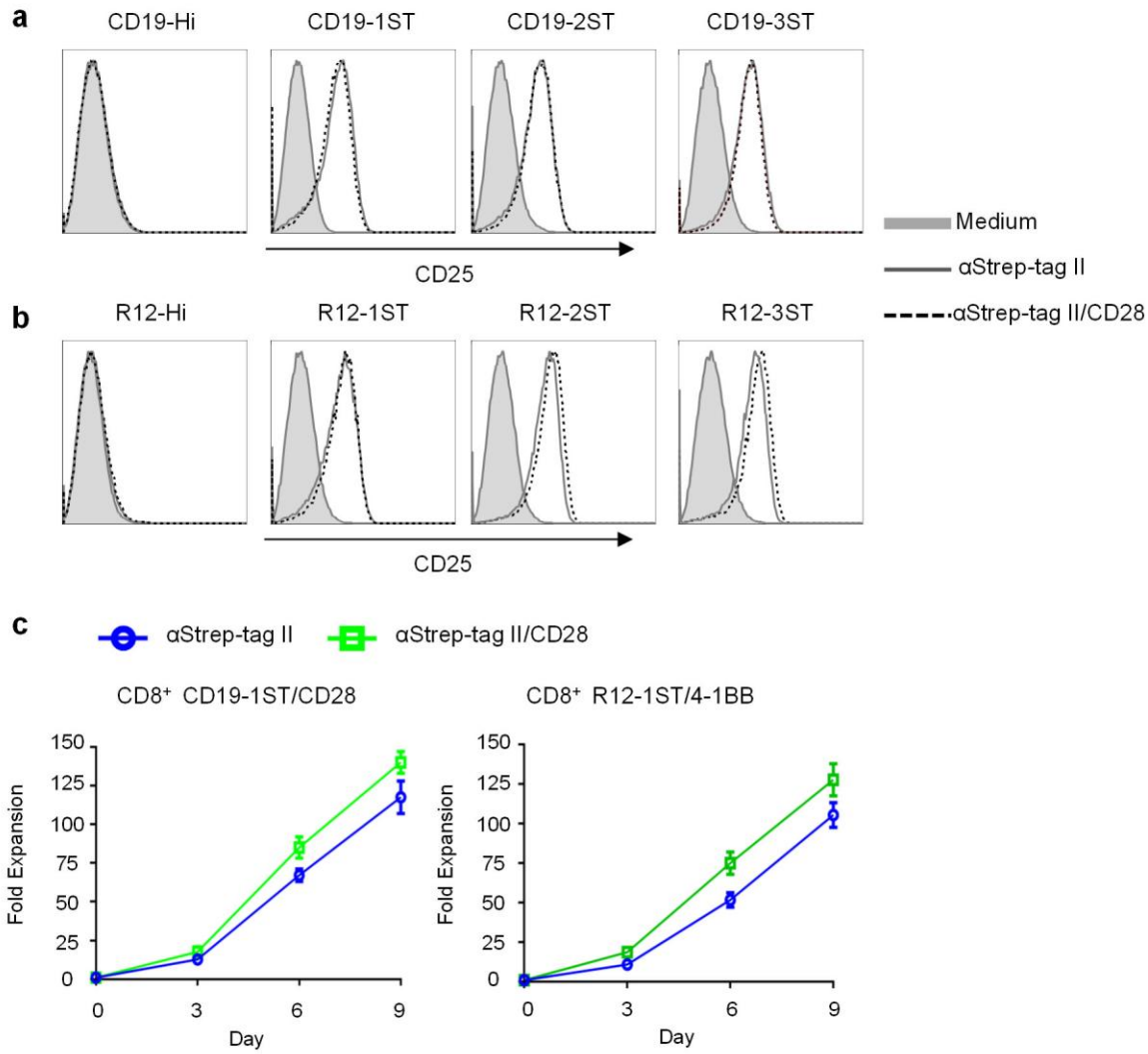
Supplementary Figure 3

Detection of *Strep*-tag CAR-T cells in human blood.

(**a**) Anti-Strep-tag II mAb and anti-EGFR staining of human peripheral blood mononuclear cells. Mononuclear cells isolated from human blood by Ficoll gradient centrifugation were stained with anti-CD3, anti-EGFR, anti-Strep-tag II mAbs and analyzed by flow cytometry. The data shows staining of $CD3^+$ and $CD3^-$ populations with the respective mAbs.

(**b**) 2×10^5 or 2×10^4 CD19 CAR-T cells containing Hi only, 1ST and 3ST spacers were spiked into $2x10^6$ PBMCs, which were then stained with anti-CD3 and anti- EGFR (top panels) or anti-Strep-tag II (bottom panels) mAbs and analyzed by flow cytometry.

(**c**) 2×10^5 or 2×10^4 CD19 CAR-T cells containing Hi only, 1ST and 3ST spacers were spiked into 200 μl human blood. The blood was then lysed using red cell lysis buffer and the cells were stained and analyzed as described in (b).



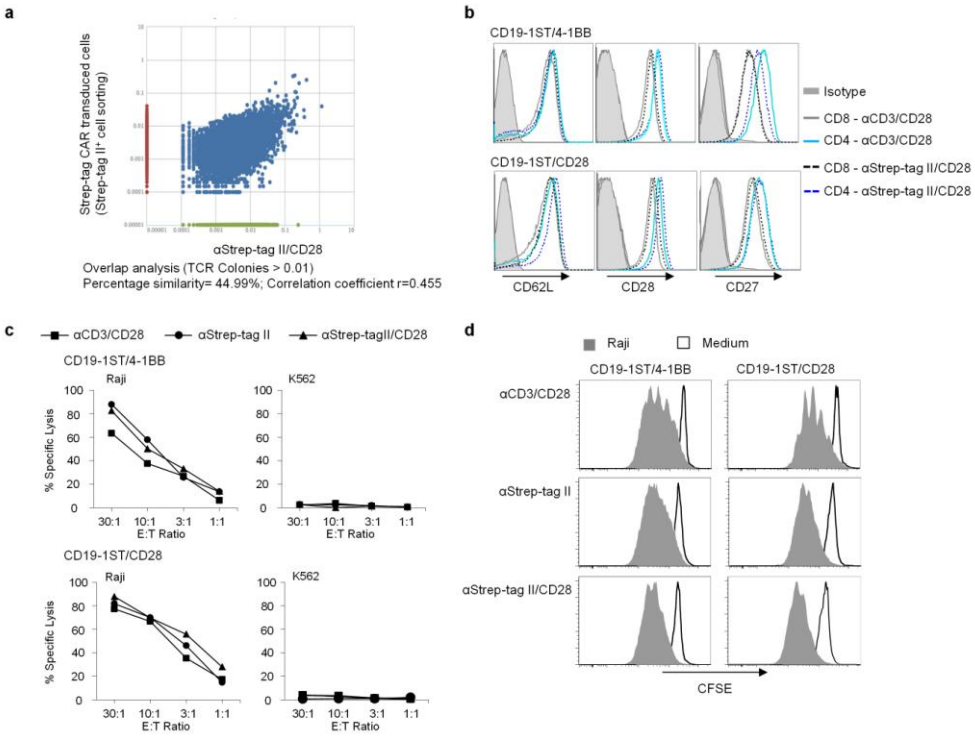
Supplementary Figure 4

Anti-*Strep* tag II mAb-coated microbeads selectively activate and expand *Strep*-tag CAR-T cells independent of scFv specificity and co-stimulatory domain.

(**a**) T cells transduced with CD19-Hi, CD19-1ST, CD19-2ST, CD19-3ST CARs containing a CD28/CD3ζ signaling domain were sorted for EGFRt expression, stimulated with anti-Strep-tag II or anti-Strep-tag II/CD28 mAb coated microbeads or left unstimulated (medium) and analyzed for CD25 expression.

(**b**) T cells transduced with R12-Hi, R12-1ST, R12-2ST, R12-3ST CARs containing a 4-1BB/CD3ζ signaling domain were sorted for EGFRt expression, stimulated with anti-Strep-tag II or anti-Strep-tag II/CD28 mAb coated microbeads, or left unstimulated (medium) and analyzed for CD25 expression. The data in a,b are representative of three experiments.

(**c**) Growth curves of $CD8^+$ Strep-tag CAR-T cells. FACS sorted $EGFR^+$ CD19-1ST/CD28/CD3ζ and R12-1ST/4-1BB/CD3ζ CAR-T cells were cultured with anti-Strep-tag II or anti-Strep-tag II/CD28 mAb coated microbeads in CTL media containing IL2 (50 U/ml) and IL15 (2 ng/ml) for 9 days. Aliquots of T cells were removed from the cultures for counting on days 3, 6, and 9 and the fold-increase in cell number determined. The data show the mean fold expansion in three experiments with T cells from different donors



Supplementary Figure 5

Strep-tag CAR-T cells retain diverse TCR clonality, expression of homing/co-stimulatory molecules, and functionality after expansion with anti-*Strep* tag II mAb-coated microbeads.

(**a**) Analysis of TCR clonality of Strep-tag CAR-T cells prior to and after expansion with anti-Strep-tag II/CD28 microbeads. CD8+ T cells were transduced with CD19-3ST/4-1BB/CD3ζ CAR and then sort-purified with anti-Strep tag II mAb. An aliquot of the sort-purified T cells was expanded on anti-Strep-tag II/CD28 microbeads for 9 days. TCR Vβ sequencing was performed on the sort-purified Strep-tag CAR-T cells prior to and after expansion to assess clonality, and the frequency of individual clonotypes in each population is shown in the log scatter plot. An overlap analysis for TCR clonotypes at a frequency of >0.01% was performed and a Pearson product-moment correlation coefficient (Pearson's r) was calculated, which revealed a high level of similarity in TCR clonality before and after expansion.

(**b**) CD62L, CD28 and CD27 expression on CD19 CAR-T cells (1ST/4-1BB/CD3ζ or 1ST/CD28/CD3ζ) after expansion with anti-CD3/CD28 or anti-Strep-tag II/CD28 beads.

(**c**) Cytolytic activity of CD19 Strep-tag CAR-T cells expanded with anti-CD3/CD28, anti-Strep-tag II, or anti-Strep-tag II/CD28. Target cells included $CD19^+$ Raji lymphoma and K562 control cells.

(**d**) Proliferation of CFSE-labeled CD19 Strep-tag CAR-T cells re-stimulated with $CD19^+$ Raji tumor cells (solid grey) or medium only (black lines) after expansion on anti-CD3/CD28, anti-Strep-tag II or anti-Strep-tag II/CD28 beads. The CAR-T cells were analyzed for CFSE dye dilution 5 days after stimulation. Data are representative of three independent experiments.

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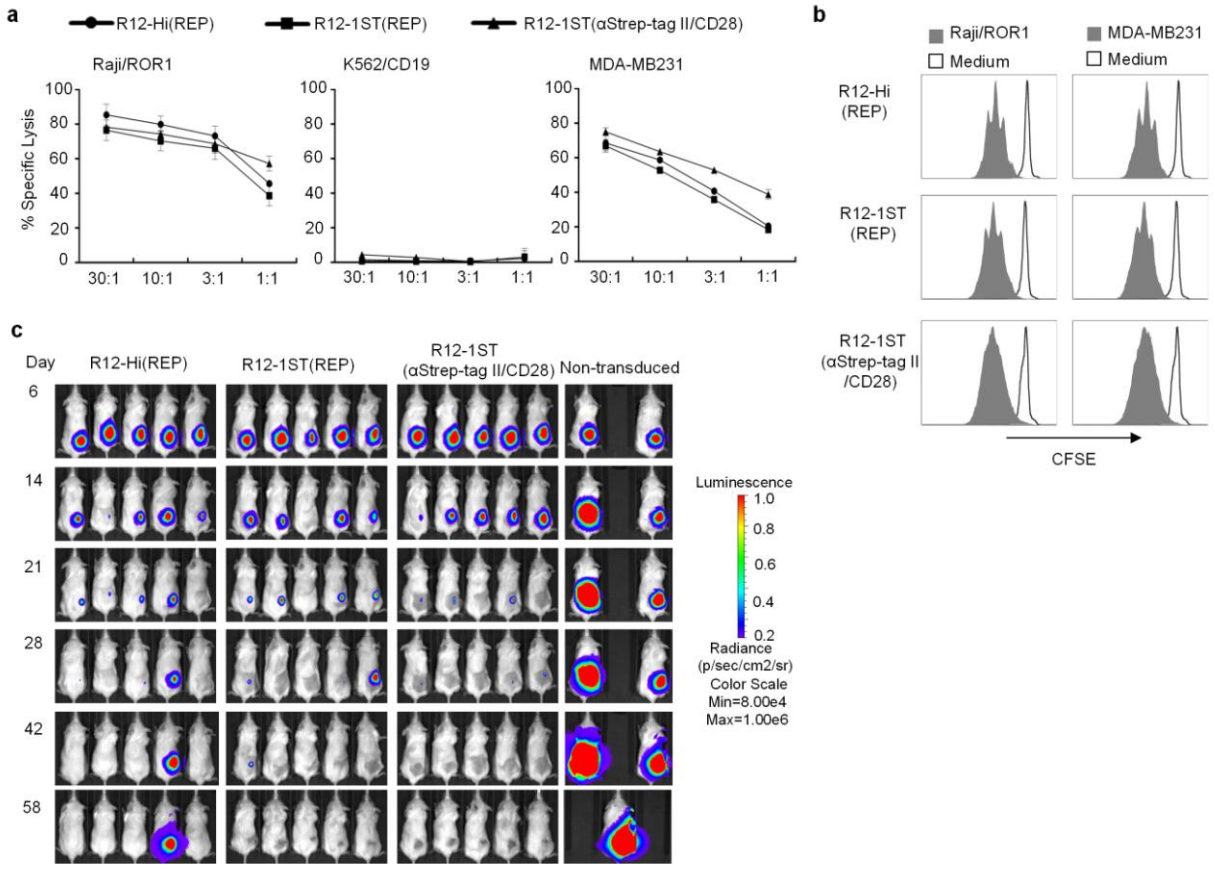


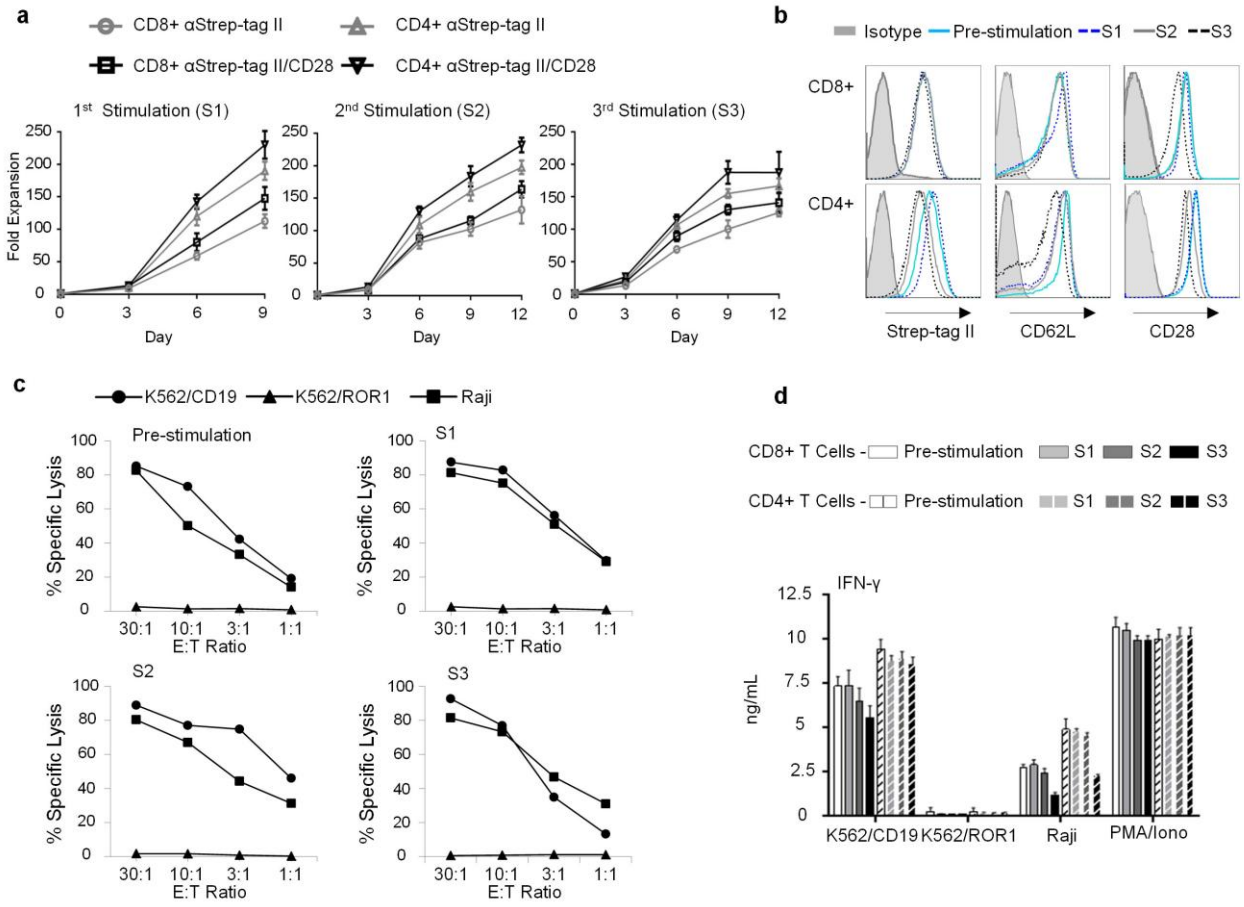
Supplementary Figure 6

Strep-tag II–modified ROR1 4-1BB/CD3ζ CAR-T cells function *in vitro* and *in vivo.*

(**a**) Cytolytic activity of $CD8^+$ R12 CAR-T cells (R12-Hi, 1ST spacer) after expansion using rapid expansion protocol (REP) or with anti-Strep-tag II/CD28 microbeads. T cells were tested for lysis of ROR1 transduced Raji lymphoma, $ROR1^+$ MDA-MB231 breast cancer and K562/CD19 cells at various effector/target (E/T) ratios.

(**b**) Proliferation of CFSE-labeled $CD8^+$ R12 CAR-T cells (R12-Hi, 1ST spacer) cultured with Raji/ROR1 or MDA-MB231 cells (solid grey) or medium only (black lines). CAR-T cells were expanded using a rapid expansion protocol (REP) or with anti-Strep-tag II/CD28 microbeads, labeled with CFSE, and analyzed for CFSE dilution 5 days after stimulation. Data are representative of two independent experiments

(**c**) Antitumor activity of R12-Hi and R12 Strep-tag CAR-T cells in NSG mice engrafted with MDA-MB231-ffluc cells. R12 $CD4^+$ and $CD8^+$ CAR-T cells were expanded separately with anti-CD3 and feeder cells (REP) or with anti-Strep-tag II/CD28 and then formulated in a CD8:CD4 ratio of 1:1 and administered at a cell dose of $10x10^6$ into NSG mice 7 days after the mice were inoculated subcutaneously with $5x10^5$ $ROR1^+$ MDA-MB231-ffluc tumor cells. Tumor progression and distribution were evaluated by serial bioluminescence imaging.



Supplementary Figure 7

Strep-tag CAR-T cells retain CAR expression and functionality after repetitive expansion with anti-*Strep*-tag II mAb-coated microbeads.

(**a**) Growth of $CD8^+$ and $CD4^+$ CD19-1ST/4-1BB/CD3ζ CAR T cells after 1 (S1), 2 (S2), and 3 (S3) cycles of stimulation with anti-Strep-tag II or anti-Strep-tag II/CD28 microbeads.

(**b**) Surface expression of the CAR, CD62L and CD28 after sequential expansion with anti-Strep-tag II/CD28 mAb beads. T cells were stained with anti-Strep-tag II, anti-CD62L, and anti-CD28 mAb and analyzed by flow cytometry.

(**c**) Cytolytic activity of $CD8^+$ CD19-1ST/4-1BB/CD3ζ CAR T cells after 1, 2, and 3 cycles of stimulation with anti-Strep-tag II/CD28 microbeads. T cells were tested for lysis of $CD19^+$ Raji and K562/CD19 or K562/ROR1 at various effector/target (E/T) ratios. The data in a-c is representative of three experiments with T cells from different donors.

(**d**) IFN-γ production by CD19-1ST/4-1BB/CD3ζ CAR T cells after 1, 2, and 3 cycles of expansion with anti-Strep-tag II/CD28 microbeads in response to K562/CD19, K562/ROR1 and Raji cells. PMA/Ionomycin stimulated CAR-T cells were used as positive control. Data are presented as mean of two independent experiments ± SD.