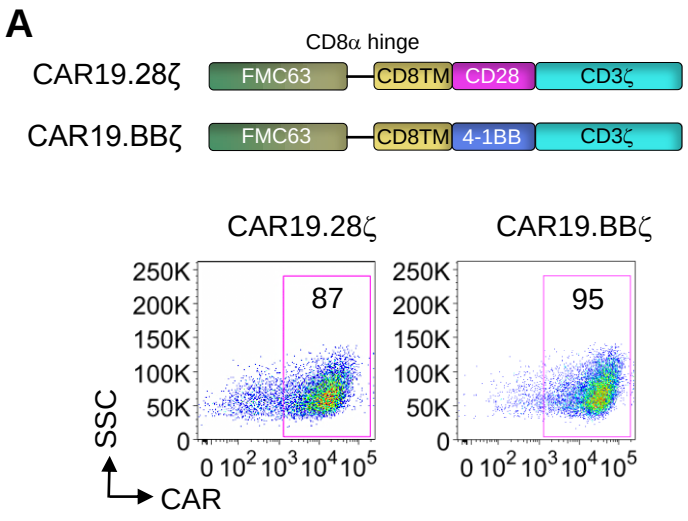
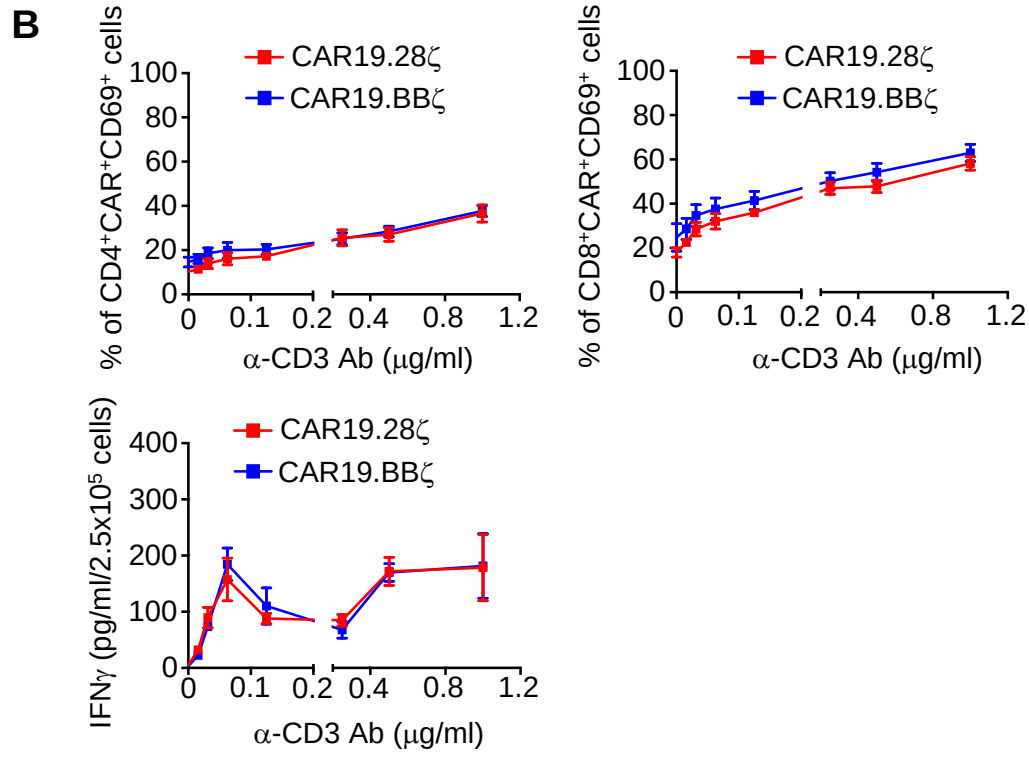
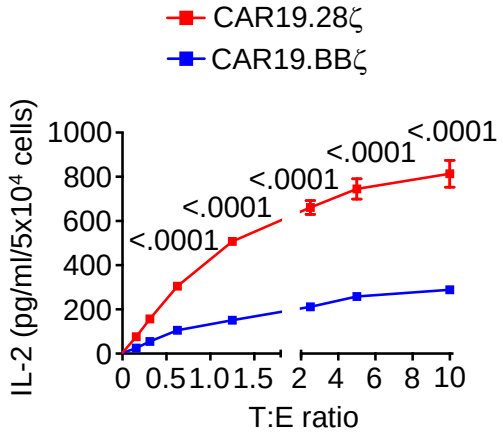
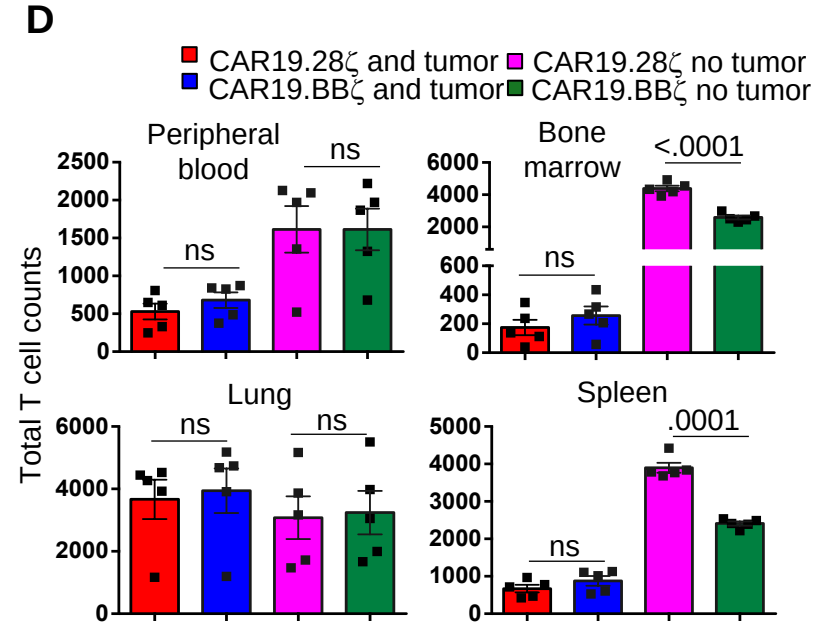
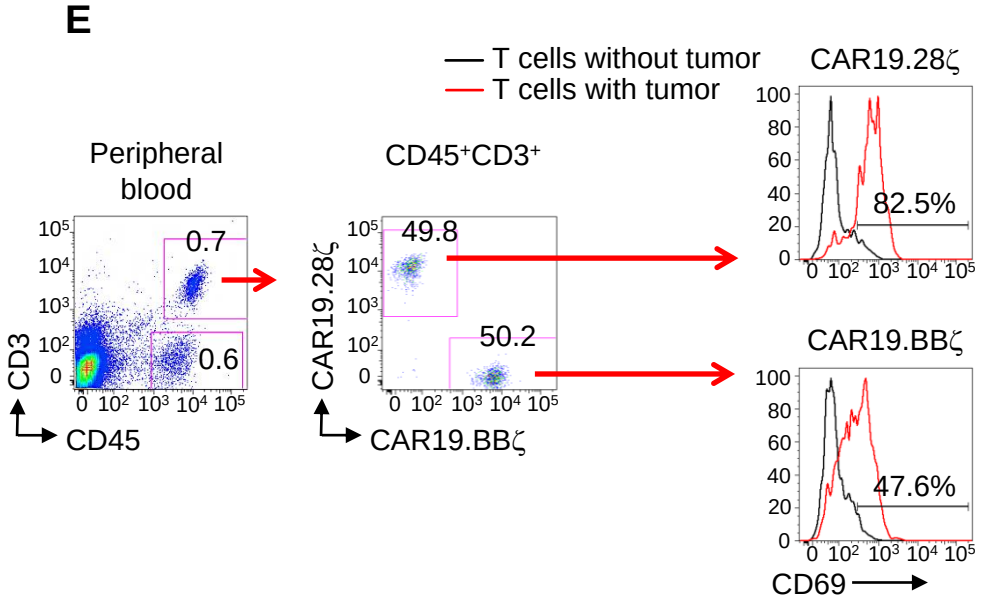
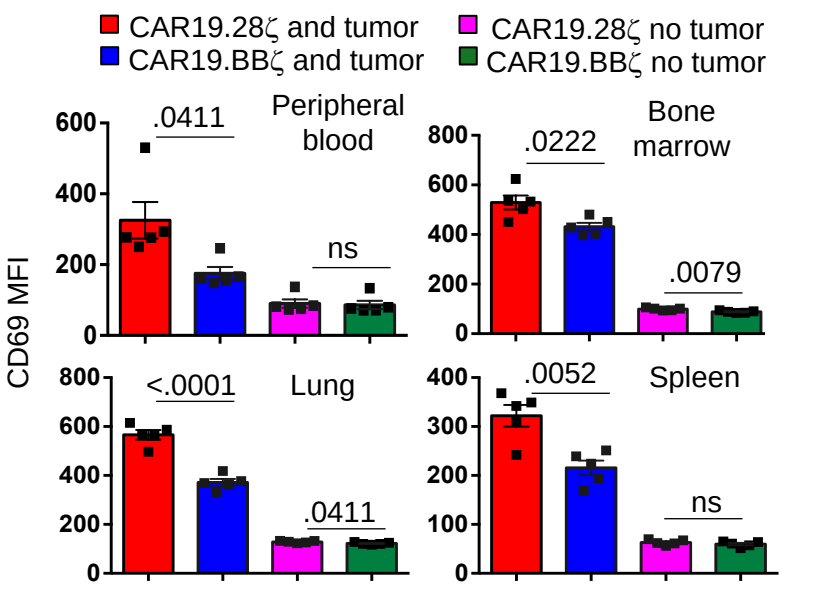

A
CD8α hinge
CAR19.28ζ
FMC63
CD8TM
CD28
CD3ζ
CAR19.BBζ
FMC63
CD8TM
4-1BB
CD3ζ
CAR19.28ζ
CAR19.BBζ
250K
200K
150K
100K
50K
0
87
95
SSC
CAR
0 10² 10³ 10⁴ 10⁵

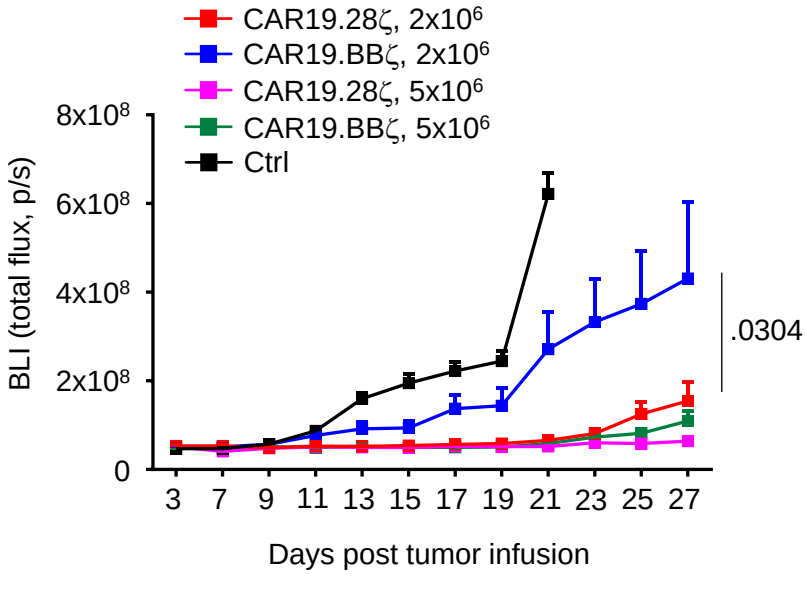

B
CAR19.28ζ
CAR19.BBζ
% of CD4⁺CAR⁺CD69⁺ cells
% of CD8⁺CAR⁺CD69⁺ cells
IFNγ (pg/ml/2.5x10⁵ cells)
α-CD3 Ab (μg/ml)
0 0.1 0.2 0.4 0.8 1.2


C
CAR19.28ζ
CAR19.BBζ
IL-2 (pg/ml/5x10⁴ cells)
<.0001
<.0001
<.0001
<.0001
<.0001
T:E ratio
0 0.5 1.0 1.5 2 4 6 8 10

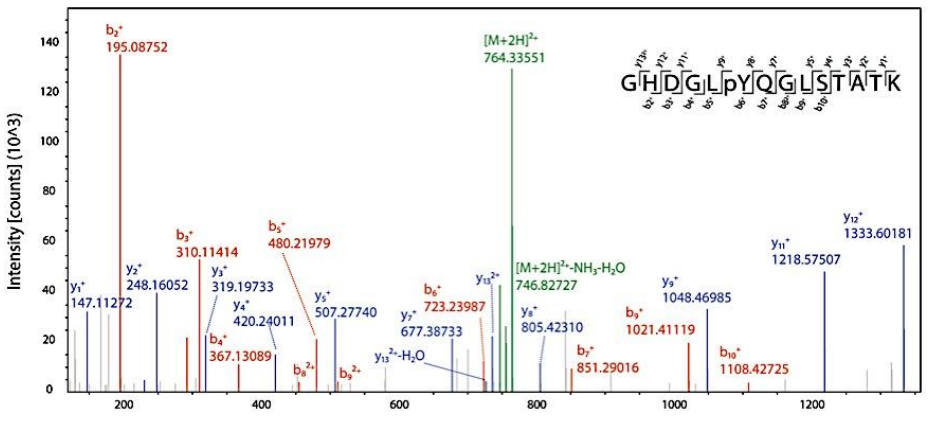

D
CAR19.28ζ and tumor
CAR19.28ζ no tumor
CAR19.BBζ and tumor
CAR19.BBζ no tumor
Peripheral blood
Bone marrow
Lung
Spleen
ns
<.0001
.0001
Total T cell counts


E
T cells without tumor
T cells with tumor
Peripheral blood
CD45⁺CD3⁺
CD3
CD45
CAR19.28ζ
CAR19.BBζ
0.7
0.6
49.8
50.2
82.5%
47.6%
CD69

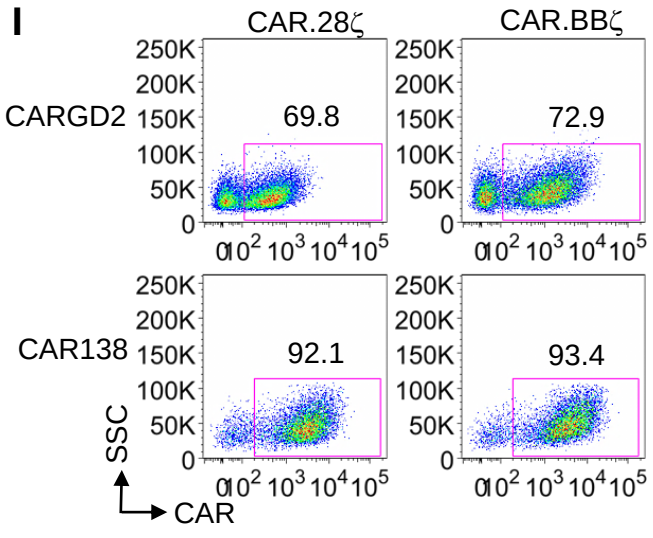

F
CAR19.28ζ and tumor
CAR19.28ζ no tumor
CAR19.BBζ and tumor
CAR19.BBζ no tumor
Peripheral blood
Bone marrow
Lung
Spleen
.0411
.0222
.0079
.0052
ns
CD69 MFI


G
CAR19.28ζ, 2x10⁶
CAR19.BBζ, 2x10⁶
CAR19.28ζ, 5x10⁶
CAR19.BBζ, 5x10⁶
Ctrl
BLI (total flux, p/s)
8x10⁸
6x10⁸
4x10⁸
2x10⁸
0
.0304
3 7 9 11 13 15 17 19 21 23 25 27
Days post tumor infusion

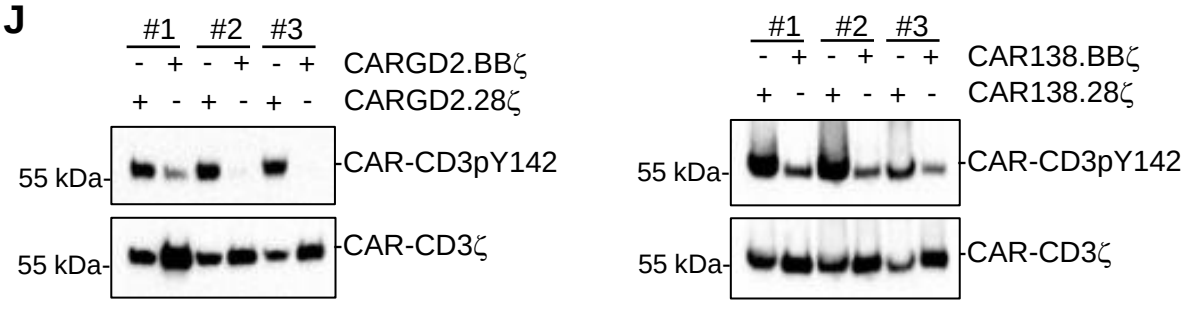
H



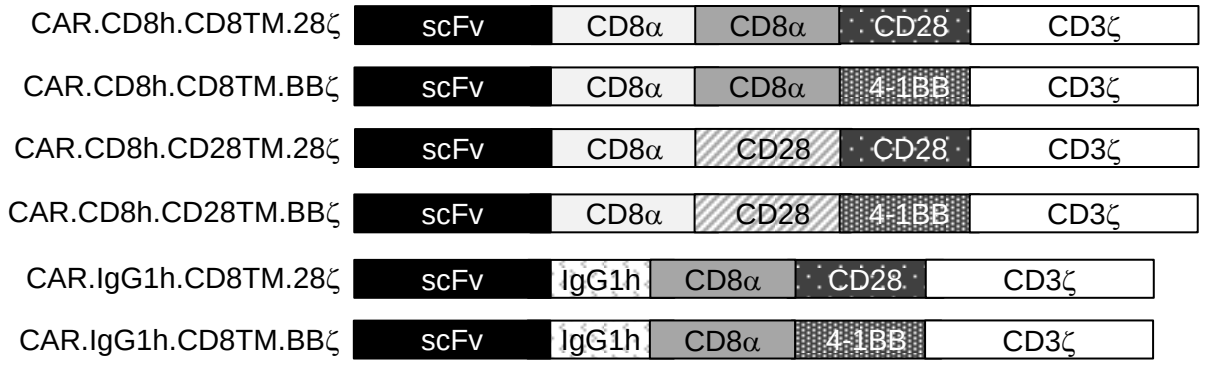
I



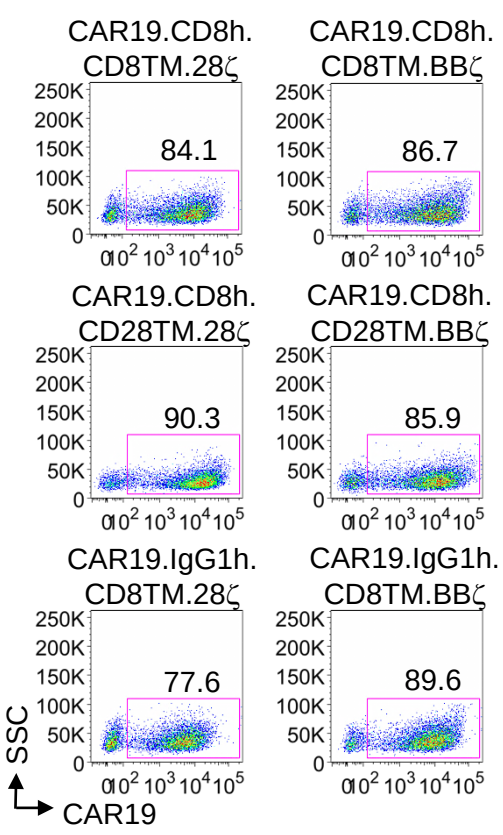
J



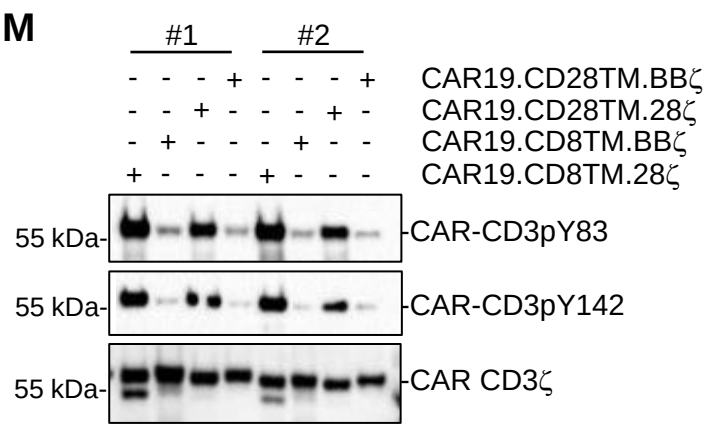
K



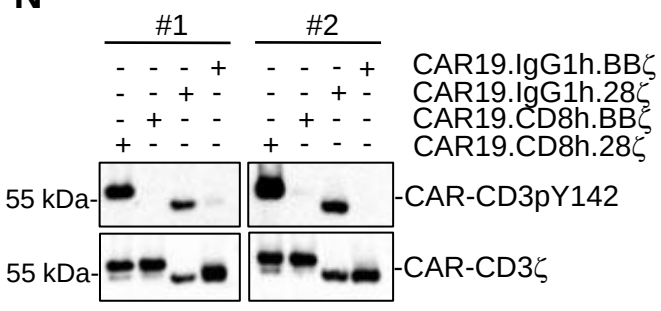
L



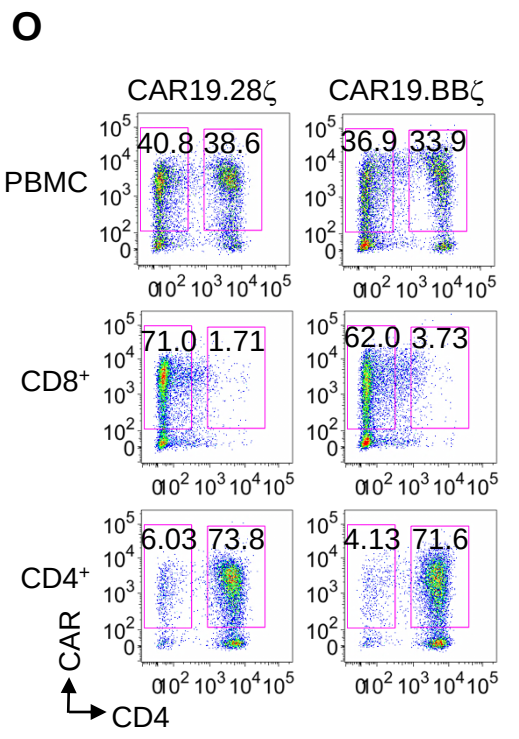
M



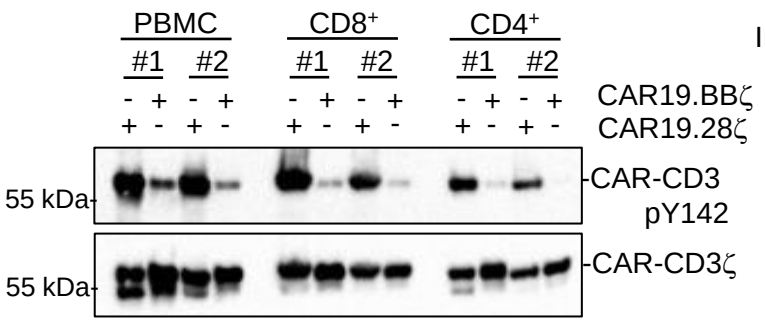
N



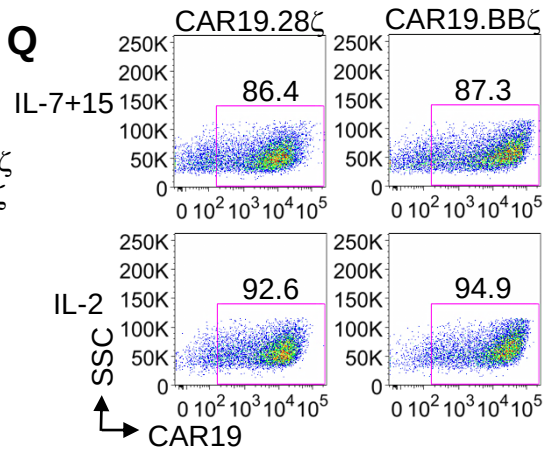
O



P



Q



R

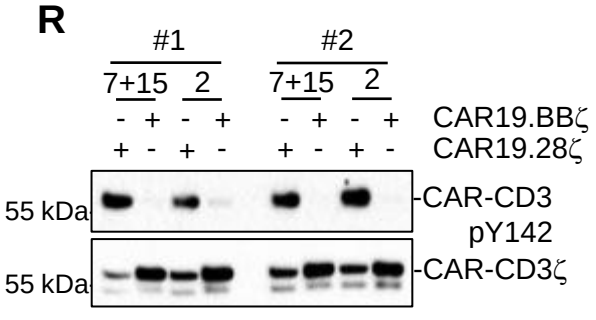


Fig. S1 (Related to Figure 1). CAR19.28ζ-T cells show higher magnitude of activation as compared to CAR19.BBζ-T cells after CAR crosslinking.

(**A**) Schematic representation of the CAR19.28ζ and CAR19.BBζ constructs (upper panel) and flow plots showing the expression of the CARs in T cells (lower panel). Representative of more than 30 donors. (**B**) CD69 expression in $CD4^+$ T cells (upper left panel) and in $CD8^+$ T cells (upper right panel), and IFNγ release (lower left panel) in CAR19.28ζ-T and CAR19.BBζ-T cells upon stimulation with αCD3 Ab (n = 11 for CD69 expression, n = 3 for IFNγ release, two-way ANOVA). (**C**) IL-2 released in the culture supernatants by CAR19.28ζ-T and CAR19.BBζ-T cells stimulated with $CD19^+$ BV173 cells at different tumor to T cell ratios (T:E) (n = 3, two-way ANOVA; representative of 6 donors). (**D-F**) NSG mice engrafted with the $CD19^+$ Daudi cell line were infused with differentially labeled CAR19.28ζ-T and CAR19.BBζ-T cells mixed at 1:1 ratio. Samples were collected 6 hr after T cell infusion. Non-tumor bearing NSG mice infused with mixed CAR19.28ζ-T and CAR19.BBζ-T cells were used as a negative control. Total T cell counts ($CD45^+CD3^+$ cells) were identified in the peripheral blood, bone marrow, lung, and spleen (**D**). Gating strategy (**E**) and CD69 expression (**F**) in T cells collected from the peripheral blood, bone marrow, lung, and spleen (n = 5, two-tailed unpaired *t*-test). (**G**) Tumor growth measured by bioluminescence in NSG mice engrafted with $CD19^+$ Daudi cells and infused with CAR19.28ζ-T and CAR19.BBζ-T cells at two different doses (Two-way ANOVA; p value between CAR19.BBζ-T cells 2 x 10^6 and CAR19.28ζ-T cells 2 x 10^6). (**H**) Tandem mass spectrum confirming Y142 phosphorylation of CAR-CD3ζ domain in 1 donor. (**I**) Representative flow plots showing the expression of CARGD2 (targeting the GD2 antigen) and CAR138 (targeting the CD138 antigen) in T cells of one representative donor at day 6 of culture. (**J**) CAR-CD3ζ Y142 phosphorylation in CARGD2-T cells and CAR138-T cells collected at day 10 of culture. Results of 3 representative donors were shown. (**K**) Schema of CAR19 constructs with different hinge (CD8α vs. IgG1h) or transmembrane (CD8α vs. CD28) domains. (**L**) Flow plots of the expression of CARs indicated in (D) in one representative donor at day 6 of culture. (**M**) CAR-CD3ζ Y142 phosphorylation of CAR-T cells with CD28 transmembrane domain as compared to those with CD8α transmembrane domain. Results of 2 representative donors were shown. (**N**) CAR-CD3ζ Y142 phosphorylation in CAR-T cells with the IgG1 hinge as compared to those with the CD8α hinge. Cells were collected at day 10 of culture. Results of 2 representative donors were shown. (**O**) CAR19.28ζ and CAR19.BBζ expression in T cells activated from total peripheral blood mononuclear cells (PBMCs) or from isolated $CD4^+$ and $CD8^+$ T cells. (**P**) CAR-CD3ζ Y142 phosphorylation in CAR19.28ζ-T and CAR19.BBζ-T cells obtained from total PBMCs or isolated $CD4^+$ and $CD8^+$ T cells at day 10 of culture. Results of 2 representative donors were shown. (**Q**) Flow plots showing CAR expression in CAR19.28ζ-T and CAR19.BBζ-T cells cultured in IL-7 and IL-15 (upper panels) or IL-2 (lower panels) at day 6 of culture. (**R**) CAR-CD3ζ Y142 phosphorylation in CAR19.28ζ-T and CAR19.BBζ-T cells expanded with different cytokines for 10 days. Results of 2 representative donors were shown. All data were presented as mean ± SEM.

Table S1 (Related to Figure 1). Phosphorylation sites identified on CAR19.28ζ-T and CAR19.BBζ-T cells by LC-MS/MS analysis.

			Peak area	
Annotated sequence	Phosphoylation site in peptide	Phosphorylation site in protein CAR19.28z/ CAR19.BBz(native CD3z)	CAR19.28z	CAR19.BBz
[R].LLHSDYMNMTPR.[R]	T10	356	2.93E+07	ND
[K].HYQPYAPPR.[D]	Y5	370	3.74E+06	ND
[R].SADAPAYQQGQNQLYNELNLGR.[R]	S1	388/389	ND	3.62E+07
[R].SADAPAYQQGQNQLYNELNLGR.[R]	Y15	402 (Y72)	4.60E+07	ND
[R].REEYDVLDKR.[R]	Y4	413 (Y83)	**	ND
[K].NPQEGLYNELQK.[D]	Y7	440/441(Y111)	2.01E+07	ND
[K].MAEAYSEIGMK.[G]	Y5	452/453 (Y123)	1.41E+07	ND
[R].GKGHDGLYQGLSTATK.[D]	Y8	471 (Y142)	4.23E+07	ND

The peptide sequence containing the phosphosite identified is listed, along with the phosphosite within the peptide and protein (CAR19.28ζ and CAR19.BBζ, or corresponding site on native CD3ζ in parentheses). The phosphoRS probability node within Proteome Discoverer 2.1 was used to localize the phosphorylation sites and only phosphopeptides with a phosphoRS probability of 100% were considered. Peak areas for each phosphopeptide were extracted using the peak area node within Proteome Discoverer 2.1. Abbreviations: ND = not detected; ** =detected, but peak area could not be extracted.

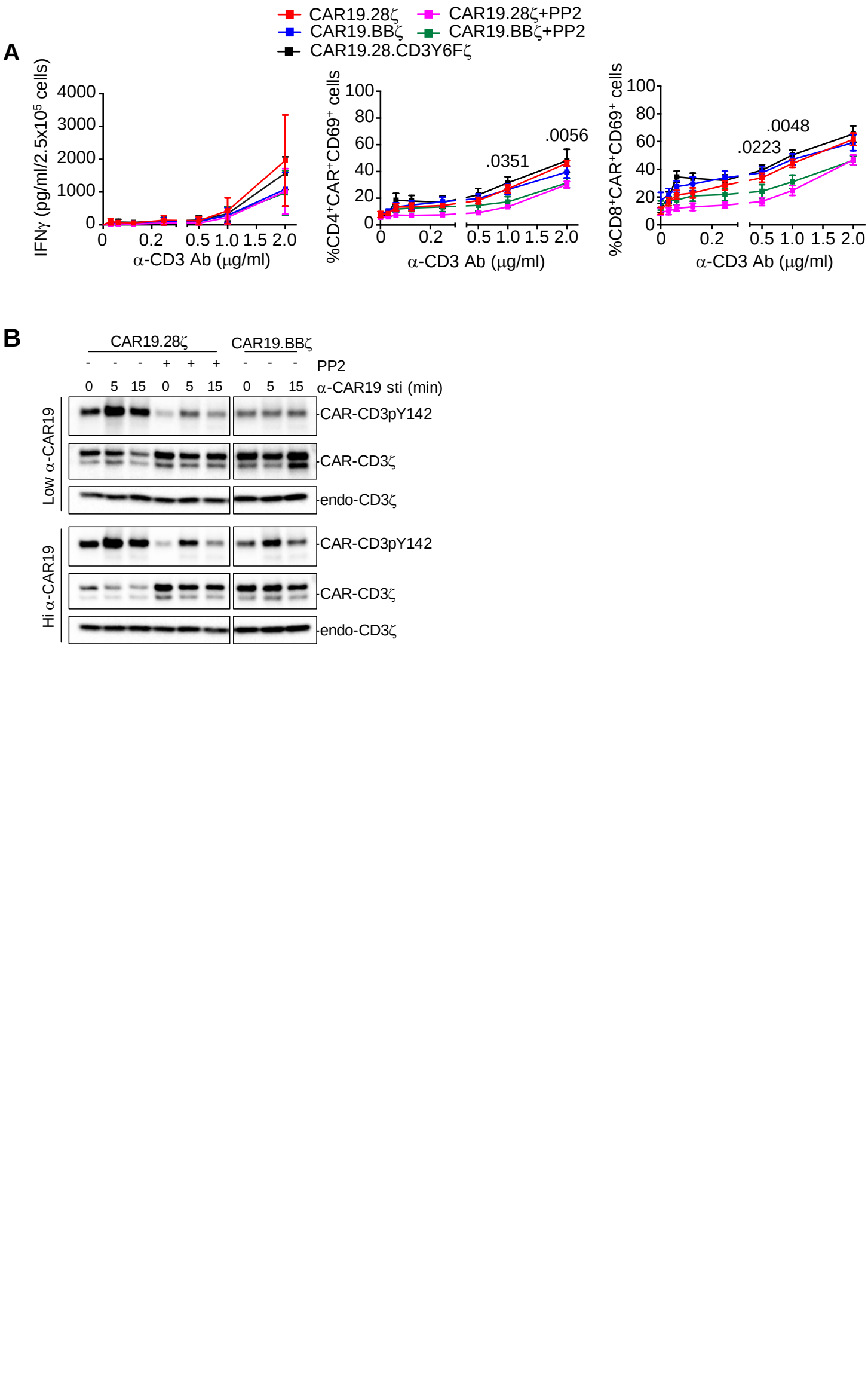
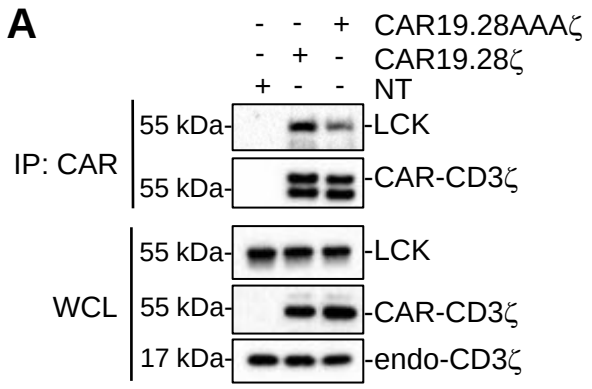
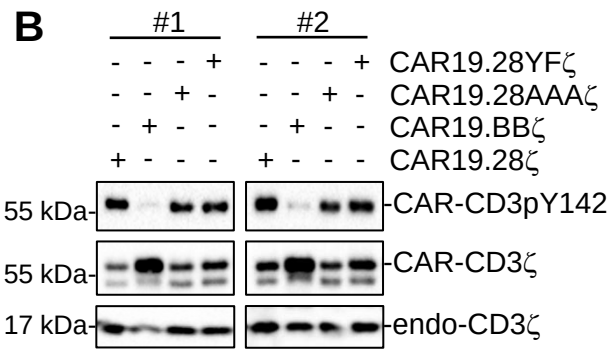
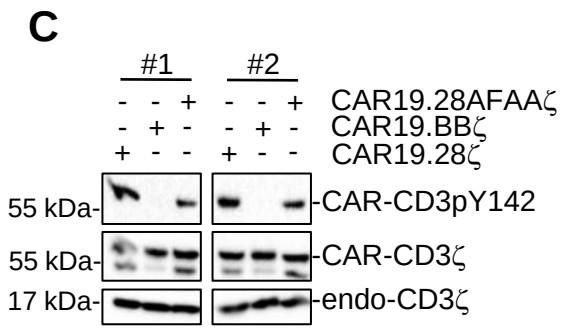

A
CAR19.28ζ
CAR19.BBζ
CAR19.28.CD3Y6Fζ
CAR19.28ζ+PP2
CAR19.BBζ+PP2
IFNγ (pg/ml/2.5x10^5 cells)
4000
3000
2000
1000
0
0 0.2 0.5 1.0 1.5 2.0
α-CD3 Ab (μg/ml)
%CD4+CAR+CD69+ cells
100
80
60
40
20
0
.0351
.0056
%CD8+CAR+CD69+ cells
.0223
.0048
B
CAR19.28ζ
CAR19.BBζ
- - - + + + - - - PP2
0 5 15 0 5 15 0 5 15 α-CAR19 sti (min)
Low α-CAR19
Hi α-CAR19
CAR-CD3pY142
CAR-CD3ζ
endo-CD3ζ

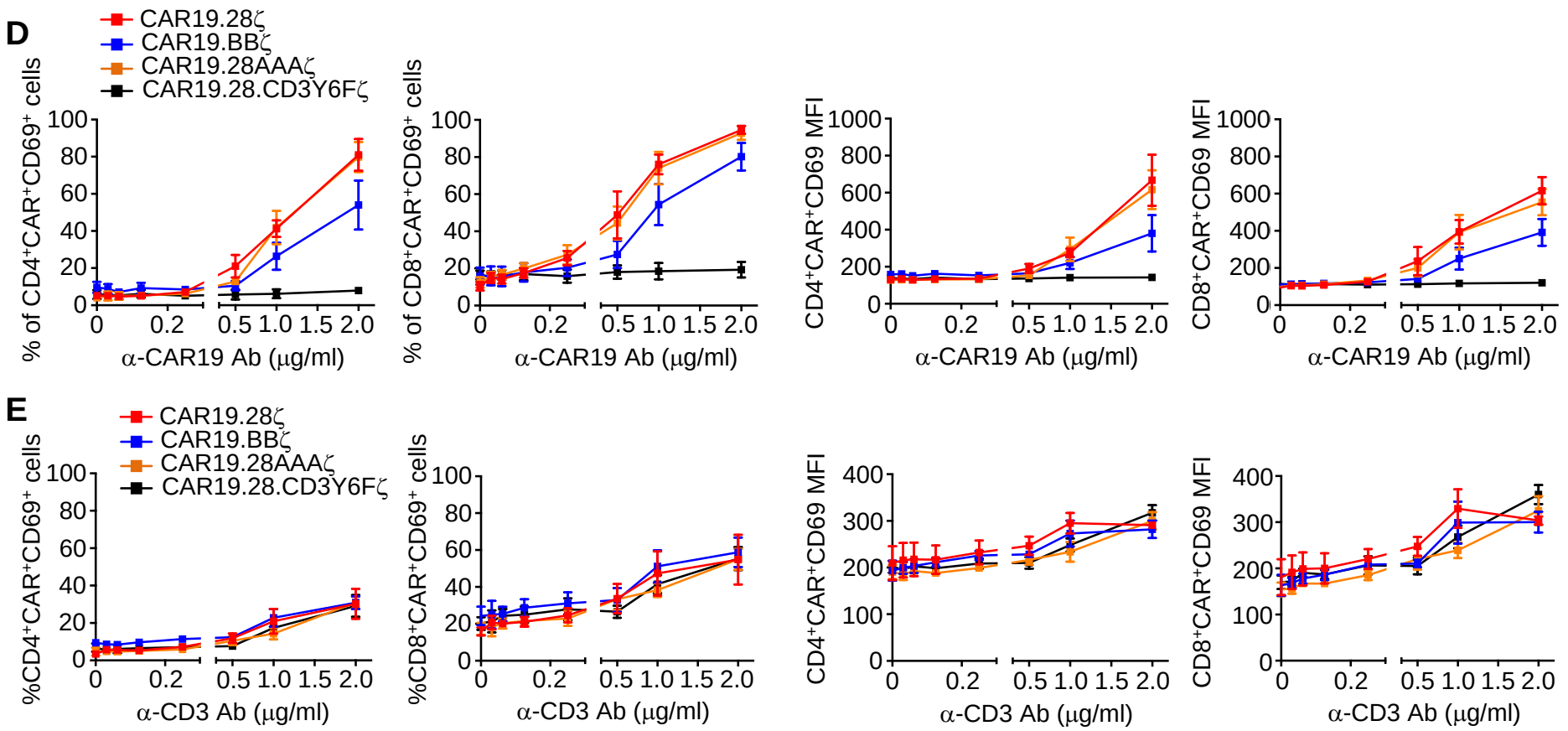
Fig. S2 (Related to Figure 2). CAR-CD3ζ basal phosphorylation causes higher magnitude of activation of CAR19.28ζ-T cells than CAR19.BBζ-T cells after CAR crosslinking.

(**A**) CAR19.28ζ-T and CAR19.BBζ-T cells at day 14 of culture were pretreated with 10 μM PP2 for 16 hr, washed 3 times, and then stimulated with α-CD3 Ab at different concentrations for 6 hr. CAR19.28ζ-T and CAR19.BBζ-T cells not pretreated with PP2 were used as control. Cytokine release and CD69 expression were quantified (n = 3 for cytokine release, n = 5 for CD69 expression, two-way ANOVA; p value between CAR19.28ζ-T and CAR19.28ζ-T cells + PP2 groups). (**B**) CAR19.28ζ-T and CAR19.BBζ-T cells were pretreated with PP2 as in (A). Cells were incubated with α-CD19 Ab followed by incubation with a goat anti-mouse IgG secondary antibody on ice. CAR-T cells were then transferred to 37° C for indicated time to be activated. Phosphorylation of CAR-CD3ζ was detected (low α-CAR19 Ab, 1 μg/ml; high α-CAR19 Ab, 10 μg/ml) (n = 2). All data were presented as mean ± SEM.


A
- - + CAR19.28AAAζ
- + - CAR19.28ζ
+ - - NT
IP: CAR
55 kDa-LCK
55 kDa-CAR-CD3ζ
WCL
55 kDa-LCK
55 kDa-CAR-CD3ζ
17 kDa-endo-CD3ζ


B
#1
#2
CAR19.28YFζ
CAR19.28AAAζ
CAR19.BBζ
CAR19.28ζ
55 kDa-CAR-CD3pY142
55 kDa-CAR-CD3ζ
17 kDa-endo-CD3ζ


C
#1
#2
CAR19.28AFAAζ
CAR19.BBζ
CAR19.28ζ
55 kDa-CAR-CD3pY142
55 kDa-CAR-CD3ζ
17 kDa-endo-CD3ζ


D
CAR19.28ζ
CAR19.BBζ
CAR19.28AAAζ
CAR19.28.CD3Y6Fζ
% of CD4+CAR+CD69+ cells
% of CD8+CAR+CD69+ cells
CD4+CAR+CD69 MFI
CD8+CAR+CD69 MFI
α-CAR19 Ab (μg/ml)

E
CAR19.28ζ
CAR19.BBζ
CAR19.28AAAζ
CAR19.28.CD3Y6Fζ
%CD4+CAR+CD69+ cells
%CD8+CAR+CD69+ cells
CD4+CAR+CD69 MFI
CD8+CAR+CD69 MFI
α-CD3 Ab (μg/ml)

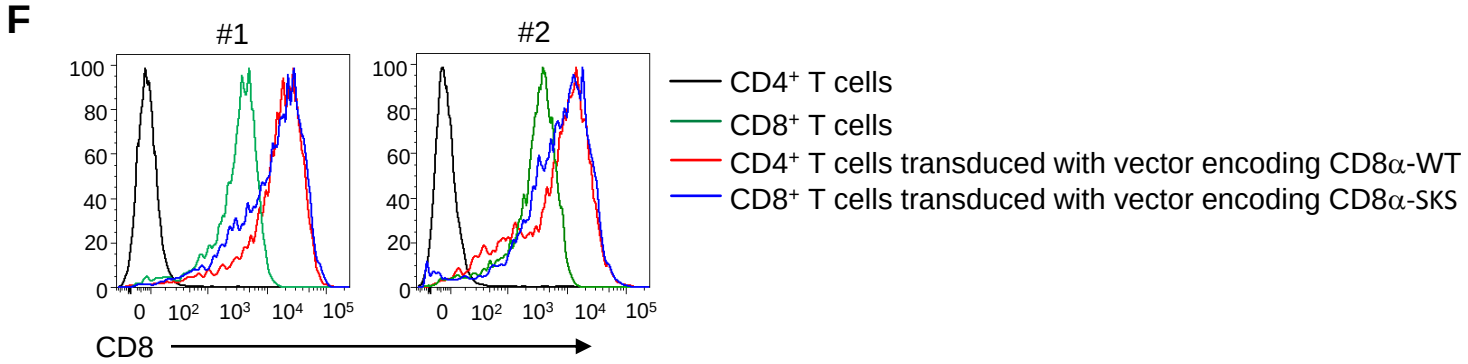

F
#1
#2
CD8
CD4+ T cells
CD8+ T cells
CD4+ T cells transduced with vector encoding CD8α-WT
CD8+ T cells transduced with vector encoding CD8α-SKS

Fig. S3 (Related to Figure 3). Co-receptors rather than CD28 bring LCK in the CAR synapse of CAR19.28ζ-T cells.

(**A**) CAR was pulled down from CAR19.28ζ-T and CAR19.28AAAζ-T cells at day 14 of culture. LCK in the IP product was evaluated by western blot using an α-LCK Ab. (**B** and **C**) CAR-T cells were collected for analysis at day 10 of culture. CAR-CD3ζ Y142 phosphorylation was assessed by western blot. Results of 2 representative donors were shown. (**D** and **E**) CD69 expression as percentage of positive cells and MFI in CAR-T cells activated by the α-CAR19 Ab (**D**) or α-CD3 Ab (**E**) at different concentrations. (n = 3). (**F**) Expression of CD8α in $CD4^+$ CAR19.28ζ-T cells transduced with the vector encoding CD8α-WT or in $CD4^+$ CAR19.BBζ-T cells transduced with the vector encoding CD8α-SKS as compared to CD8α expression in $CD8^+$ T cells. Results of 2 representative donors were shown. All data were presented as mean ± SEM.

Table S2 (Related to Figure 3). Proteomics results for selected proteins in IP samples of CAR19.28ζ-T and CAR19.BBζ-T cells.

		CAR19.28z				CAR19.BBz			
Accession	Gene symbol	Peptides	Coverage	Area	SC	Peptides	Coverage	Area	SC
P06239	LCK	12	32%	6.84E+08	60	13	34%	4.48E+08	61
Q8N1K5	THEMIS	2	3%	1.89E+07	9	2	4%	3.78E+07	14
P27986	PIK3R1	11	17%	2.30E+08	40	1	2%	1.51E+07	3
O00329	PIK3CD	12	13%	1.79E+08	39	2	2%	5.10E+06	2
Q13077	TRAF1	-	-	-	-	4	15%	2.31E+08	16
Q12933	TRAF2	-	-	-	-	6	14%	5.52E+07	12
P01732	CD8A	7	32%	1.59E+08	17	6	32%	7.34E+08	16

The number of peptides identified and percent coverage for each protein is averaged across three biological replicates. A peptide false discovery rate of 5% was used to filter all data. Areas and spectral counts (SC) were used as abundance measures. Areas are summed across all biological replicates and were calculated by averaging the peak areas of the top 3 (or less) unique peptides for each protein. SCs were calculated by summing the number of identified peptide spectrum matches for each protein across all biological replicates. Values not identified are denoted by “-”.

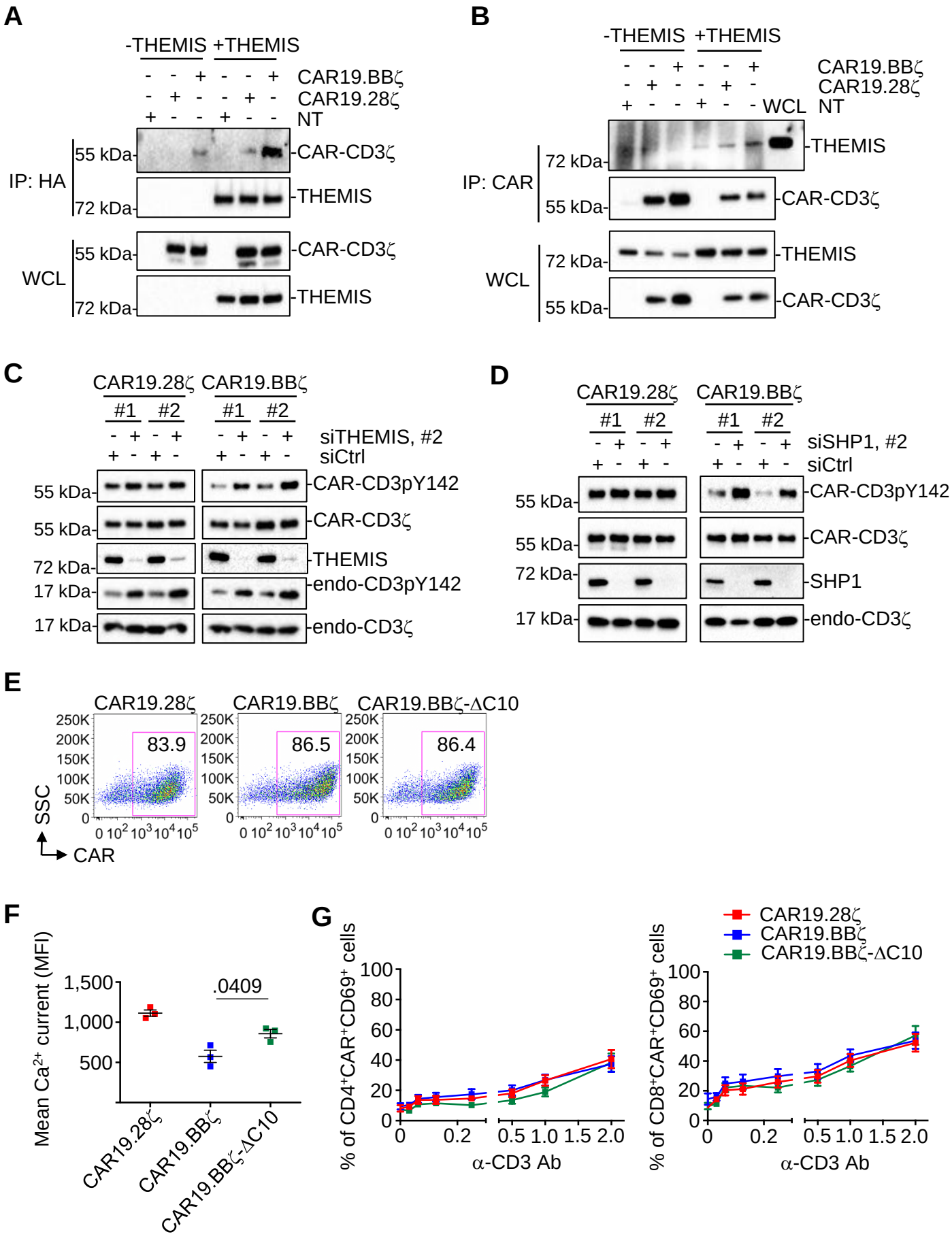
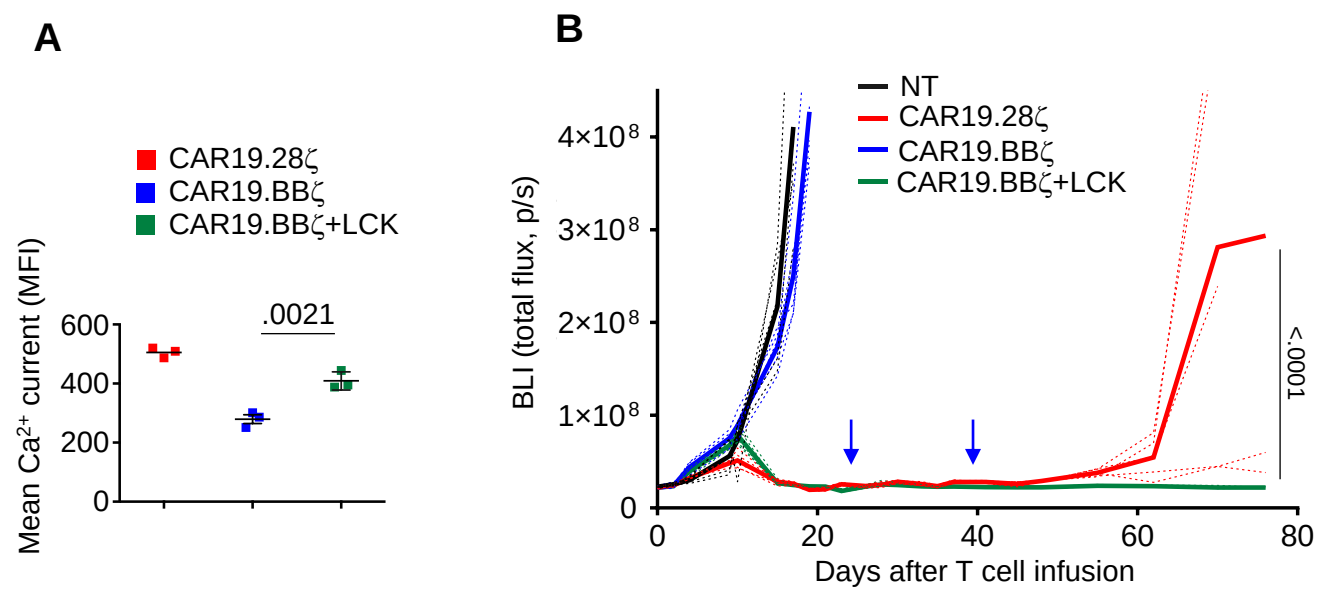

A
-THEMIS +THEMIS
CAR19.BBζ
CAR19.28ζ
NT
IP: HA
55 kDa
72 kDa
CAR-CD3ζ
THEMIS
WCL
B
-THEMIS +THEMIS
CAR19.BBζ
CAR19.28ζ
NT
WCL
IP: CAR
72 kDa
55 kDa
THEMIS
CAR-CD3ζ
C
CAR19.28ζ CAR19.BBζ
#1 #2 #1 #2
siTHEMIS, #2
siCtrl
55 kDa
72 kDa
17 kDa
CAR-CD3pY142
CAR-CD3ζ
THEMIS
endo-CD3pY142
endo-CD3ζ
D
CAR19.28ζ CAR19.BBζ
#1 #2 #1 #2
siSHP1, #2
siCtrl
55 kDa
72 kDa
17 kDa
CAR-CD3pY142
CAR-CD3ζ
SHP1
endo-CD3ζ
E
CAR19.28ζ
CAR19.BBζ
CAR19.BBζ-ΔC10
83.9
86.5
86.4
SSC
CAR
F
Mean Ca2+ current (MFI)
.0409
CAR19.28ζ
CAR19.BBζ
CAR19.BBζ-ΔC10
G
CAR19.28ζ
CAR19.BBζ
CAR19.BBζ-ΔC10
% of CD4+CAR+CD69+ cells
% of CD8+CAR+CD69+ cells
IFNγ (pg/ml/2.5x10^5 cells)
α-CD3 Ab

Fig. S4 (Related to Figure 4). THEMIS-SHP1 complex attenuates CAR-CD3ζ basal phosphorylation of CAR19.BBζ-T cells.

(**A**) CAR19.28ζ-T or CAR19.BBζ-T cells co-transduced to express HA-tagged THEMIS were collected at day 14 of culture and THEMIS molecules were pulled down by HA IP. CAR in the IP product was evaluated by western blot using the α-CD3ζ Ab. (**B**) Jurkat cells engineered to express CAR19.28ζ and CAR19.BBζ were co-transduced with the lentiviral vector encoding THEMIS with Strep-tag II (THEMIS-STPII). These cells were then used for CAR immune precipitation. The presence of THEMIS-STPII in the CAR IP products was determined by western blot using the α-THEMIS Ab. (**C**) CAR19.28ζ-T and CAR19.BBζ-T cells co-transduced with lentiviral vector encoding a second shRNA specific for THEMIS (siTHEMIS, #2) and selected by puromycin were collected at day 10 of culture. CAR-CD3ζ pY142 and THEMIS levels were evaluated by western blot. Results of 2 representative donors were shown. (**D**) CAR19.28ζ-T and CAR19.BBζ-T cells were co-transduced with vectors encoding shRNA (#2) specific for SHP1 and selected by puromycin. CAR-CD3ζ Y142 phosphorylation and SHP1 were measured by western blot. Results of 2 representative donors were shown. (**E**) Expression of CAR19.BBζ and its mutant CAR19.BBζ-ΔC10 (deletion of 10 AA at the COOH of 4-1BB) in T cells. Representative of 3 donors. (**F**) Ca^{2+} influx of CAR19.28ζ-T cells, CAR19.BBζ-T cells or CAR19.BBζ-ΔC10-T cells upon stimulation with the α-CAR19 Ab. The panel illustrates quantitative analysis of the mean Ca^{2+} current (n = 3, one-way ANOVA). (**G**) CAR19.28ζ-T cells, CAR19.BBζ-T cells, or CAR19.BBζ-ΔC10-T cells were stimulated α-CD3 Ab. CD69 expression and IFNγ release were assessed by flow cytometry and ELISA, respectively (n = 3 for IFNγ release, n = 5 for CD69 expression, two-way ANOVA; p value between CAR19.BBζ-T cells and CAR19.BBζ-ΔC10-T cell groups). All data were presented as mean ± SEM.


A
CAR19.28ζ
CAR19.BBζ
CAR19.BBζ+LCK
Mean Ca2+ current (MFI)
600
400
200
0
.0021
B
NT
CAR19.28ζ
CAR19.BBζ
CAR19.BBζ+LCK
BLI (total flux, p/s)
4×10^8
3×10^8
2×10^8
1×10^8
0
<.0001
0
20
40
60
80
Days after T cell infusion

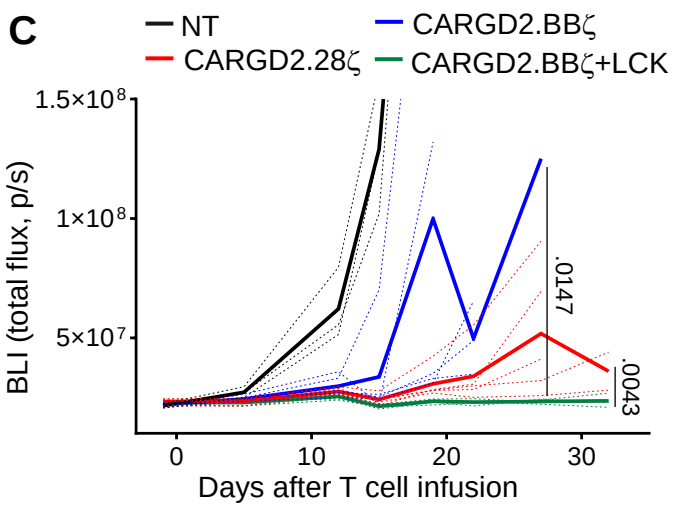

C
NT
CARGD2.28ζ
CARGD2.BBζ
CARGD2.BBζ+LCK
BLI (total flux, p/s)
1.5×10^8
1×10^8
5×10^7
.0147
.0043
0
10
20
30
Days after T cell infusion

Fig. S5 (Related to Figure 5). Engineering LCK kinase in CAR-T cells encoding 4-1BB enhances antitumor activity.
(**A**) Quantification of Ca^{2+} influx in CAR19.28ζ-T or CAR19.BBζ-T cells with or without co-expression of LCK (n = 3, one-way ANOVA). (**B**) Tumor growth monitored by BLI in NSG mice engrafted with the $CD19^{+}$ Daudi cells and infused with suboptimal dose of CAR19.28ζ-T or CAR19.BBζ-T cells with or without LCK (n = 5, two-way ANOVA). (**C**) Tumor growth monitored by BLI in NSG mice engrafted with the neuroblastoma tumor cell line CHLA-255 and infused with suboptimal dose of either CARGD2.28ζ-T or CARGD2.BBζ-T cells with or without LCK (n = 3 in NT group, n = 5 in other groups, two-way ANOVA). All data were presented as mean ± SEM.

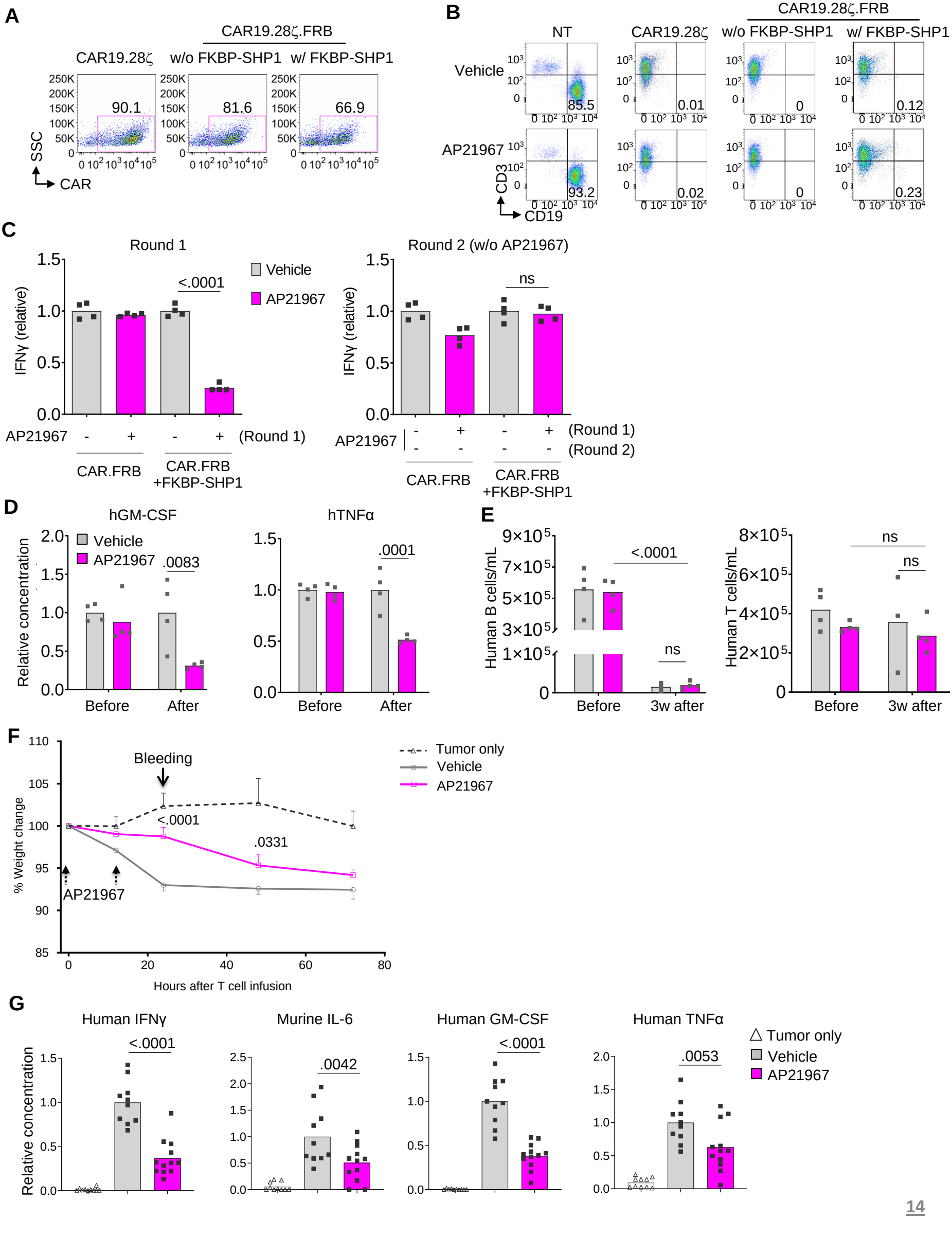

A
CAR19.28ζ.FRB
CAR19.28ζ
w/o FKBP-SHP1
w/ FKBP-SHP1
90.1
81.6
66.9
SSC
CAR
B
NT
CAR19.28ζ
w/o FKBP-SHP1
w/ FKBP-SHP1
Vehicle
AP21967
85.5
0.01
0
0.12
93.2
0.02
0
0.23
CD3
CD19
C
Round 1
Round 2 (w/o AP21967)
IFNγ (relative)
Vehicle
AP21967
<.0001
ns
AP21967
(Round 1)
(Round 2)
CAR.FRB
CAR.FRB +FKBP-SHP1
D
hGM-CSF
hTNFα
Relative concentration
.0083
.0001
Before
After
E
Human B cells/mL
Human T cells/mL
<.0001
ns
Before
3w after
F
Bleeding
Tumor only
Vehicle
AP21967
<.0001
.0331
% Weight change
Hours after T cell infusion
G
Human IFNγ
Murine IL-6
Human GM-CSF
Human TNFα
<.0001
.0042
<.0001
.0053
Relative concentration
Tumor only
Vehicle
AP21967

Fig. S6 (Related to Figure 6). Engineering SHP1 phosphatase in CAR-T cells encoding CD28 tunes down cytokine release syndrome.

(**A**) Representative flow plots of the expression of CARs in CAR19.28ζ-T or CAR19.28ζ.FRB.FLAG-T cells with or without co-expression of FKBP-SHP1. (**B**) $CD19^+$ BV173 tumor cells were co-cultured with NT, CAR19.28ζ-T and CAR19.28ζ.FRB.FLAG-T cells with or without FKBP-SHP1 for 3 days. Vehicle or AP21967 (1 μM) was added to the co-cultures every day. At day 3, cells were collected to evaluate the presence of T cells and tumor cells by flow cytometry. The experiment was replicated in 3 donors. (**C**) IFNγ release in the supernatant by CAR19.28ζ.FRB-T cells alone or co-expressing FKBP-SHP1 and incubated with the $CD19^+$ BV173 tumor cell line at a 1:5 ratio. CAR-T cells were co-cultured in the presence of vehicle or AP21967 during round #1. Vehicle or AP21967 were added to the co-cultures every day. At day 3, cells were washed and transferred into a new well with BV173 cells without AP21967 during round #2 (n = 4, values were normalized to average values in vehicle group, two-way ANOVA; representative of 2 donors). (**D** and **E**) Cytokine production (**D**) and cell counts (**E**) in humanized mice infused with CAR-T cells. Mice were treated as in Fig.6G. Human GM-CSF and TNFα were detected before (Day 1) and after (Day 2) the administration of vehicle or AP21967. Human B and T cells in the peripheral blood were counted by flow cytometry before and 3 weeks after CAR-T cell infusion (n = 4, values were normalized to average values in vehicle groups in (D), two-way ANOVA). (**F** and **G**) Weight change (**F**) and cytokine detection (**G**) in tumor bearing SCID-beige mice infused with CAR-T cells. Raji-FFluc tumor cells were injected intraperitoneally at day -21. Vehicle or AP21967 was injected i.p. 1 hr before and 12 hr after CAR19.FRB+FKBP-SHP1-T cell infusion. Human IFNγ, GM-CSF, TNFα, and murine IL-6 were detected in the plasma at 24 hr (n = 5 - 7 in (F), mouse weight was normalized to starting weight before CAR-T cell infusion, two-way ANOVA, p value between vehicle and AP21967 groups; n = 10 - 12 in (G), values were normalized to average values in vehicle group, one-way ANOVA). All data were presented as mean ± SEM.