Supplementary Data for

Modular chimaeric cytokine receptors with leucine zipper enhance the antitumor activity of CAR T cells via JAK/STAT signalling

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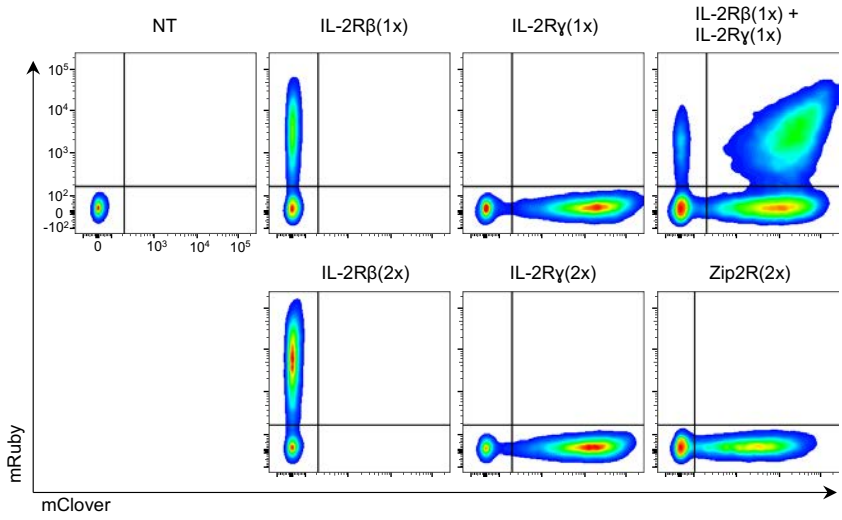
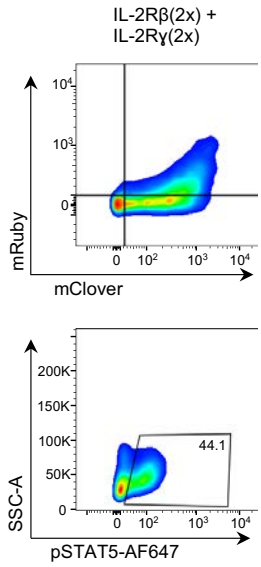
a


NT
IL-2Rβ(1x)
IL-2Rγ(1x)
IL-2Rβ(1x) +
IL-2Rγ(1x)
IL-2Rβ(2x)
IL-2Rγ(2x)
Zip2R(2x)
mRuby
mClover

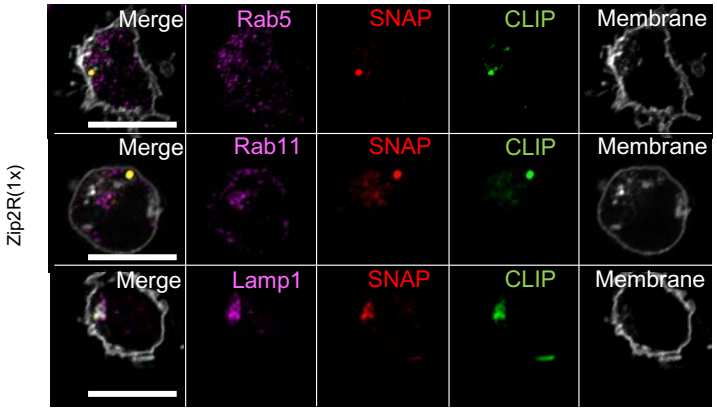
b


IL-2Rβ(2x) +
IL-2Rγ(2x)
mRuby
mClover
SSC-A
pSTAT5-AF647
44.1

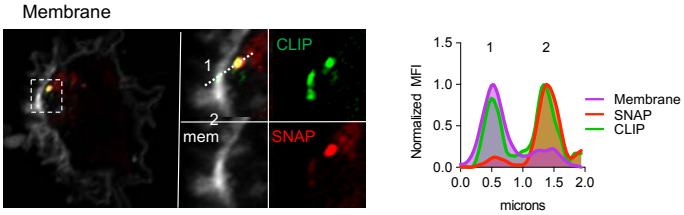
19
20

Supplementary Data Figure 1: Representative flow cytometry plots.
(**a**) Representative flow cytometry plots for primary T cell transduction (corresponding to Figure 1c). (**b**) Transduction and pSTAT5 expression with double transduction of IL-2Rβ(2x) and IL-2Rɣ(2x).

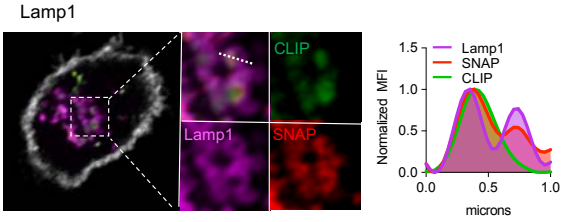
a


Merge
Rab5
SNAP
CLIP
Membrane
Merge
Rab11
SNAP
CLIP
Membrane
Merge
Lamp1
SNAP
CLIP
Membrane
Zip2R(1x)

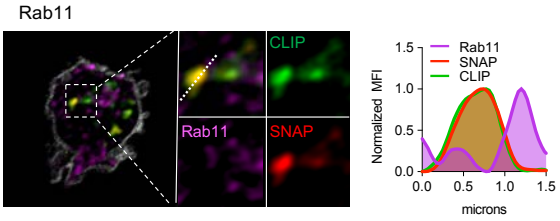
b


Membrane
CLIP
1
2
mem
SNAP
Normalized MFI
microns
Membrane
SNAP
CLIP

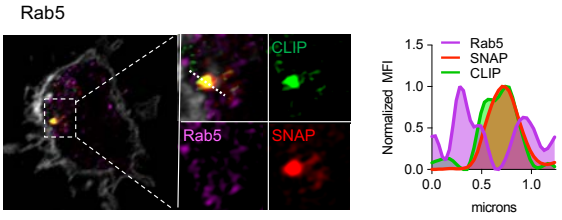
c


Lamp1
CLIP
Lamp1
SNAP
Normalized MFI
microns
Lamp1
SNAP
CLIP

d

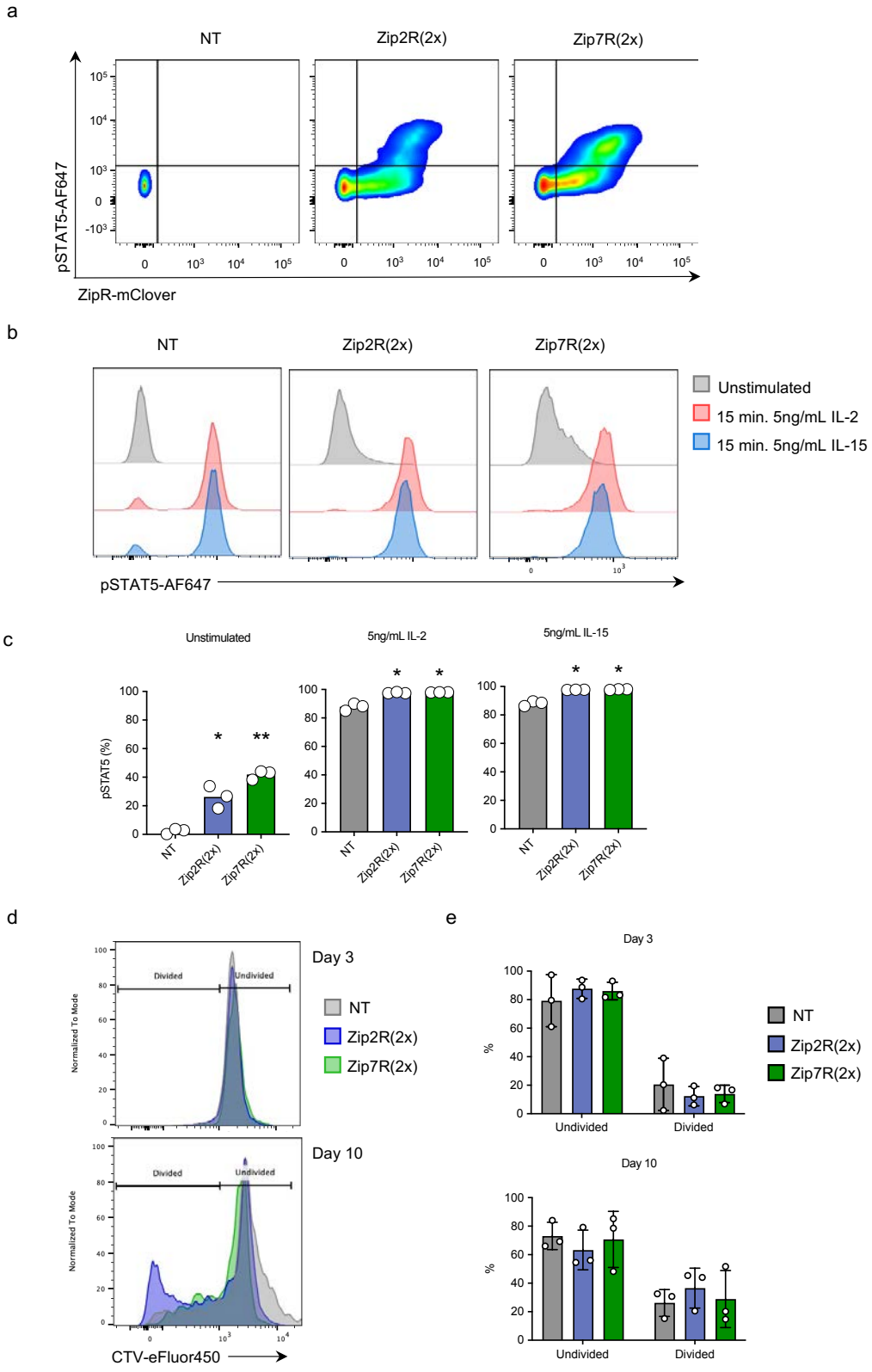

Rab11
CLIP
Rab11
SNAP
Normalized MFI
microns
Rab11
SNAP
CLIP

e


Rab5
CLIP
Rab5
SNAP
Normalized MFI
microns
Rab5
SNAP
CLIP

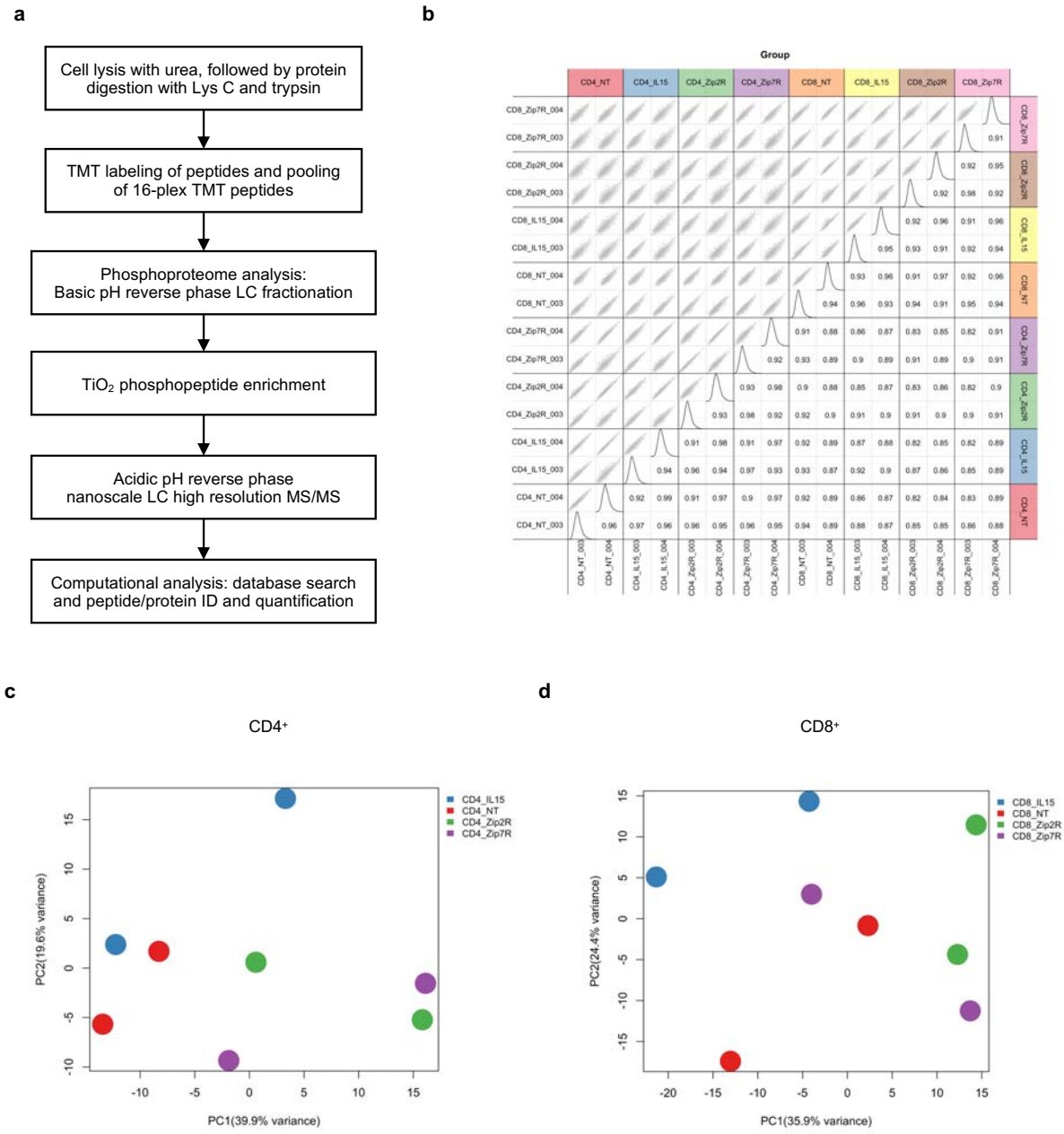
25
26

Supplementary Data Figure 2: SNAP/CLIP tag confocal microscopy of Zip2R(1x).
(**a**) Representative images of Zip2R(1x) with endosomal markers. Scale bar = 10μm. (**b-e**) Representative images of Zip2R(1x) colocalization with (**b**) cell membrane, (**c**) Lamp1, (**d**) Rab11, or (**e**) Rab5.


a
NT
Zip2R(2x)
Zip7R(2x)
pSTAT5-AF647
ZipR-mClover
b
NT
Zip2R(2x)
Zip7R(2x)
Unstimulated
15 min. 5ng/mL IL-2
15 min. 5ng/mL IL-15
pSTAT5-AF647
c
Unstimulated
5ng/mL IL-2
5ng/mL IL-15
pSTAT5 (%)
NT
Zip2R(2x)
Zip7R(2x)
d
Day 3
Day 10
Divided
Undivided
Normalized To Mode
CTV-eFluor450
NT
Zip2R(2x)
Zip7R(2x)
e
Day 3
Day 10
%
Undivided
Divided
NT
Zip2R(2x)
Zip7R(2x)

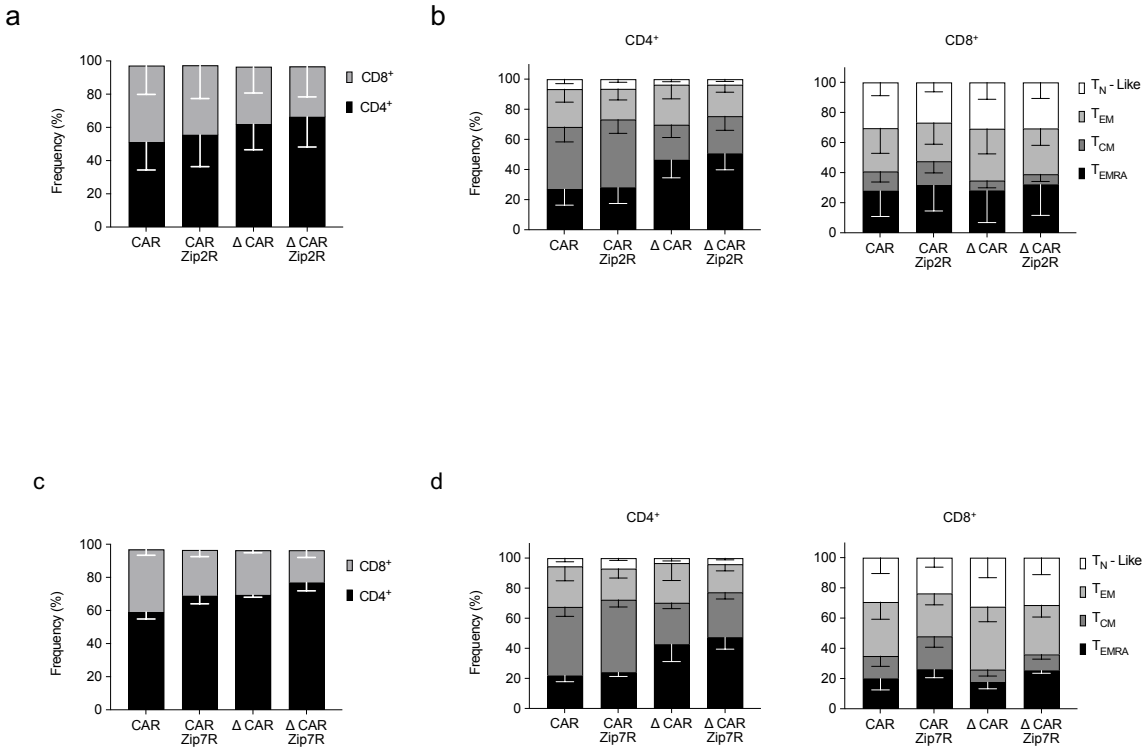
31
32

Supplementary Data Figure 3: STAT5 induction and cell proliferation of ZipRs
(**a**) pSTAT5 expression in NT, Zip2R(2x), and Zip7R(2x) T cells. (**b**) Representative histograms of unstimulated, IL-2, or IL-15 stimulated samples. (**c**) Quantification of pSTAT5 expression in unstimulated, IL-2, or IL-15 stimulated samples (N=3 biological replicates, *p<0.05, **p<0.01, one-way ANOVA with Tukey's multiple comparisons test). (**d**) Representative histograms of cell proliferation dye (CTV-eFluor 450) labelled NT, Zip2R(2x), or Zip7R(2x) T cells at day 3 or 10. (**e**) Quantification of divided and undivided cells at day 3 and day 10 (N=3 biological replicates).


a
Cell lysis with urea, followed by protein digestion with Lys C and trypsin
TMT labeling of peptides and pooling of 16-plex TMT peptides
Phosphoproteome analysis: Basic pH reverse phase LC fractionation
TiO2 phosphopeptide enrichment
Acidic pH reverse phase nanoscale LC high resolution MS/MS
Computational analysis: database search and peptide/protein ID and quantification
b
Group
CD4_NT
CD4_IL15
CD4_Zip2R
CD4_Zip7R
CD8_NT
CD8_IL15
CD8_Zip2R
CD8_Zip7R
c
CD4+
CD4_IL15
CD4_NT
CD4_Zip2R
CD4_Zip7R
PC2(19.6% variance)
PC1(39.9% variance)
d
CD8+
CD8_IL15
CD8_NT
CD8_Zip2R
CD8_Zip7R
PC2(24.4% variance)
PC1(35.9% variance)

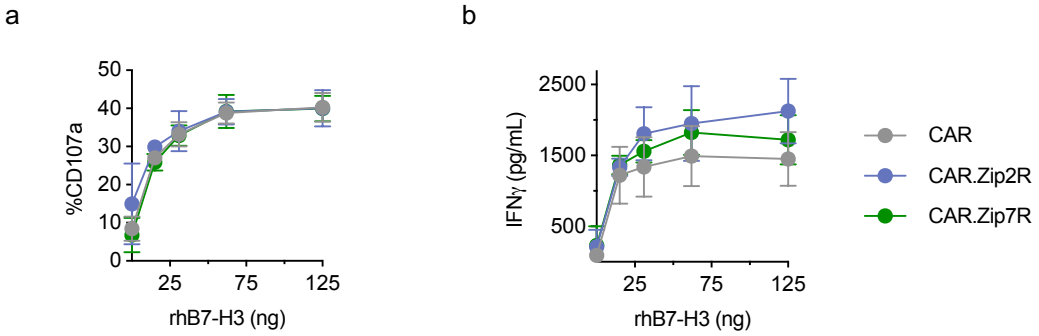
Supplementary Data Figure 4: Phosphoproteomics experimental workflow and sample quality.

(**a**) Phosphoproteomics sample preparation workflow. (**b**) Dataset characteristics. (**c**) Pearson correlation analysis of biological replicates. (**d,e**) Principal component analysis of top 700 DE proteins of $CD4^+$ (**d**) and $CD8^+$ (**e**) T cells.



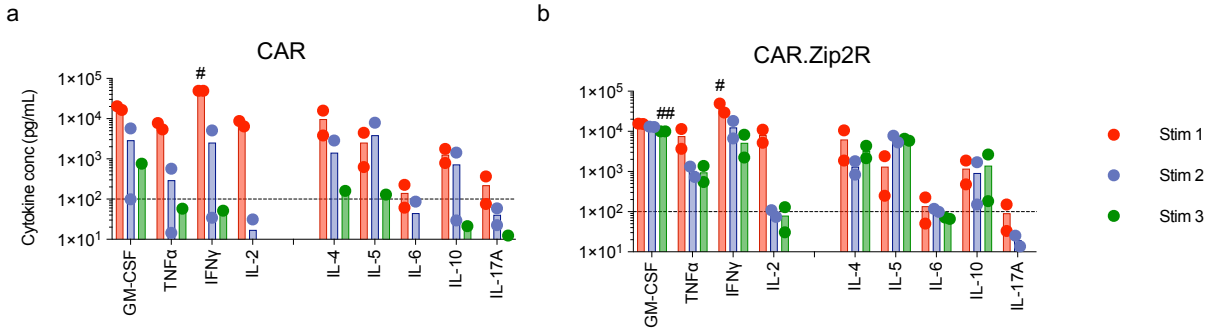
Supplementary Data Figure 5: Phenotypic analysis of CAR.ZipR T cells.

(**a**) CD4:CD8 ratio of CAR.Zip2R T cells with indicated constructs (N=3-4, mean ± SD). (**b**) Immunophenotype of $CD4^+$ (left) or $CD8^+$ (right) CAR.Zip2R T cells with indicated constructs (T_N-Like : $CCR7^+$ $CD45RA^+$, T_{EM} : $CCR7^-$ $CD45RA^-$, T_{CM} : $CCR7^+$ $CD45RA^-$, T_{EMRA} : $CCR7^-$ $CD45RA^+$, N=3-4, mean±SD). (**c**) CD4:CD8 ratio of CAR.Zip7R T cells with indicated constructs (N=3-4, mean ± SD). (**d**) Immunophenotype of $CD4^+$ (left) or $CD8^+$ (right) CAR.Zip7R T cells with indicated constructs (N=3-4, mean±SD).



Supplementary Data Figure 6: Antigen sensitivity of CAR.ZipR T cells

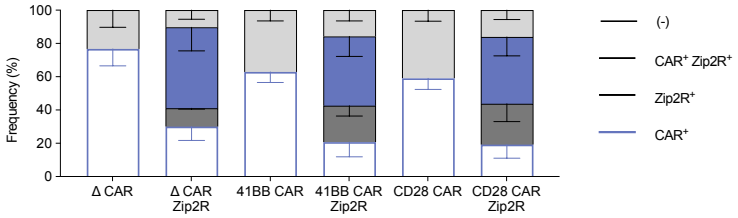
(**a**) Percentage of CD107a positive cells after 4-hour stimulation with indicated concentration of plate-bound recombinant human B7-H3 (rhB7-H3) (N=3 biological replicates, mean ± SD). (**b**) Concentration of IFNγ in cell culture supernatant after 24-hour stimulation with rhB7-H3 (N=3 biological replicates, mean ± SD).



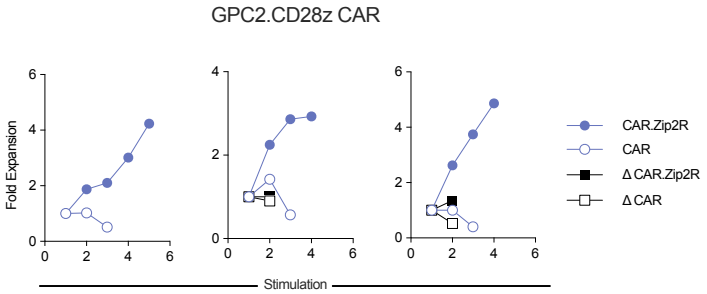
Supplementary Data Figure 7: Repetitive cytokine production of B7-H3-CAR and B7-H3-CAR.Zip2R T cells

Quantification of (**a**) B7-H3-CAR or (**b**) B7-H3-CAR.Zip2R T cell cytokine production following 1, 2, or 3 stimulations with A549 WT cells (N=2 biological replicates). One (#) or two (##) cytokine concentrations were greater than the limit of detection (LOD) and the LOD was plotted (IFNγ: 50,000 pg/mL; GM-CSF: 10,000 pg/mL).

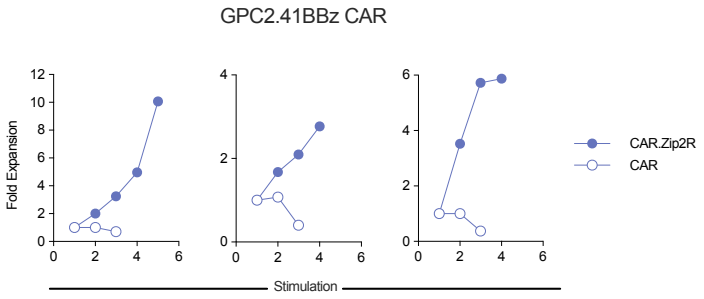
a


Frequency (%)
100
80
60
40
20
0
Δ CAR
Δ CAR Zip2R
41BB CAR
41BB CAR Zip2R
CD28 CAR
CD28 CAR Zip2R
(-)
CAR+ Zip2R+
Zip2R+
CAR+

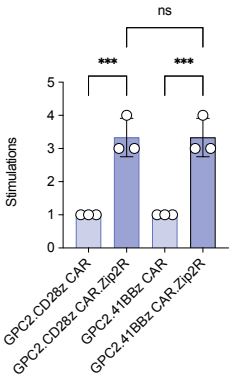
b


GPC2.CD28z CAR
Fold Expansion
Stimulation
CAR.Zip2R
CAR
Δ CAR.Zip2R
Δ CAR

c

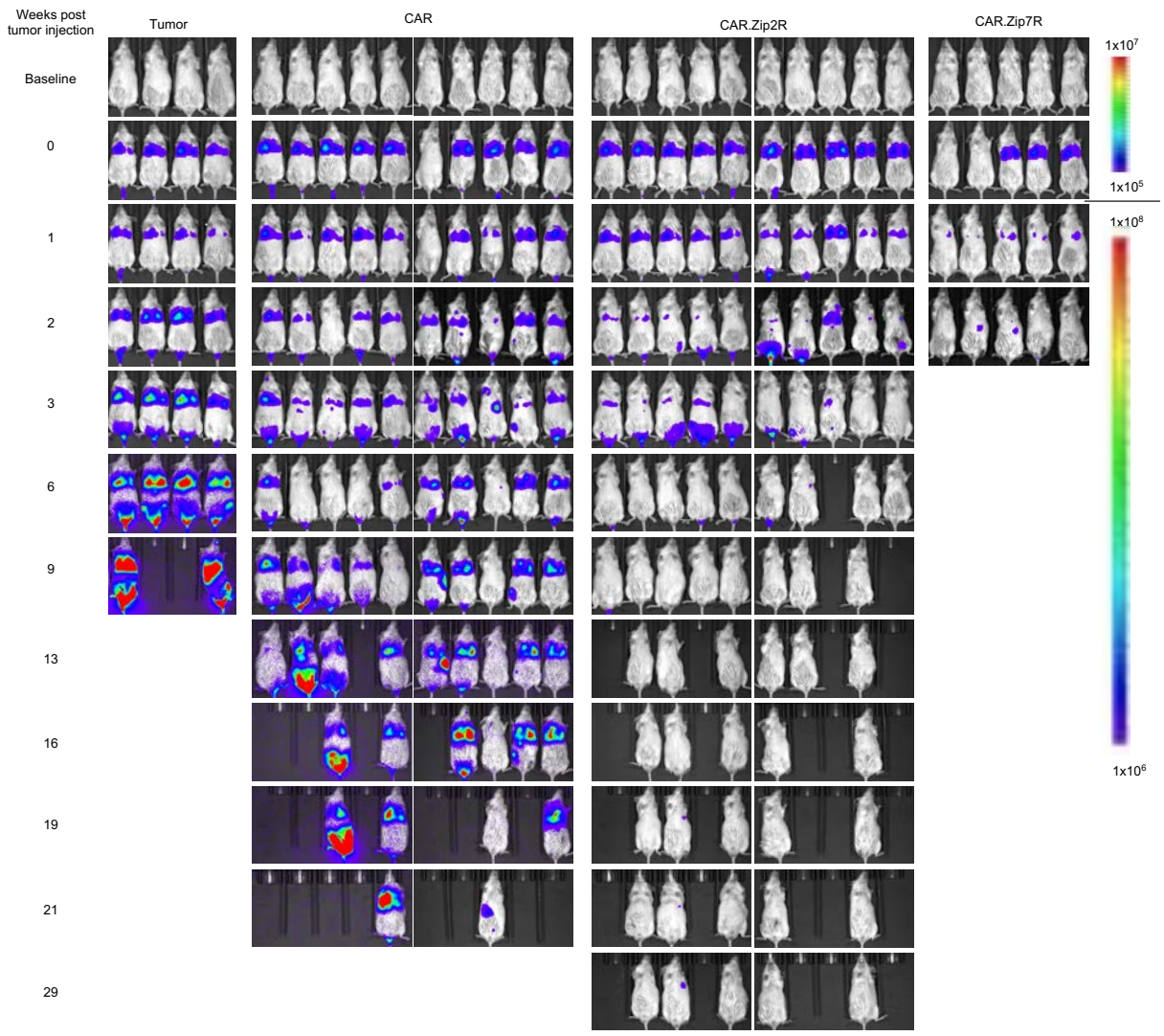

GPC2.41BBz CAR
Fold Expansion
Stimulation
CAR.Zip2R
CAR

d

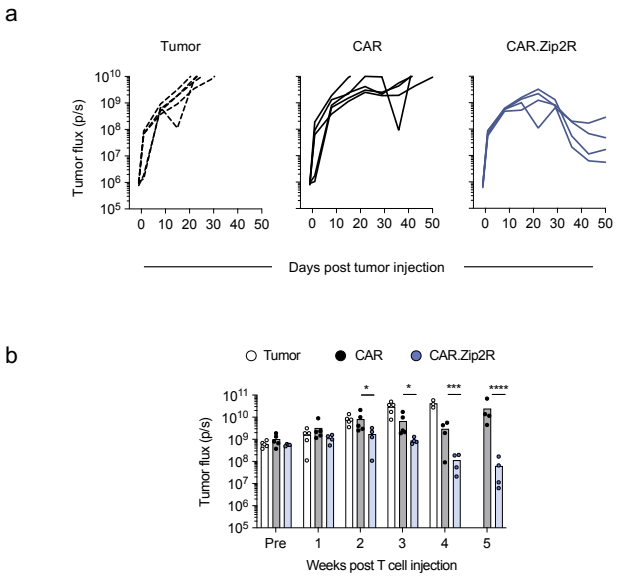

ns
***
***
Stimulations
GPC2.CD28z CAR
GPC2.CD28z CAR.Zip2R
GPC2.41BBz CAR
GPC2.41BBz CAR.Zip2R

Supplementary Data Figure 8: Zip2R improves GPC2.CD28z and GPC2.41BBz CAR T cell antitumor activity

(**a**) Transduction efficiency of GPC2 CAR and Zip2R (N=2-3 biological replicates). (**b**) 7-day repeat stimulation assay with GPC2.CD28z CAR T cells and LAN1 tumor cells at 2:1 E:T. (**c**) 7-day repeat stimulation assay with GPC2.41BBz CAR T cells and LAN1 tumor cells at 2:1 E:T. (**d**) Stimulations of tumor cell killing in 7-day repeat simulation assay with LAN1 WT cells and CAR T cells at 2:1 E:T ratio (N=3 biological replicates, mean±SD, ***p<0.001, ns = non-significant, one-way ANOVA).

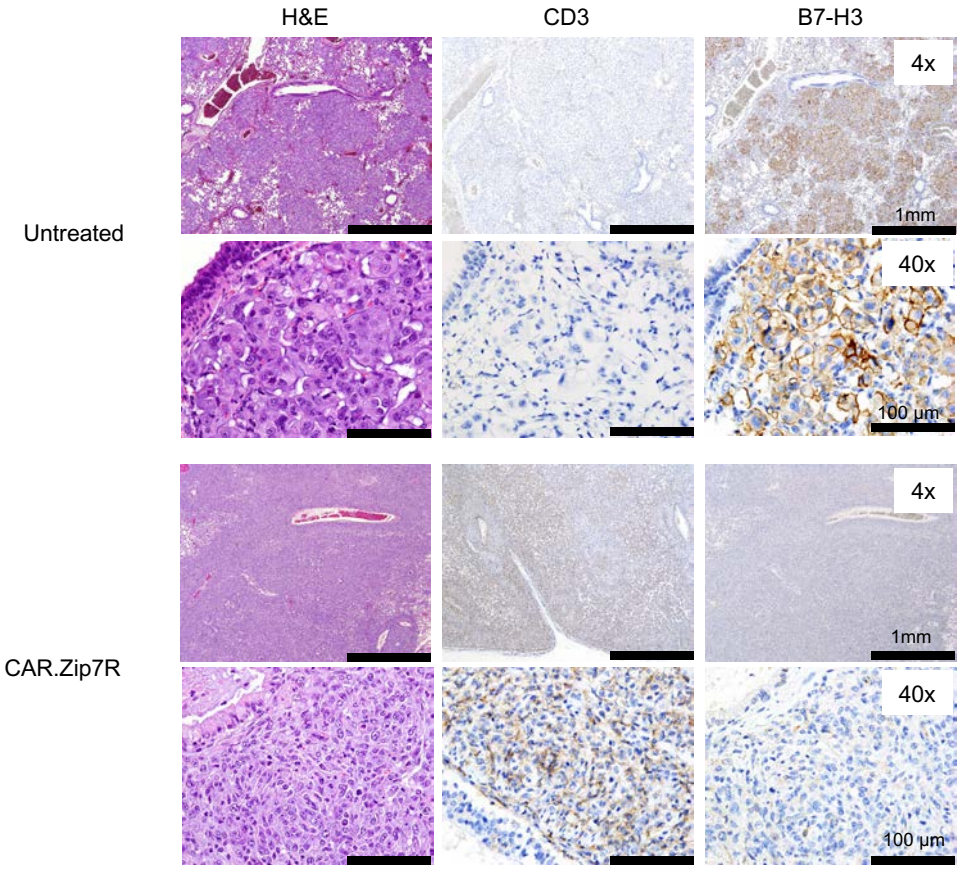


Supplementary Data Figure 9: Representative bioluminescence images of mice from Fig. 4b. See Figure legend 4a,b for details.



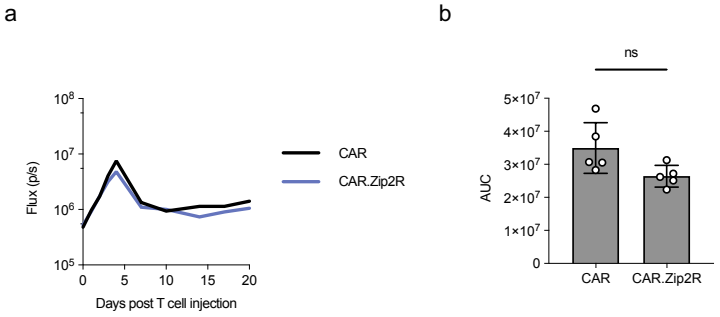
Extended Data Figure 10: Zip2R augments the antitumor activity of B7-H3-CAR.Zip2R T cells in the A549 model.

Same experimental scheme as described in Figure 4a. Mice received a single i.v. dose of $3x10^5$ CAR T cells on day 7 post A549.GFP.ffLuc cell injection. Data for a 3rd donor (N=4-5). (**a**) Tumor burden in the lungs as determined by serial bioluminescence imaging. (**b**) Quantification of tumor flux in the lungs of treated mice (mean±SD, *p<0.05, ***p<0.001, ****p<0.0001, two-way ANOVA of log transformed BLI data).

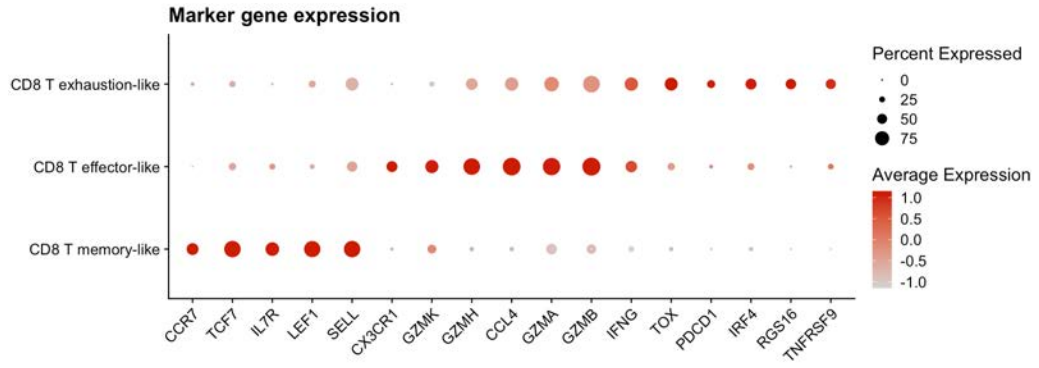


Supplementary Data Figure 11: Representative IHC images of lungs from untreated or CAR.Zip7R T cell treated mice.

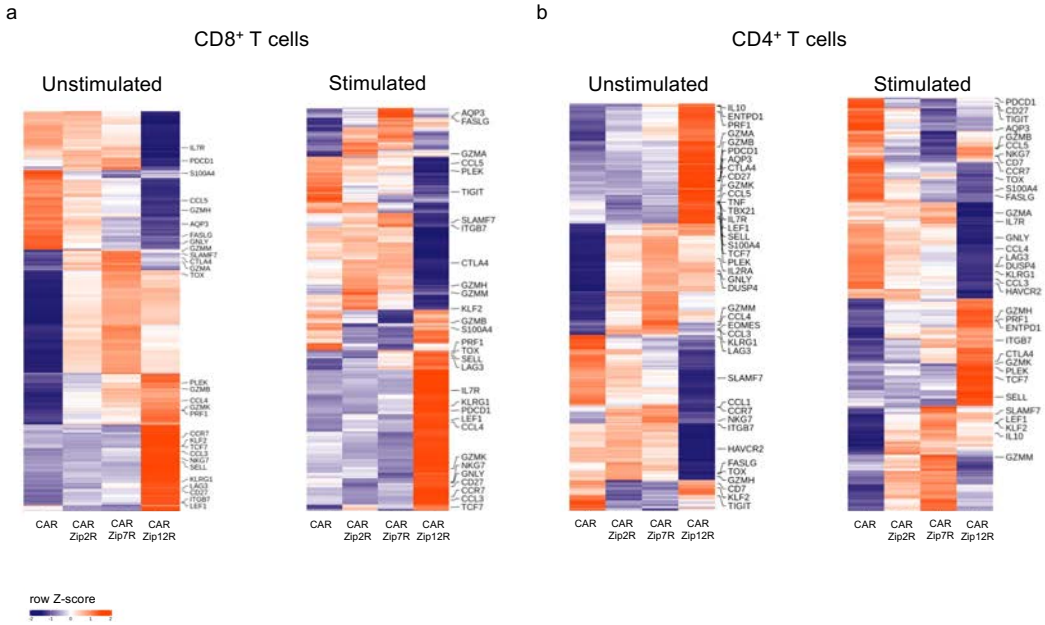
Immunohistochemistry (IHC) of lungs from mice from the indicated treatment groups. Untreated mice were analyzed on day 28 post tumor injection. CAR.Zip7R T cell treated mice were analyzed on day 26 post tumor injection (day 19 post T cell injection). Hematoxylin and eosin (H&E) staining, anti-CD3, and anti-B7-H3 staining is shown. Representative images. Top panel for untreated and CAR.Zip7R T cells: (4x); scale bar: 1 mm. Bottom panel for untreated and CAR.Zip7R T cells: (40x); scale bar: 100 μm.



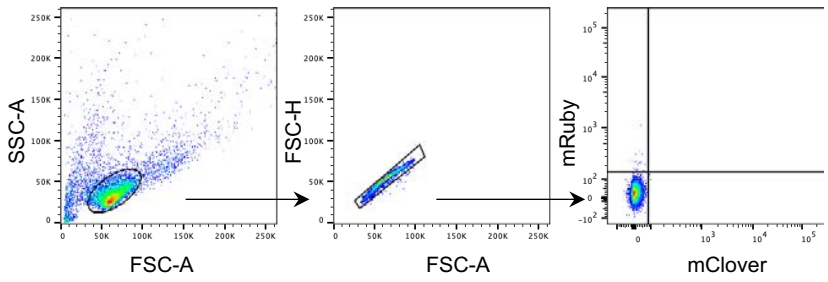
Supplementary Data Figure 12: CAR.Zip2R expansion *in vivo.*
Same experimental scheme as described in Figure 4a. Mice received a single i.v. dose of $1x10^6$ ffLuc-expressing CAR T cells on day 7 post A549 cell injection.
(**a**) BLI of CAR.ffLuc and CAR.Zip2R.ffLuc T cells in A549 WT tumor bearing mice. (**b**) Area under the curve (AUC) of flux from day 0-20.



Supplementary Data Figure 13: Marker gene expression corresponding to Fig. 4e,f
Expression of marker genes defining CD8 exhaustion-like, CD8 effector-like, and CD8 memory-like CAR T cells.



Supplementary Data Figure 14: DEGs from CD8$^+$ or CD4$^+$ CAR.ZipR T cells.
Corresponding to Figure 6. Global differentially expressed genes in unstimulated and stimulated CD8$^+$ and CD4$^+$ CAR T cells.



Supplementary Data Figure 15: Example gating strategy.
Left panel: Live cell gate FSC vs SSC. Middle panel: Doublets were excluded using FSC-H vs FSC-A as cells displaying double the signal of singlets. Right panel:Gates were based on non-transduced T cells to account for any background binding.

Supplementary Data Figure 16: Phosphoproteome dataset. See separate Excel spreadsheet.

Supplementary Data Figure 17: ZipReceptor sequences. See separate Word document.