SUPPLEMENTARY MATERIALS FOR:

Enhanced Solid Tumor Recognition and Persistent Anti-tumor Activity with SynNotch CAR Circuits

Axel Hyrenius-Wittsten, Yang Su, Minhee Park, Julie M. Garcia, Josef Alavi, Nathaniel Perry, Garrett Montgomery, Bin Liu*, Kole T. Roybal*

*Correspondence: kole.roybal@ucsf.edu (KT Roybal), bin.liu@ucsf.edu (B Liu)

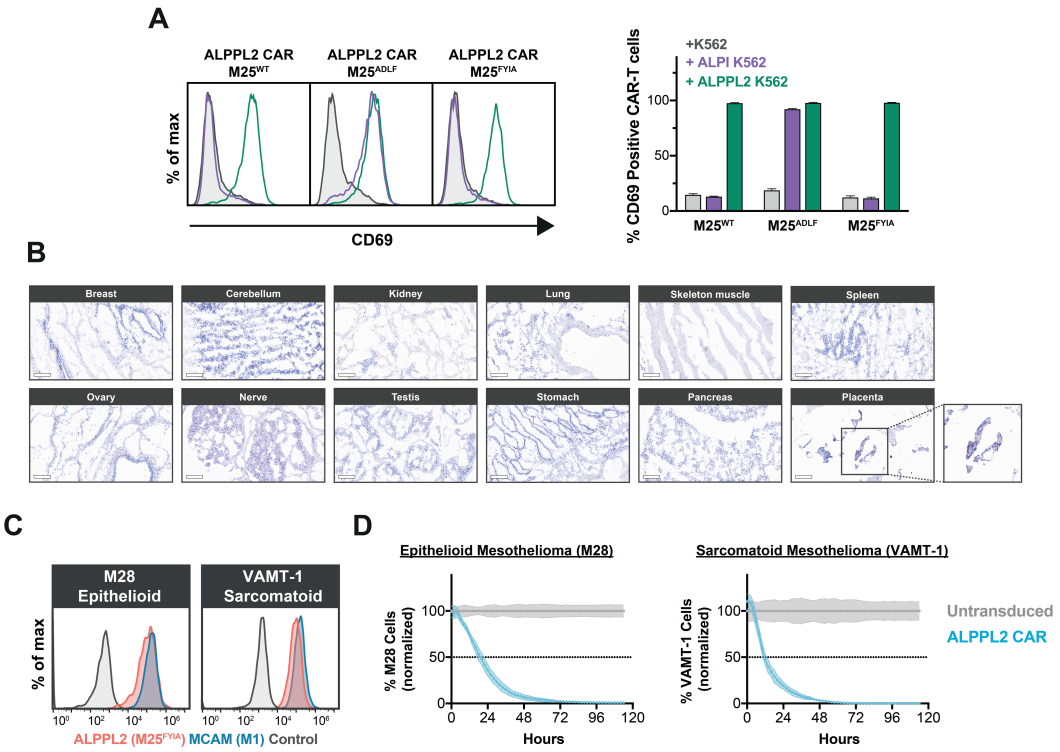

Fig S1. Characterization of ALPPL2 tissue expression and ALPPL2 CAR specificity. (**A**) Expression of the early activation marker CD69 in CD8$^+$ CAR-T cells expressing a BBζ CAR with either a M25^{WT}, M25^{ADLF}, or M25^{FYIA} scFv 24 hours after stimulation with K562 leukemia cells expressing either ALPI or ALPPL2 (n=3 for all groups, data from one experiment). (**B**) Immunohistochemistry (IHC) of ALPPL2 expression in normal human frozen tissues using the M25^{FYIA} scFv. Binding was only observed in placental trophoblasts. Scale bar: 100μm (representative from two independent experiments). (**C**) Cell surface co-expression of ALPPL2 and MCAM in an epithelioid mesothelioma cell line M28 and a sarcomatoid cell line VAMT-1 using M1 and M25^{FYIA} scFvs (representative from three independent experiments). (**D**) Killing kinetics of epithelioid (M28) and sarcomatoid (VAMT-1) mesothelioma tumor cells by CD8$^+$ T cells expressing an ALPPL2 CAR (M25^{FYIA}) (n=3 for both groups, representative of at least two independent experiments). CAR, chimeric antigen receptor.

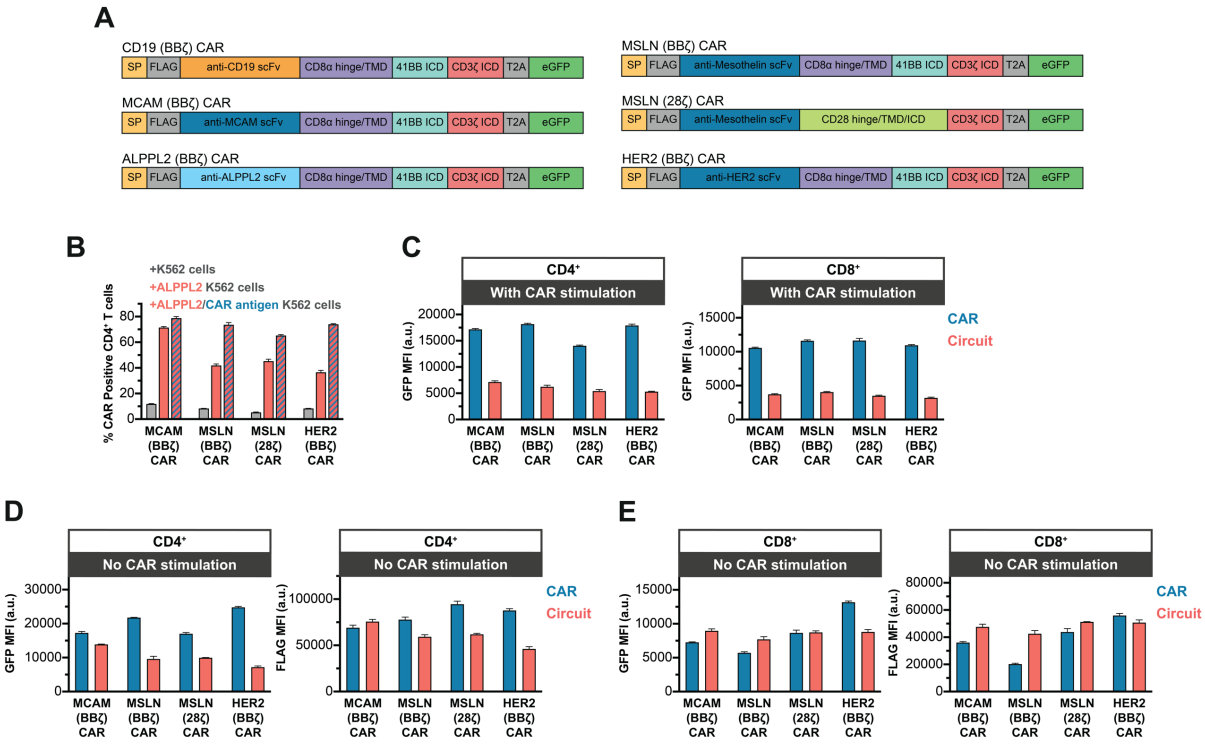

Fig. S2. Characterization of ALPPL2 synNotch-regulated CAR expression. (**A**) Design of CD19, MCAM, ALPPL2, Mesothelin (MSLN), and HER2 CARs included in the study. SP, signaling peptide; FLAG, FLAG-tag; ICD, intracellular domain; TMD, transmembrane domain. (**B**) Antigen-specific CAR expression in $CD4^+$ T cells was determined by green fluorescent protein (GFP) expression after 48 hours of stimulation with K562 cells expressing MCAM, mesothelin, and HER2 ± ALPPL2 (representative of two independent experiments). (**C**) CAR expression in $CD4^+$ or $CD8^+$ T cells engineered to express MCAM (BBζ) CAR, Mesothelin (MSLN, BBζ or 28ζ) CAR, or HER2 (BBζ) CAR constitutively or under ALPPL2 synNotch transcriptional regulation 48 hours post-stimulation with K562 target cells expressing ALPPL2, MCAM, mesothelin, and HER2, as determined by GFP mean fluorescence intensity (MFI). (**D,E**) CAR expression in $CD4^+$ (D) or $CD8^+$ (E) T cells engineered to express MCAM (BBζ) CAR, MSLN (BBζ or 28ζ) CAR, or HER2 (BBζ) CAR constitutively or under ALPPL2 synNotch transcriptional regulation 48 hours post stimulation with K562 target cells expressing solely ALPPL2, as determined by GFP expression

and FLAG-tag surface staining by MFI. (**B-E**; n=3 for all groups, representative of two independent experiments) Data are shown as mean±SD.

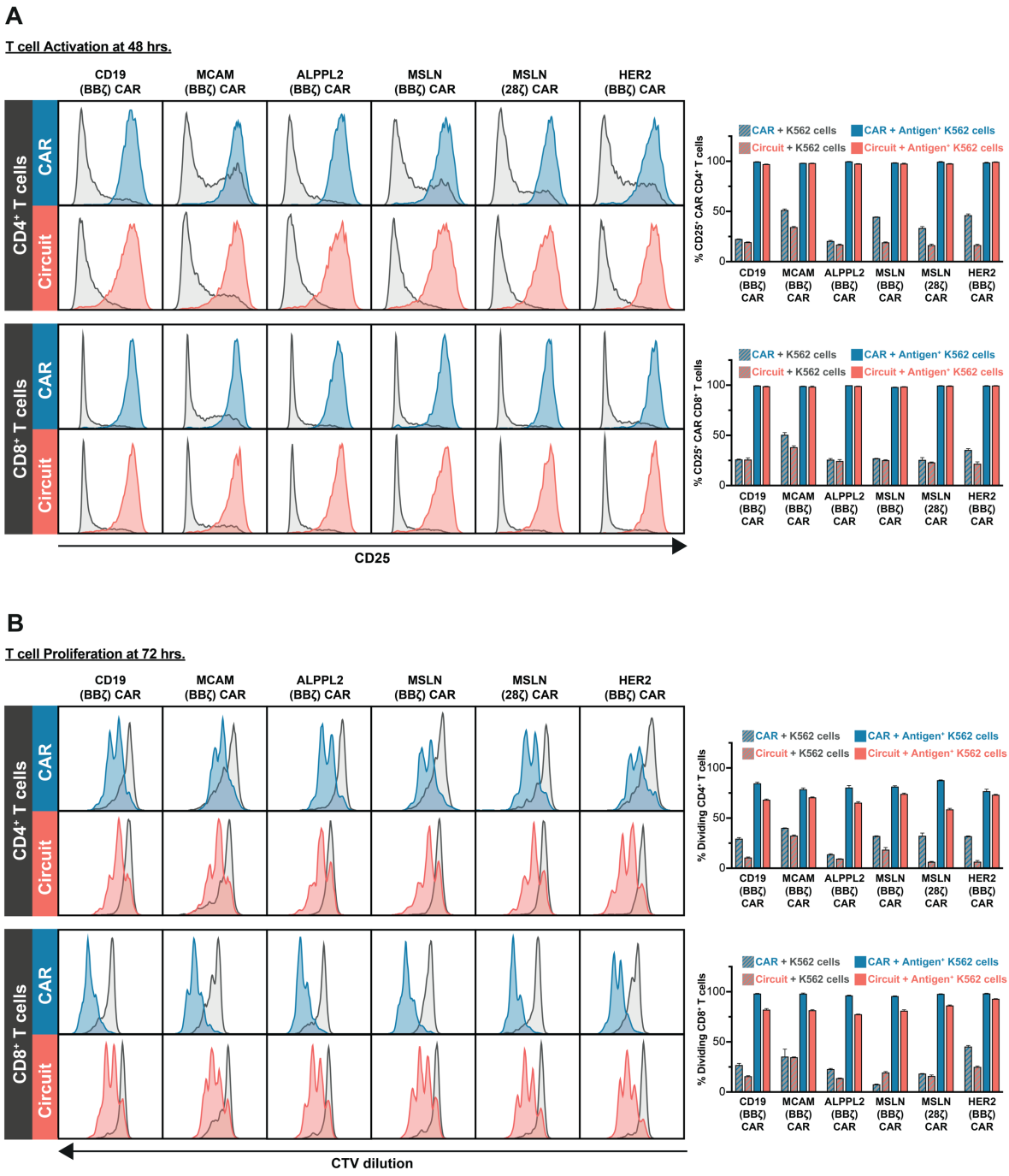

Fig. S3. Activation and proliferation of antigen-stimulated ALPPL2 synNotch CAR circuits. (**A**) CD4$^+$ or CD8$^+$ T cells were engineered to express an ALPPL2 synNotch together with a genetic circuit encoding for either a CD19 (BBζ), MCAM (BBζ), ALPPL2 (BBζ), mesothelin (MSLN, BBζ or 28ζ), or HER2 (BBζ) CAR and were analyzed for CD25 expression in CAR positive cells after 48 hours of stimulation with K562 cells ± cognate antigens (n=3 for all groups,

representative of two independent experiments). (**B**) Proliferation of cell trace violet (CTV)-labelled $CD4^+$ or $CD8^+$ T cells expressing ALPPL2 synNotch CAR circuits three days after stimulation with K562 cells ± cognate antigens (n=3 for all groups, representative of two independent experiments). Data are shown as mean±SD. For flow histograms, y-axis shows % of max.

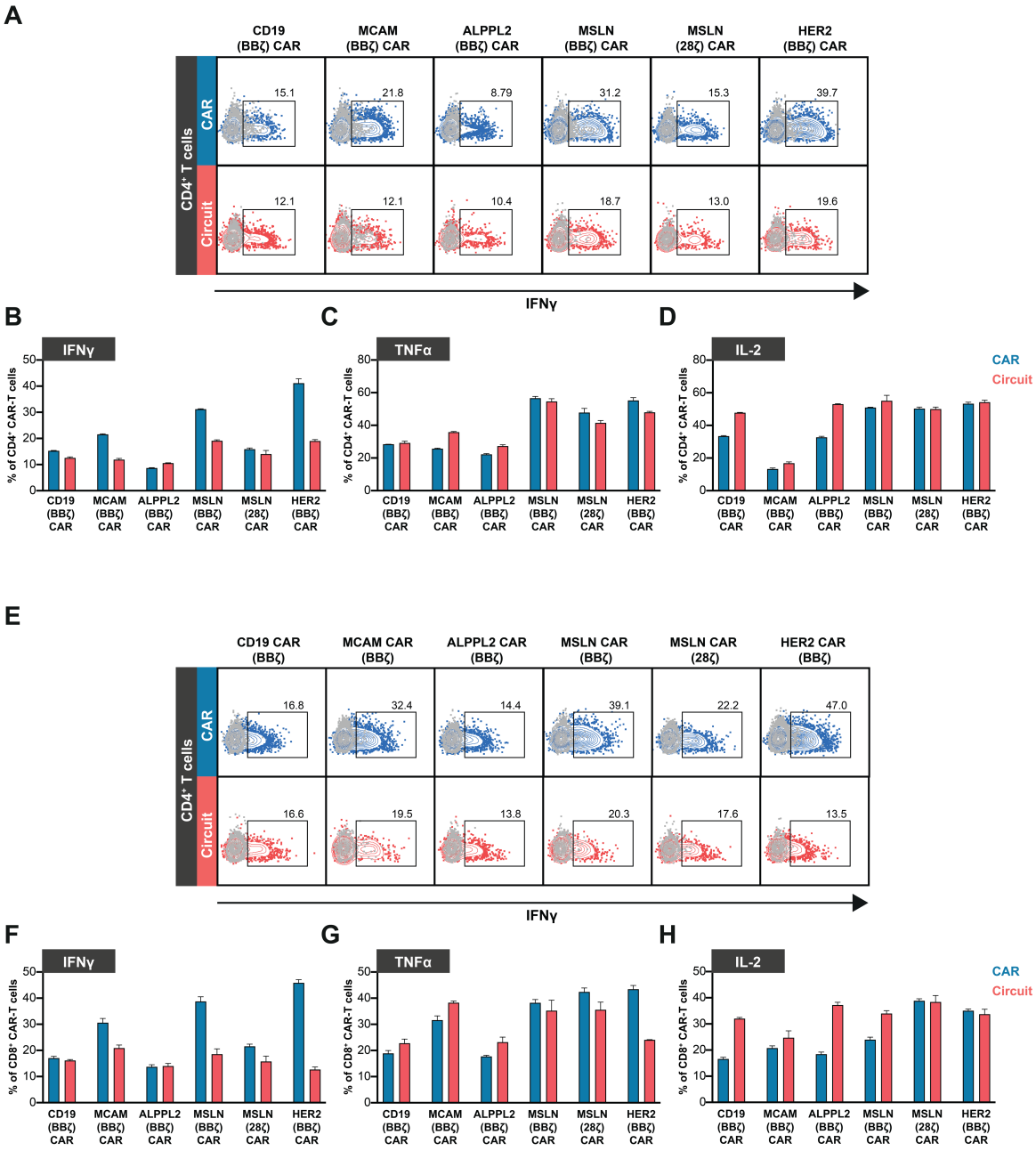

Fig. S4. Cytokine production by CAR and synNotch CAR circuit T cells. (**A**) Contour plots showing interferon gamma (IFNγ) production in CD4$^+$ T cells engineered to express a CD19 (BBζ), MCAM (BBζ), ALPPL2 (BBζ), mesothelin (MSLN, BBζ or 28ζ), or HER2 (BBζ) CAR constitutively or through ALPPL2-synNotch CAR circuits after stimulation with K562 cells ± cognate antigens. SynNotch CAR circuits were primed to express the CAR prior to antigen stimulation using myc-beads to allow for instant CAR stimulation. (**B-D**) Summarized cytokine production in CD4$^+$ T cells

for interferon gamma (IFNγ, **B**), tumor necrosis factor alpha (TNFα, **C**), or interleukin (IL)-2 (**D**) after 6 hours of CAR antigen stimulation (n=3 for all groups, data from one experiment). (**E**) Contour plots showing IFNγ production in $CD8^+$ T cells engineered to express a CD19 (BBζ), MCAM (BBζ), ALPPL2 (BBζ), MSLN (BBζ or 28ζ), or HER2 (BBζ) CAR constitutively or through ALPPL2-synNotch CAR circuits after stimulation with K562 cells ± cognate antigens. CAR circuits were primed to express the CAR prior to antigen stimulation using myc-beads. (**F-H**) Summarized cytokine production in $CD8^+$ T cells for IFNγ (**F**), TNFα (**G**), and IL-2 (**H**) after 6 hours of CAR antigen stimulation (n=3 for all groups, data from one experiment). Data are shown as mean±SD.

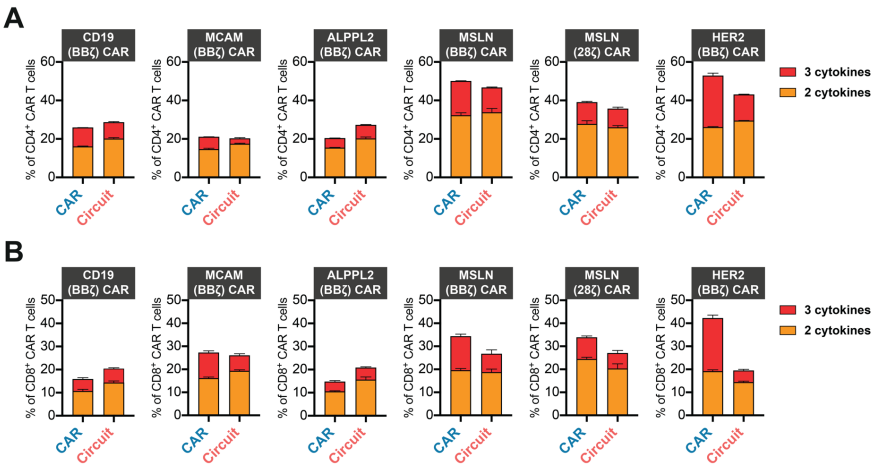

Fig. S5. Polyfunctionality of CAR and synNotch CAR circuit T cells. (**A and B**) $CD4^+$ (**A**) or $CD8^+$ (**B**) T cells engineered to express a CD19 (BBζ), MCAM (BBζ), ALPPL2 (BBζ), mesothelin (MSLN, BBζ or 28ζ), or HER2 (BBζ) CAR constitutively or through ALPPL2-synNotch CAR circuits were stimulated with K562 cells expressing the cognate CAR antigen and ALPPL2. All T cells were cultured in the presence myc-beads for 24 hours prior to the assay in order to prime SynNotch CAR circuits to express the CAR prior to antigen stimulation for instant CAR activation for all groups. Polyfunctional $CD4^+$ or $CD8^+$ T cells were determined by simultaneous expression of two or three of cytokines measured (IFNγ, TNFα, or IL-2) after 6 hours of CAR antigen stimulation (n=3 for all groups, data from one experiment). Data are shown as mean±SD.

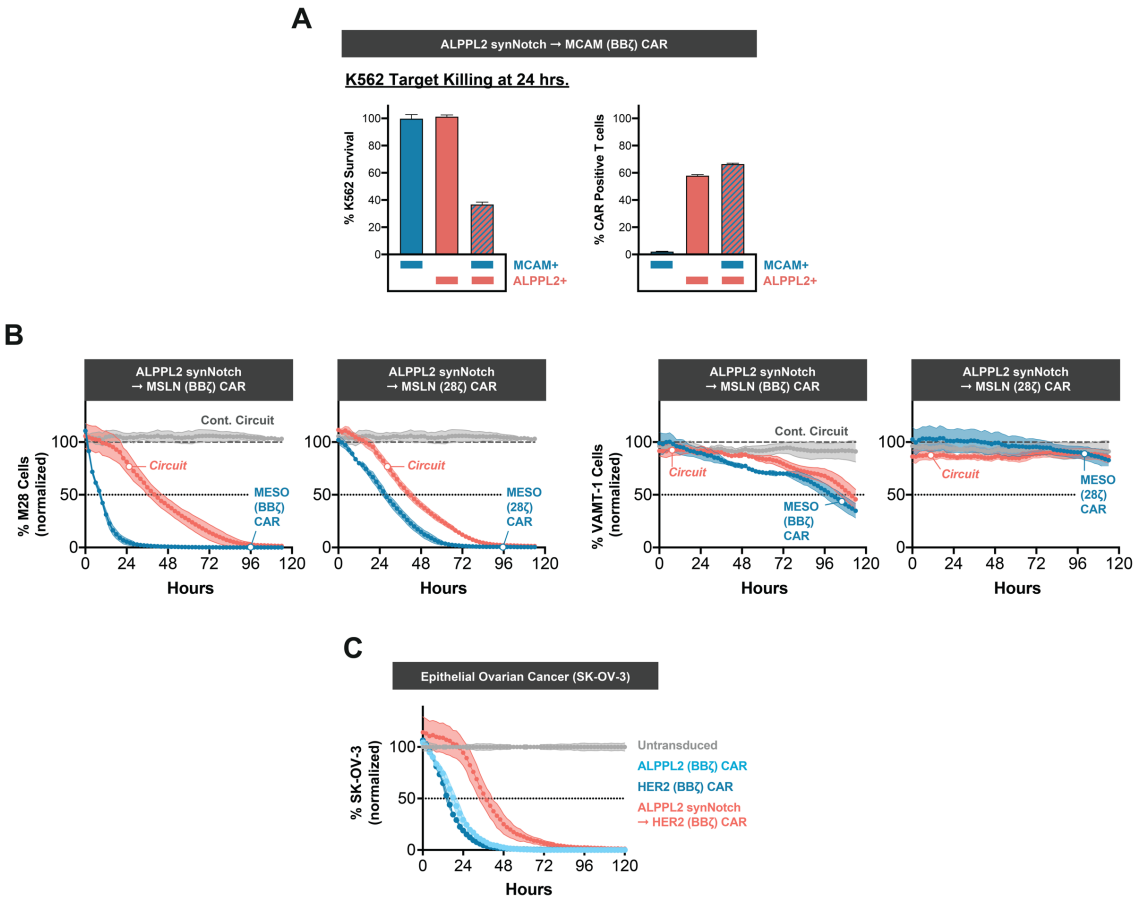

Fig. S6. SynNotch CAR circuit T cells exhibit multi-antigen specificity and paced tumor elimination. (**A**) Target killing of K562 cells expressing MCAM, ALPPL2, or the combination of the two by CD8$^+$ T cells expressing an ALPPL2 synNotch MCAM (BBζ) CAR circuit after 24 hours of co-culture. Percentage CAR positive T cells was determined by GFP expression (n=3 for all groups, representative of at least two independent experiments). (**B**) Incucyte assay showing killing kinetics of epithelioid (M28) and sarcomatoid (VAMT-1) mesothelioma tumor cells by CD8$^+$ T cells expressing a BBζ mesothelin (MSLN) or 28ζ MSLN CAR constitutively or through an ALPPL2-synNotch (n=3 for all groups, representative of two independent experiments). (**C**) Incucyte assay showing killing kinetics of ALPPL2$^+$ SK-OV-3 ovarian tumor cells by CD8$^+$ T cells expressing an ALPPL2 (BBζ) CAR or a HER2 (BBζ) CAR constitutively or HER2 (BBζ) CAR through an ALPPL2-synNotch CAR circuit (n=3 for all groups, data from one experiment). Data are shown as mean±SD.

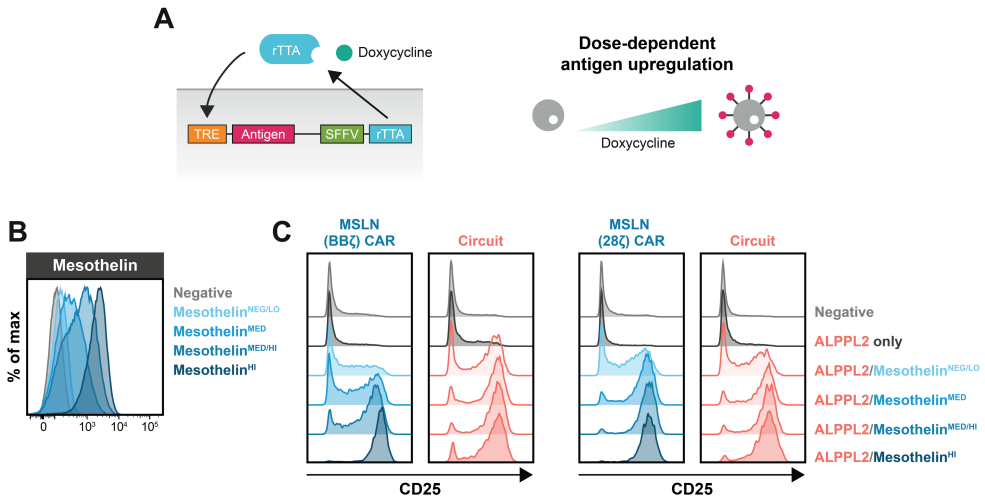

Fig. S7. Development of tumor cell lines expressing titratable antigen densities and synNotch CAR circuit sensitivity toward mesothelin. (**A**) Genetic circuit for doxycycline inducible ligand surface expression. TRE, tetracycline response element; rTTA, reverse tetracycline-controlled transactivator. (**B**) ALPPL2^{+} K562 cells were constructed to display dose-dependent expression of mesothelin upon treatment with doxycycline. Mesothelin^{HI} cells express mesothelin driven by the spleen focus-forming virus (SFFV) promoter. (**C**) CD8^{+} T cells engineered with a mesothelin (MSLN, BBζ or 28ζ) CAR either under a constitutive SFFV promoter or ALPPL2 synNotch circuit were challenged with doxycycline-treated K562 target cells and assessed for CD25 expression after 48 hours (data from one experiment). For flow histograms, y-axis shows % of max.

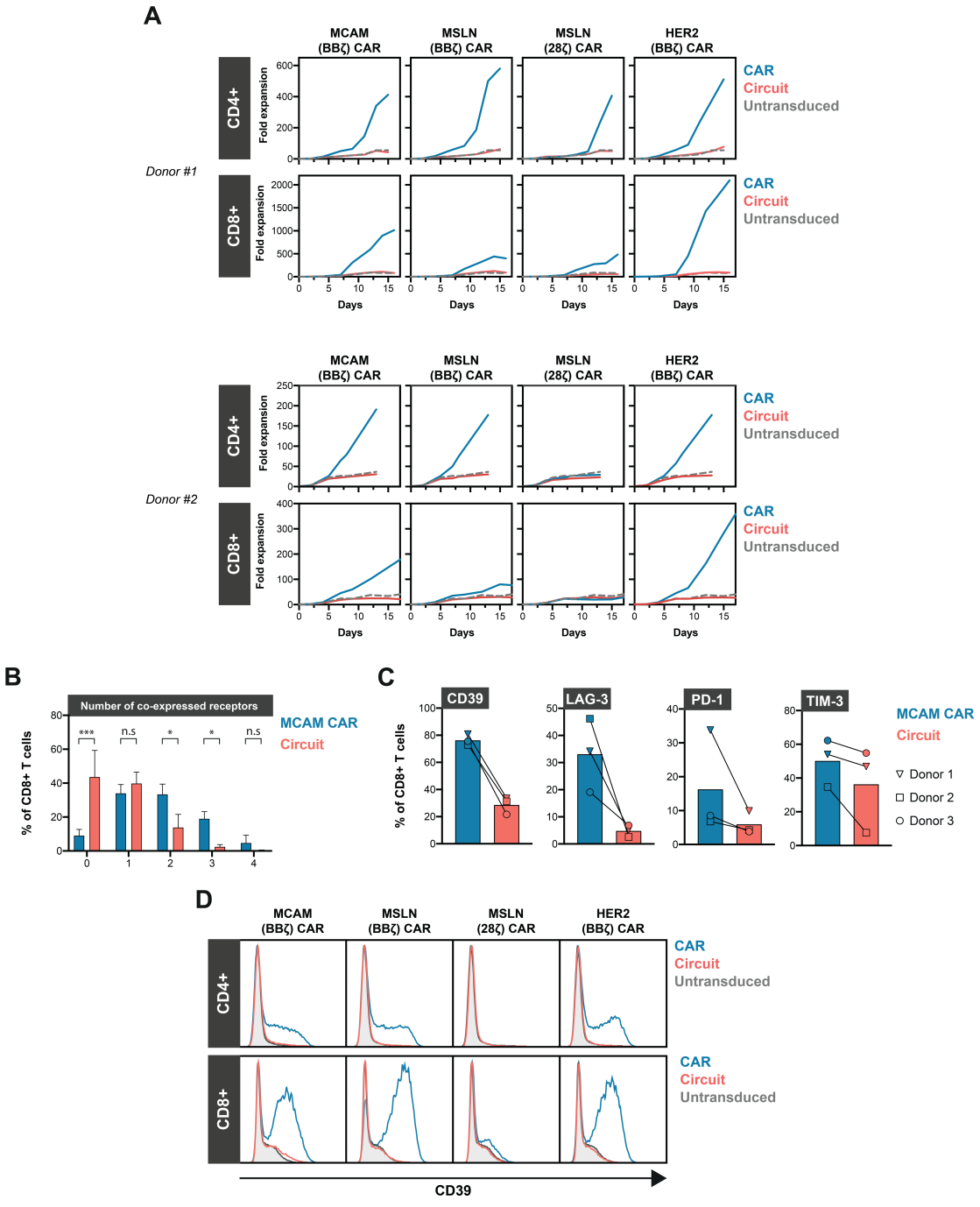

Fig. S8. Phenotypic analysis of CAR and synNotch CAR T cells prior to antigen exposure. (**A**) Expansion of engineered CD4$^+$ or CD8$^+$ T cells after removal of CD3/CD28 Dynabead stimulation in two separate donors. (**B**) Co-expression pattern and (**C**) fraction of cells positive for CD39, lymphocyte-activation gene 3 (Lag-3), programmed cell death protein 1 (PD-1), or T-cell immunoglobulin and mucin-domain containing-3 (Tim-3) in non-antigen exposed CD8$^+$ T cells

from three different donors engineered to express a MCAM (BBζ) CAR either constitutively or through ALPPL2 synNotch circuit 14 days post initial CD3/CD28 Dynabead stimulation. (**D**) Expression of CD39 in $CD4^+$ or $CD8^+$ T cells engineered to express a MCAM (BBζ), mesothelin (MSLN, BBζ or 28ζ), or HER2 (BBζ) CAR either constitutively or through ALPPL2 synNotch circuit 14 days post initial CD3/CD28 Dynabead stimulation (representative of two independent experiments). Statistics were calculated using a two-way ANOVA with Šidák's post-hoc test. Data is shown as mean±SD. For flow histograms, y-axis shows % of max. *$P \leq 0.05$; ***$P \leq 0.001$; n.s.; not significant.

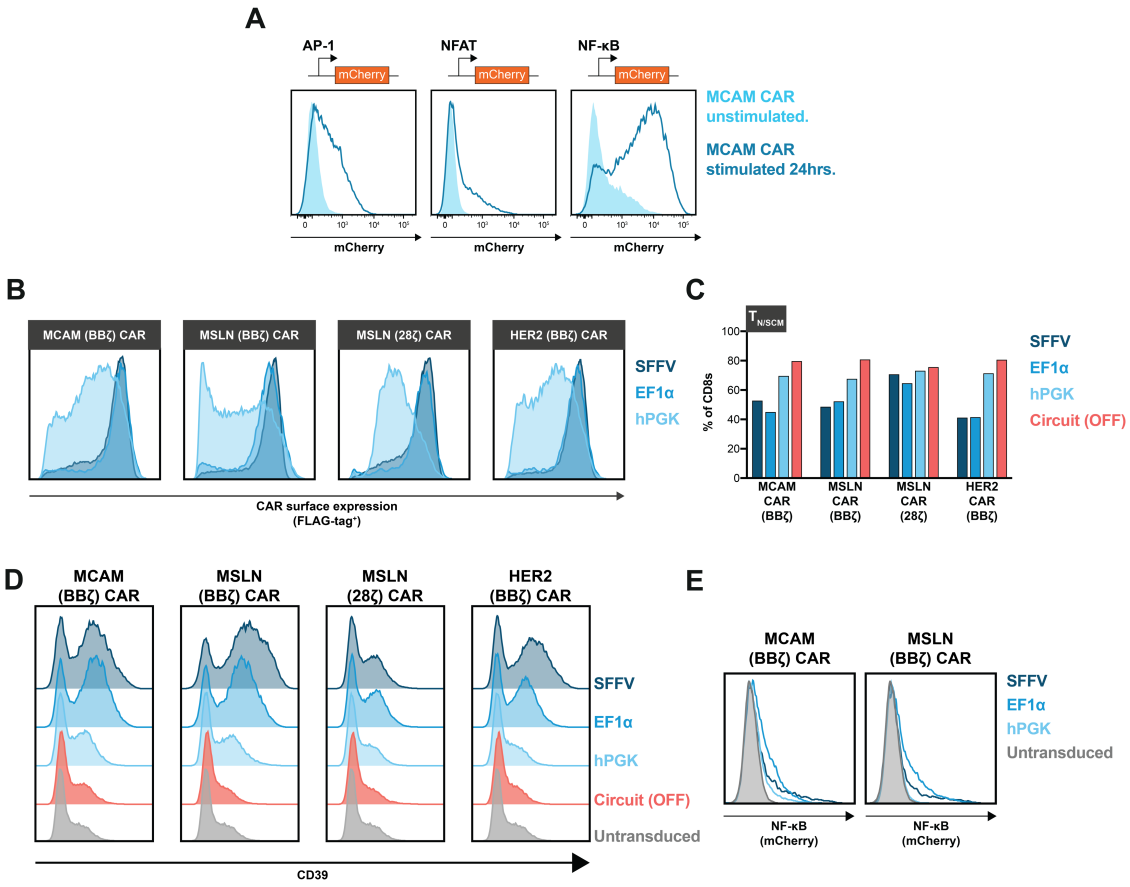

Fig. S9. Impact of CAR expression on tonic signaling. (**A**) Activator protein 1 (AP-1), nuclear factor of activated T cells (NFAT), or nuclear factor kappa B (NF-κB) response element activity in Jurkat cells constitutively expressing a MCAM (BBζ) CAR 24 hours after stimulation with MCAM positive K562 cells (data from one experiment). (**B**) Expression of MCAM (BBζ), mesothelin (MSLN, BBζ or 28ζ), or HER2 (BBζ) CAR driven by either a SFFV, elongation factor 1-alpha (EF1α), or human phosphoglycerate kinase (hPGK) promoter in $CD8^+$ T cells after removal of CD3/CD28 Dynabead stimulation as determined by surface FLAG-tag expression (data from one experiment). (**C**) Fraction of $CD8^+$ displaying a $T_{N/SCM}$ (naïve/stem cell memory-like, $CD45RA^+CD62L^+$) phenotype and (**D**) CD39 expression when expressing an MCAM (BBζ), MSLN (BBζ or 28ζ), or HER2 (BBζ) CAR by either a SFFV, EF1α, or hPGK promoter (data from one experiment). (**E**) NF-κB response element regulated mCherry expression in Jurkat cells carrying

an MCAM (BBζ) or MSLN (BBζ) CAR driven by either a SFFV, EF1α, or hPGK promoter (data from one experiment). For flow histograms, y-axis shows % of max.

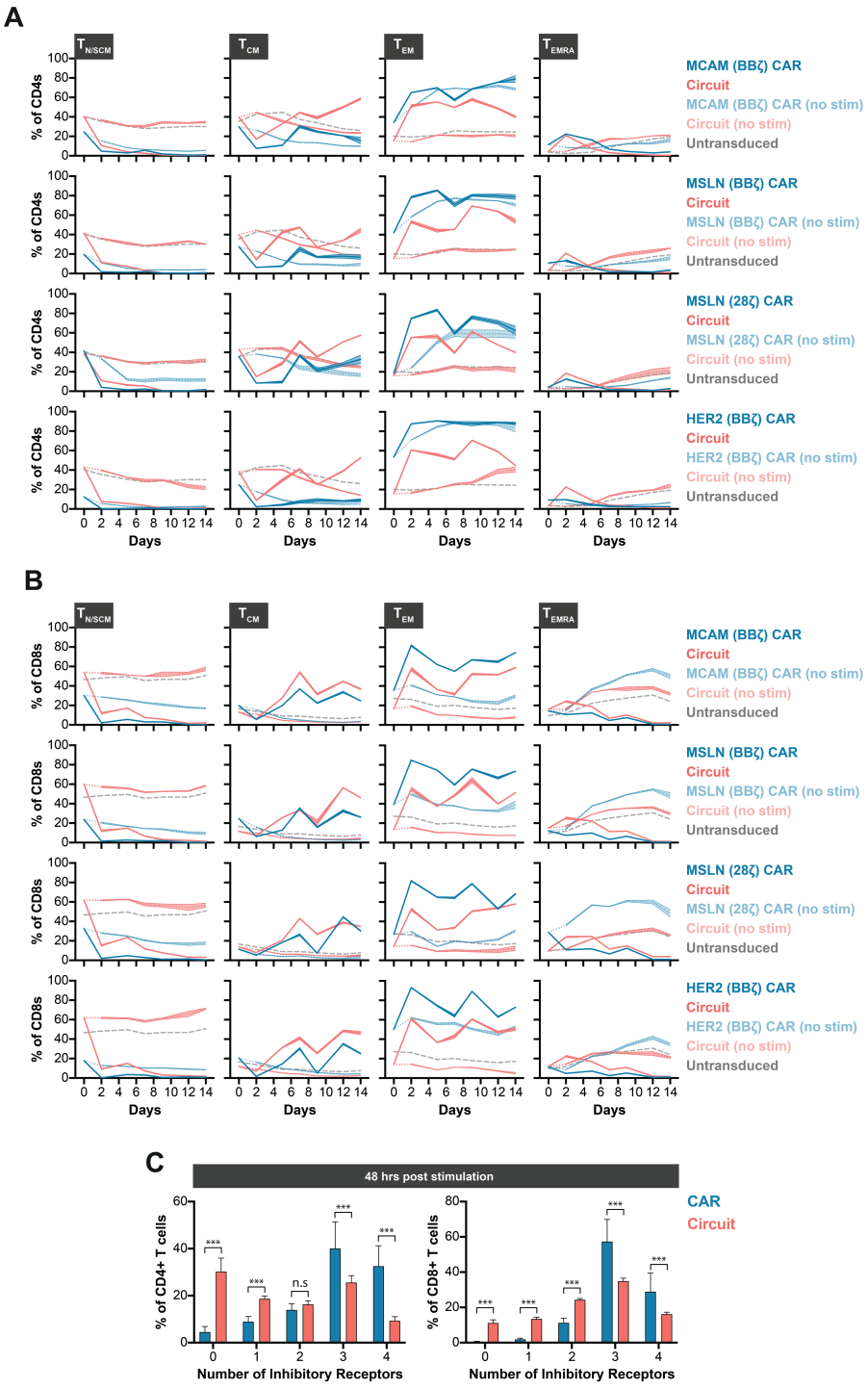

Fig. S10. SynNotch CAR circuits regulate the evolution of T cell differentiation pre and post antigen stimulation. (**A**, **B**) Phenotypic evolvement for engineered CD4$^+$ (**A**) or CD8$^+$ (**B**) T cells expressing MCAM (BBζ), mesothelin (MSLN, BBζ or 28ζ), or HER2 (BBζ) CAR constitutively or through an ALPPL2-synNotch circuit upon culture without or after two stimulations of ALPPL2$^+$

K562 cells expressing the cognate CAR antigens (data from one experiment). (**C**) Co-expressional pattern of CD39, Lag-3, PD-1, and Tim-3 in $CD4^+$ or $CD8^+$ T cells engineered to express a MCAM (BBζ), MSLN (BBζ or 28ζ), or HER2 (BBζ) CAR constitutively or through an ALPPL2-synNotch circuit 48 hours after stimulation with $ALPPL2^+$ K562 cells expressing the cognate CAR antigens (n=12 for both groups, data from one experiment). Data is presented as mean±SEM (**A**,**B**) or mean±SD (**C**) and statistics were calculated using two-way ANOVA with Šidák's post-hoc test. ***P ≤0.001; n.s.; not significant. T_{CM}, central memory-like T cells ($CD45RA^-$ $CD62L^+$); T_{EM}, effector memory-like T cells ($CD45RA^-CD62L^-$); T_{EMRA}, effector memory-like T cells re-expressing CD45RA ($CD45RA^+CD62L^-$).

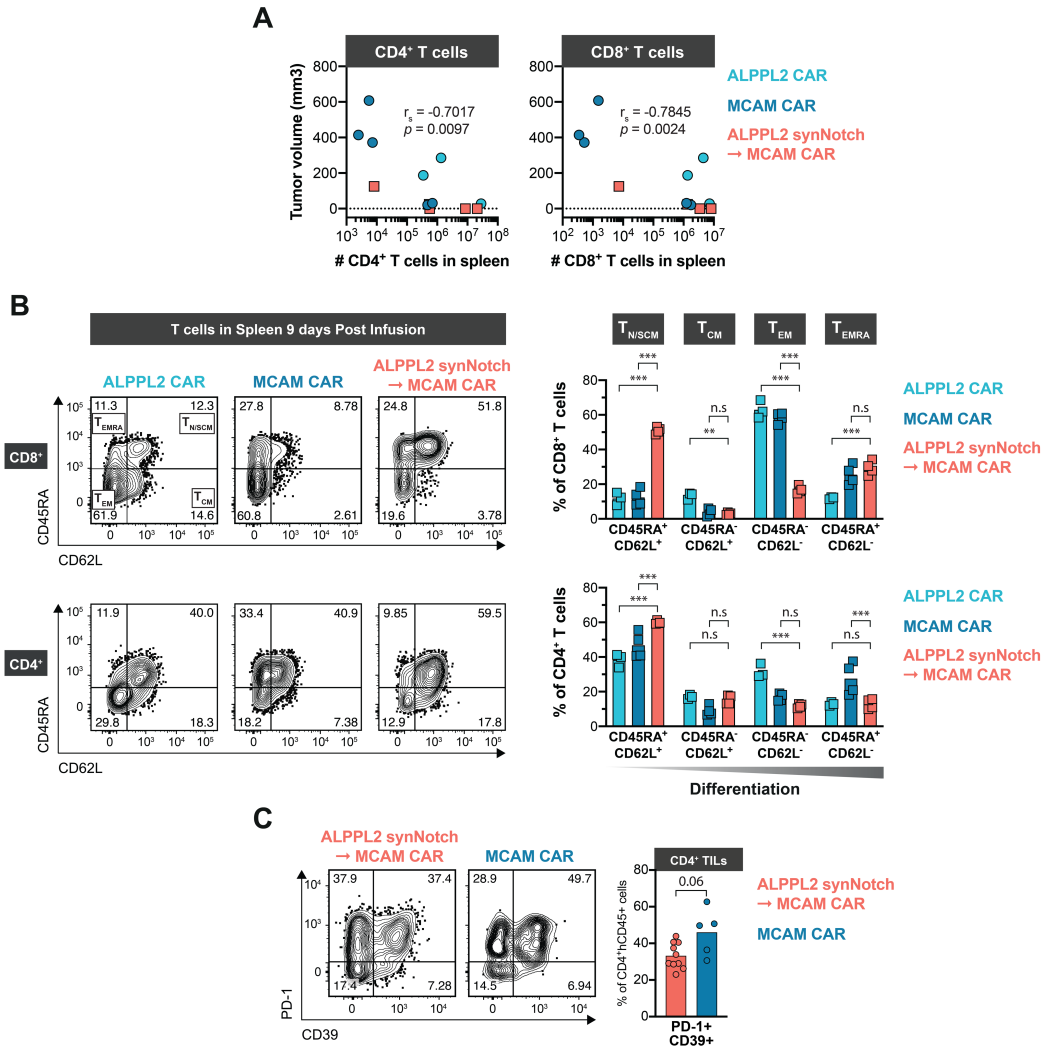

Fig. S11. Persistence of CAR T cells and synNotch CAR circuit T cells in treated M28 tumor-bearing mice. (**A**) Correlation between final tumor volume and number of human $CD4^+$ or $CD8^+$ T cells in the spleen at the end of the experiment. Correlation was determined using Spearman's rank correlation coefficient (data from one experiment). (**B**) Percentage of T cell memory subsets in human $CD8^+$ and $CD4^+$ T cells in the spleen of M28 tumor-bearing NOD.Cg-*Prkdc*^{scid}*Il2rg*^{tm1Wjl}*/SzJ* (NSG) mice 9 days after T cell infusion (data from one experiment). (**C**) Expression of PD-1 and CD39 in $CD4^+$ tumor-infiltrating lymphocytes (TILs) expressing a MCAM (BBζ) CAR either constitutively or through an ALPPL2 synNotch (representative of two

independent experiments). Statistics were calculated using two-way ANOVA with Tukey's post-hoc test (**B**) or Mann-Whitney U test (**C**). **P ≤ 0.01; ***P ≤ 0.001; n.s.; not significant.

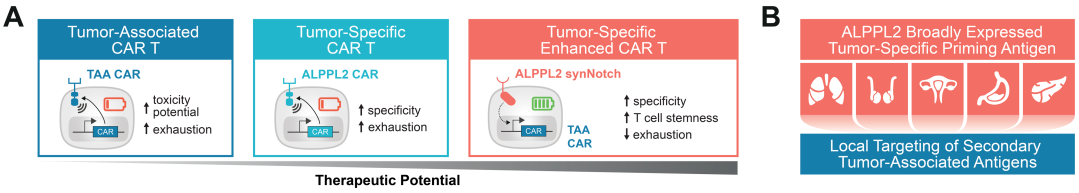

Fig. S12 SynNotch enhancements for CAR T therapy. (**A**) SynNotch CAR circuits allow for safe targeting of tumor-associated antigens and enhances immunotherapeutic potential by avoiding tonic CAR signaling outside tumor tissues. TAA, Tumor-associated antigen. (**B**) ALPPL2 is a highly tumor-specific antigen expressed in mesothelioma, seminoma, gastric-, ovarian-, and pancreatic cancer that allows for safe targeting of other less specific tumor-associated antigens.

Table S1. **ALPPL2 expression in solid tumors.** IHC was performed on formalin-fixed paraffin-embedded (FFPE) tumor tissues using a mouse monoclonal antibody (mAb, LifeSpan clone SPM593, catalogue# LS-C390148-20). The number and percentage of ALPPL2 positive cases are indicated. ALPPL2 is expressed in all seminoma cases studied and the expression appears to be uniformly high. For other tumors studied, a fraction of the cases showed ALPPL2 expression. For those cases, graded ALPPL2 expression is seen for individual specimens. Within the same specimen, expression variation exists to a limited extent (less than 50%) for mesothelioma, ovarian cancer, and gastric cancer. Greater intra-specimen variation is observed for pancreatic cancer compared with other tumors studied. For reference, we also analyzed a public protein expression database, the Human Protein Atlas (https://www.proteinatlas.org), and listed the result of ALPPL2 expression by IHC using the validated rabbit polyclonal antibody (pAb, MilliporeSigma HPA038764). NA, not available.

Tumor Category	This study	Protein Atlas
Mesothelioma	39/91 (42.86%)	N/A
Ovarian cancer	36/60 (60%)	7/12 (58.33%)
Pancreatic cancer	18/50 (36%)	2/10 (20%)
Gastric cancer	13/72 (18.06%)	3/11 (27.27%)
Seminoma	11/11(100%)	9/11 (81.82%)

Table S2. **Tissue specificity of ALPPL2 expression.** The biotin-labeled M25^{FYIA} scFv and a biotin-labeled non-binding scFv (control) were used to stain normal human frozen tissue arrays. Each tissue type has duplicated cores on the array. Staining results of the M25^{FYIA} scFv were compared with that of the control scFv to determine positive or negative staining. Other than placenta (which expresses ALPPL2), all other normal tissues showed uniformly negative staining. N: negative staining.

Organ	Core 1	Core 2
Salivary	N	N
Esophagus	N	N
Rectum	N	N
Stomach	N	N
Kidney	N	N
Skeletal muscle	N	N
Skin	N	N
Testis	N	N
Placenta	Positive	Positive
Breast	N	N
Cervix	N	N
Uterus	N	N
Spleen	N	N
Lung	N	N
Cerebellum	N	N
Nerve	N	N
Thyroid gland	N	N
Pancreas	N	N
Ovary	N	N
Prostate	N	N

Table S3. **IHC of MCAM expression in FFPE mesothelioma tissue arrays**. The number and percentage of each type of staining patterns are indicated. MCAM was detected by both mAb (Abcam clone EPR3208, catalogue# ab75769) and pAb (Abcam, catalogue# ab228487). The difference in percentage between mAb and pAb may reflect difference in sensitivity of antigen/epitope detection in FFPE tissues.

Staining Result	**Anti-MCAM** (Rabbit mAb)	**Anti-MCAM** (Rabbit pAb)
Positive	31 (38.27%)	73 (75.26%)
Negative	50 (61.73%)	24 (24.74%)
Total tissue cores studied	81	97

Table S4. **MCAM co-expression in ALPPL2 positive mesothelioma.** IHC was performed on FFPE tumor tissue arrays using anti-ALPPL2 mouse mAb (LifeSpan clone SPM593) and anti-MCAM rabbit mAb (Abcam clone EPR3208) or pAb (Abcam). Antibody pair 1: Anti-ALPPL2 mouse mAb + Anti-MCAM rabbit mAb. Antibody pair 2: Anti-ALPPL2 mouse mAb + Anti-MCAM rabbit pAb. The number and percentage of each type of staining patterns are indicated.

Staining Result	Antibody pair 1	Antibody pair 2
ALPPL2 positive/MCAM positive	46 (52.27%)	58 (80.56%)
ALPPL2 positive/MCAM negative	42 (47.73%)	14 (19.44%)
Total ALPPL2 positive tissue cores studied	88	72

Table S5. Antibodies used for immunophenotypic analysis. APC, allophycocyanin; PE, phycoerythrin; FITC, fluorescein isothiocyanate; BV, brilliant violet; AF, Alexa Fluor; BUV, brilliant ultraviolet

Antibody	Source	Dilution	Identifier
anti-CD146 APC (clone 541-10B2)	Miltenyi Biotec	1:200	Cat#: 130-097-942, RRID:AB_2660769
anti-CD146 PE (clone P1H12)	BioLegend	1:200	Cat#: 361005, RRID:AB_2562980
anti-CD19 FITC (clone HIB19)	eBioscience	1:200	Cat#: 11-0199-41, RRID:AB_10668005
anti-CD197 PE/Cy7 (clone G043H7)	BioLegend	1:200	Cat#: 353226, RRID:AB_11126145
anti-CD223 BV785 (clone 11C3C65)	BioLegend	1:200	Cat#: 369322, RRID:AB_2716127
anti-CD27 APC/Cy7 (clone M-T271)	BioLegend	1:200	Cat#: 356424, RRID:AB_2566773
anti-CD279 PE (clone EH12.2H7)	BioLegend	1:200	Cat#: 329906, RRID:AB_940483
anti-CD279 APC (clone EH12.2H7)	BioLegend	1:200	Cat#: 329908, RRID:AB_940475
anti-CD279 BV421 (clone EH12.2H7)	BioLegend	1:200	Cat#: 329920, RRID:AB_10960742
anti-CD3 BV711 (clone UCHT1)	BioLegend	1:200	Cat#: 300463, RRID:AB_2566035
anti-CD340 AF647 (clone 24D2)	BioLegend	1:200	Cat#: 324412, RRID:AB_2262300
anti-CD366 PE (clone 7D3)	BD Biosciences	1:200	Cat#: 563422, RRID:AB_2716866
anti-CD366 BV421 (clone F38-2E2)	BioLegend	1:200	Cat#: 345007, RRID:AB_10900073
anti-CD366 APC/Cy7 (clone F38-2E2)	BioLegend	1:200	Cat#: 345026, RRID:AB_2565717
anti-CD39 BV711 (clone A1)	BioLegend	1:200	Cat#: 328228, RRID:AB_2632894
anti-CD4 BV650 (clone SK3)	BD Biosciences	1:200	Cat#: 563875, RRID:AB_2744425
anti-CD4 PE (clone SK3)	BioLegend	1:200	Cat#: 344606, RRID:AB_1937246
anti-CD45 APC/Cy7 (clone 2D1)	BioLegend	1:200	Cat#: 368515, RRID:AB_2566375
anti-CD45 BUV395 (clone HI30)	BD Biosciences	1:200	Cat#: 563792, RRID:AB_2869519
anti-CD45RA APC (clone HI100)	BioLegend	1:200	Cat#: 304150, RRID:AB_2564158
anti-CD45RA BV711 (clone HI100)	BioLegend	1:200	Cat#: 304138, RRID:AB_2563815
anti-CD45RO BUV395 (clone UCHL1)	BD Biosciences	1:200	Cat#: 564291, RRID:AB_2744410
anti-CD62L BV785 (clone DREG-56)	BioLegend	1:200	Cat#: 304830, RRID:AB_2629555
anti-CD62L PE/Cy7 (clone DREG-56)	BioLegend	1:200	Cat#: 304822, RRID:AB_830801
anti-CD69 BUV395 (clone FN50)	BD Biosciences	1:200	Cat#: 564364, RRID:AB_2738770
anti-CD69 APC (clone FN50)	BioLegend	1:200	Cat#: 310910, RRID:AB_314845
anti-CD8a BUV395 (clone RPA-T8)	BD Biosciences	1:200	Cat#: 563796
anti-CD8a BV786 (clone RPA-T8)	BD Biosciences	1:200	Cat#: 563823, RRID:AB_2687487
anti-CD8a PE (clone SK1)	BioLegend	1:200	Cat#: 344706, RRID:AB_1953244
anti-CD8a APC (clone OKT8)	eBioscience	1:200	Cat#: 17-0086-42, RRID:AB_10667892
anti-CD95 BV711 (clone DX2)	BioLegend	1:200	Cat#: 305644, RRID:AB_2632623
anti-FLAG (DYKDDDDK) tag PE (clone L5)	BioLegend	1:200	Cat#: 637310, RRID:AB_2563148
anti-FLAG (DYKDDDDK) tag AF647 (clone L5)	BioLegend	1:200	Cat#: 637315, RRID:AB_2716154
anti-FLAG (DYKDDDDK) tag BV421 (clone L5)	BioLegend	1:200	Cat#: 637322, RRID:AB_2750061
anti-HA tag PE (clone GG8-1F3.3.1)	Miltenyi Biotec	1:200	Cat#: 130-120-786, RRID:AB_2784360
anti-IFN-γ BV786 (clone 4S.B3)	BD Biosciences	1:200	Cat#: 563731, RRID:AB_2738391
anti-IL-2 PE/Cy7 (clone MQ1-17H12)	BD Biosciences	1:200	Cat#: 560707, RRID:AB_1727542
anti-Mesothelin APC (clone REA1057)	Miltenyi Biotec	1:200	Cat#: 130-118-096, RRID:AB_2733436
anti-Myc tag AF647 (clone 9B11)	Cell Signaling Technology	1:200	Cat#: 2233S, RRID:AB_823474
anti-TNF BUV395 (clone MAb11)	BD Biosciences	1:200	Cat#: 563996, RRID:AB_2738533