Supplementary Materials

MATERIALS AND METHODS

T cells retroviral transduction

γδ and αβ T cell obtained from PBMCs stimulation were transduced using RetroNectin® coated 6-well plates (20 μg/mL). Briefly, after coating the plates with 1.5 mL/well of RetroNectin® for two hours at room temperature, the wells were blocked with 2 mL of PBS + 2% BSA for 30 min at room temperature. After, wells were carefully washed with PBS; then, 2 mL of viral supernatant + 2 mL of DMEM (supplemented with 10%FBS and antibiotic) were added into each well. Spinoculation of the virus onto the wells was done by centrifuging the plates for 2 hours at 2000 g, 32 °C. Immediately after, the supernatant of each well was discarded and 4 mL of either γδ ($3.5x10^6$ cells/well in RMPI) or αβ T cell ($2.5x10^6$ cells/well in X-Vivo) were plated in each well. The plates were then centrifuged for 10 min at 1000 rpm, 32 °C to maximize T cell-virus interaction. A second transduction of the same cells was performed 24 hours later. Cells were kept at 37°C, 5% CO_2 for their time in culture.

CAR expression efficiency, as well as γδ T cell content were assessed by flow cytometry 4-6 days after transduction. The staining protocol included CAR staining either with previous incubation with biotinylated-protein L, followed by surface staining of Streptavidin-PerCP/Cy5.5 or using an anti-G4S linker antibody. We included antibodies to determine CD3 to gate for T cells, TCRVd2, TCRα/β to segregate αβ from γδ T cells and other markers, including a viability dye, as indicated in the pertinent figures. Flow cytometry was performed on a LSRII flow cytometer using FACSDiva software (BD Biosciences) at the Moffitt Cancer Center Flow Cytometry Core and further analyzed with FlowJo software (BD Biosciences). When necessary, the total number of CAR expressing cells was normalized by adding untransduced cells to the

sample with the highest expression of CAR until the percentages of the CAR-T cells to be compared were similar.

Phosphoproteomics analysis by stable isotope labeling by amino acids in cell culture (SILAC) MS.

C4-2B PSCA^{+} cells were cultured in RPMI media supplemented with heavy lysine and arginine amino acids for cell labeling ($^{13}C_6$ $^{14}N_4$ L-arginine 200 mg/L, and $^{13}C_6$ L-lysine 40 mg/L) replacing the media three times per week. After four weeks, >95% of cell protein was labeled with heavy amino acids. Twenty million tumor cells per sample were cocultured with T cells at a ratio of 1:2 total T cells, or 2.8:1 CAR-T cells (37°C, 5%CO_2). Six different conditions were prepared by triplicates to account for: basal levels of protein phosphorylation in (1) untransduced (UT) γδ or (2) αβ T cells after 1 hour of coculture, basal levels of CAR-induced phosphorylation in (3) γδ or (4) αβ CAR-T cells (0 hours of coculture), or antigen-CAR-recognition-induced phosphorylation events in γδ (5) or αβ (6) CAR-T cells after 1 hour of coculture. Cell mixtures were washed twice with cold PBS and centrifuged at 4 ˚C, 300 g for 5 min post incubation times (1h, or immediately for 0h). The supernatant was discarded, and pellets were fast-frozen in dry ice for further protein extraction and MS as previously described*(82)*. Briefly, cells were lysed in denaturing buffer containing 8 M urea, 20 mM HEPES (pH 8), 1 mM sodium orthovanadate, 2.5 mM sodium pyrophosphate and 1 mM β-glycerophosphate with needle sonication, followed by centrifugation to pellet cell debris. Bradford assays determined the protein concentration for each sample. Aliquots of 2.8 mg and 200 mg were prepared for tyrosine-phosphorylation and global phosphorylation/expression, respectively. An additional pooled sample with 24 mg total protein served as boosting sample or Bulk. Protein disulfides were reduced with 4.5 mM DTT at 60 °C for 30 minutes and then cysteines were alkylated with 10 mM iodoacetamide for 20 minutes in the dark at room temperature. Trypsin digestion was carried out at room temperature overnight with enzyme to substrate ratio of 1:20, and tryptic peptides were acidified with

aqueous 1% trifluoroacetic acid (TFA) and desalted with C18 Sep-Pak cartridges according to the manufacturer's procedure and lyophilized.

pY Enrichment.

Following lyophilization, peptide pellets were re-dissolved in immunoaffinity purification (IAP) buffer containing 50 mM MOPS pH 7.2, 10 mM sodium phosphate and 50 mM NaCl. Phosphotyrosine-containing peptides (pY) were immunoprecipitated with p-Tyr-1000 beads (Cell Signaling Technology #8803S), according to the manufacturer's instructions.

Peptide labeling with Isobaric tags.

Phospho-Tyrosine (pY) enriched samples were labeled with TMT11plex and for global phosphorylation/expression, 200 mg of each tryptic digest were TMT labeled as well, following manufacture's protocol. Label incorporation was verified to be >95% by LC-MS/MS and spectral counting (Protein Discoverer, Thermo). The samples were then quenched with aqueous 5% hydroxylamine, pooled according to each Plex and lyophilized. The TMT channel layouts were: Plex1-126C γδ CAR-T cells, 0 hour_rep1, Plex1-127N αβ CAR-T cells, 0 hour_rep1, Plex1-127C γδ CAR-T cells, 1 hour_rep1, Plex1-128N αβ CAR-T cells, 1 hour_rep1, Plex1-128C γδ CAR-T cells, 1 hour_rep2, Plex1-129N αβ CAR-T cells, 1 hour_rep2, Plex1-129C γδ Untransduced T cells, 1 hour_rep1, Plex1-130N αβ Untransduced T cells, 1 hour_rep1, Plex1-131N γδ Untransduced T cells, 1 hour_rep2, Plex1-131C bulk, and, Plex2-126 γδ CAR-T cells, 0 hour_rep2, Plex2-127N αβ CAR-T cells, 0 hour_rep2, Plex2-127C γδ CAR-T cells, 0 hour_rep3, Plex2-128N αβ CAR-T cells, 0 hour_rep3, Plex2-128C γδ CAR-T cells, 1 hour_rep3, Plex2-129N αβ CAR-T cells, 1 hour_rep3, Plex2-129C γδ Untransduced T cells, 1 hour_rep3, Plex2-130N αβ Untransduced T cells, 1 hour_rep2, Plex2-131N αβ Untransduced T cells, 1 hour_rep3, Plex2-131C bulk. Channel 130C is left empty to avoid interferences from bulk sample.

After TMT labeling. pY enriched samples were pooled into Plex1 and Plex 2, respectively and lyophilized to purify and remove non-phosphopeptides. Peptides were re-dissolved in aqueous 85% acetonitrile (ACN) containing 0.1% TFA immobilized metal affinity chromatography (IMAC) loading buffer; 20 µl of IMAC magnetic beads (Cell Signaling Technology # 20432) were washed 3x with aqueous 80% ACN, 0.1% TFA prior to incubation with immunoprecipitated peptides for 30 minutes at room temperature. Next, beads were washed 3x with aqueous 80% ACN, 0.1% TFA. Phosphopeptides were eluted twice with aqueous 50% ACN with 2.5% ammonia. Eluted peptides were dry and re-suspended in 20 µL of aqueous 2% acetonitrile/0.1% formic acid and inject 5 µL into mass spectrometer.

Basic reversed phase liquid chromatography.

After lyophilization, for global phosphorylation samples, TMT-labeled peptides were redissolved in 250 mL of aqueous 20 mM Ammonium Formate, (pH 10.0), and subject for high pH reversed phase liquid chromatography (bRPLC) separation, performed on a XBridge 4.6 mm x 100 mm column packed with BEH C18 resin, 3.5 µm particle size, 130 Å pore size (Waters). bRPLC Solvent A was aqueous 2% ACN with 5 mM Ammonium Formate, pH 10.0. Peptides were eluted by: 5% bRPLC B (aqueous 90% acetonitrile with 5 mM Ammonium Formate, pH 10.0) for 10 minutes, 5% - 15% B in 5 minutes, 15-40% B in 47 minutes, 40-100% B in 5 minutes and 100% B held for 10 minutes, followed by re-equilibration at 1% B. The flow rate was 0.6 ml/min, and 12 concatenated fractions were collected for phosphorylation enrichment. Vacuum centrifugation (Speedvac, Thermo) dried the samples.

IMAC enrichment.

Concatenated fractions of TMT-labeled peptides samples were re-dissolved IMAC loading buffer containing aqueous 0.1% TFA and 85% acetonitrile. Phosphopeptides were enriched using IMAC resin (Cell Signaling Technology # 20432) on a Kingfisher (Thermo). IMAC resin was washed with loading

buffer. Peptides were incubated with 20 mL of resin for 30 minutes at room temperature with gentle agitation. The IMAC resin was washed twice with loading buffer followed by wash buffer (aqueous 80% ACN, 0.1% TFA). Phosphopeptides were eluted with 100 µL aqueous 50% ACN, 2.5% Ammonia. Samples were dry via vacuum centrifugation and resuspended in 20 µL of 20% acetonitrile/0.01% formic acid.

LC-MS/MS

A nanoflow ultra-high performance liquid chromatograph and nanoelectrospray orbitrap mass spectrometer (Dionex RSLC nano and Q Exactive HF-X, Thermo) were used for LC-MS/MS. The sample was loaded onto a pre-column (C18 PepMap100, 2 cm length x 100 µm ID packed with C18 reversed-phase resin, 5 µm particle size, 100 Å pore size) and washed for 8 minutes with aqueous 2% acetonitrile and 0.1% formic acid. Trapped peptides were eluted onto the analytical column, (C18 PepMap100, 25 cm length x 75 µm ID, 2 µm particle size, 100 Å pore size, Thermo). A 120-minute gradient was programmed as: 95% solvent A (aqueous 2% acetonitrile + 0.1% formic acid) for 8 minutes, solvent B (aqueous 90% acetonitrile + 0.1% formic acid) from 5% to 38.5% in 90 minutes, then solvent B from 50% to 90% B in 7 minutes and held at 90% for 5 minutes, followed by solvent B from 90% to 5% in 1 minute and re-equilibration for 10 minutes using a flow rate of 300 nL/min. Spray voltage was 1900 V. Capillary temperature was 275 °C. S lens RF level was set at 40. The top 20 tandem mass spectra were collected in a data-dependent manner. Settings for MS acquisition were 60,000 resolution, 3E6 AGC target, 45 ms Max IT, and recording m/z 440-2000. The settings for tandem mass spectrometry data acquisition were 45,000 resolution, 1E5 AGC target, 86 ms Max IT for global phosphorylation and 300 ms Max IT for pY samples, isolation window 0.8 with 0.2 offset, fixed first mass at m/z 100, and 2 normalized collision energy (NCE) values of 24 and 30.

Data Analysis

MaxQuant(*85*) (version 1.6.14.0) was used to identify peptides using the UniProt human database (June 2020) and quantify the TMT reporter ion intensities. Up to 2 missed trypsin cleavages were allowed. The mass tolerance was 20 ppm first search and 4.5 ppm main search. Reporter ion mass tolerance was set to 0.003 Da. Carbamidomethyl cysteine was set as fixed modification. Phosphorylation on Serine/Threonine/Tyrosine and Methionine oxidation were set as variable modifications. Both peptide spectral match (PSM) and protein false discovery rate (FDR) were set at 0.05. Match between run features were activated to carry identifications across samples. Reporter ion intensities were used for the relative quantification of each peptide in the TMT global pSTY data and pY data. Both data sets were normalized using IRON (iron_generic–proteomics(*83*)) against the 5x pooled bulk sample channels within each plex (Plex1-131C and Plex2-131C). The normalized bulk sample channels were then used to remove systematic differences between plexes. Each of these replicates was run twice. Results from both runs from the same isobaric tag in each Plex were averaged together. Then, the three injection replicates in different Plex were average together to calculate Log_2 ratios between conditions by subtracting averaged sample replicates. Welch's t-test was used to determine statistical significance in the difference between the log_2 ratio. Data normalization was evaluated with scatterplots, and PCA analysis separated samples by the groups expected on the experimental design.

***In vivo* mouse model**

All animal experiments were performed under University of South Florida Institutional Animal Care and Use Committee (IACUC) approval (14112R) Animals were maintained on a 12-hour light/dark schedule and fed ad libitum. Male, 6-week-old, NSG mice, purchased from The Jackson Laboratory), were intratibially inoculated with 20µl of luciferase expressing C4-2B $PSCA^+$ cells ($5x10^5$ cells per leg diluted in PBS) as

described previouslyTumor growth was monitored measuring bioluminescence twice a week. Briefly, mice were sedated and injected intraperitoneally with 150µg/g body weight of D-luciferin-potassium (GoldBio, St Louis, MO, USA), after five minutes, a ventral image of them was captured for 120sec exposure. Two weeks after tumor inoculation, mice were randomized in five groups to be treated with retro orbital injections of PBS (as untreated control), UT, CAR, CAR/RANK-CD27 or CAR/RANK41BB γδ T cells T cells ($1.2x10^7$ cells/mouse, in 100µl of total volume). Mice were supplemented IP with IL-2 (100IU in 100µl per mouse) every 48 hours for two weeks after T cell injection. At endpoint, mice were euthanized in a CO_2 chamber, and spleen, blood, and hind limbs were collected. T cells were isolated from spleen, and tibia bone marrow. Briefly, spleens were mechanically dissociated, and the cells were strained through a 40µm Nailon strainer. In parallel, tibias were cut open, and the bone marrow was extracted by centrifugationPelleted cells were washed with PBS and then resuspended in 2mL of ACK buffer to eliminate RBCs. Finally, isolated T cells were cryopreserved for further flow cytometry analysis.

FIGURES AND TABLES

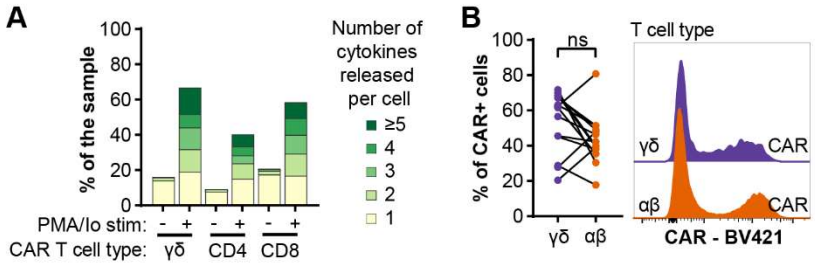

Figure S1. Phenotypic differences between γδ and αβ CAR T-cells. (A) Analysis of CAR T cell polyfunctionality, a measure of the percentage of single cells in a T cell population able to release more than one cytokine. T cells were either resting or stimulated by PMA/Ionomycin. **(B)** Surface expression (mean ±SD) of CAR in γδ and αβ T cells. Each symbol represents T cells from an independent healthy donor. Representative flow cytometry histograms to the right (paired t-test, gated on lymphocytes/singlets/live/CD3+/ either Vδ2+ or αβTCR+ cells).

Protein Accession Number	Gene Symbol	Phospho sites	FC	p values
Q8TD55	PLEKHO2	S 389	3.088	0.043
P08567	PLEK	S 117	3.072	0.037
Q49A26	GLYR1	S 132	2.451	0.021
Q9Y2D9	ZNF652	T 203	2.444	0.010
P16403	H1-2	S 36	2.417	0.009
P10412	H1-4	S 36	2.339	0.014
P20701	ITGAL	S 1165	2.316	0.006
O95936	EOMES	S 646	2.294	0.000
Q49A26	GLYR1	S 130	2.274	0.004
Q13185	CBX3	S 176	2.267	0.002
Q16539	MAPK14	Y 182	2.226	0.013
Q7Z591	AKNA	T 51	2.189	0.025
Q14789	GOLGB1	S 1744	2.189	0.006
Q14789	GOLGB1	S 1747	2.189	0.006
Q14789	GOLGB1	S 1751	2.189	0.006
Q8NEY8	PPHLN1	S 201	2.145	0.001
Q8NEY8	PPHLN1	T 200	2.145	0.001
Q86YV0	RASAL3	T 17	2.068	0.036
Q9HCH5	SYTL2	S 493	2.047	0.013
Q7Z591	AKNA	S 52	2.007	0.034
Q6W2J9	BCOR	S 422	1.963	0.021
Q9BPY8	HOPX	S 70	1.932	0.045
Q7Z4V5	HDGFL2	S 369	1.932	0.002
Q7Z4V5	HDGFL2	S 370	1.932	0.002
P23497	SP100	S 476	1.913	0.022

Protein Accession Number	Gene Symbol	Phospho sites	FC	p values
O43561	LAT	S 40	1.905	0.027
O43561	LAT	S 43	1.905	0.027
Q9NRY4	ARHGAP35	Y 1105	1.904	0.024
P22102	GART	Y 348	1.888	0.001
Q6PD62	CTR9	S 1072	1.883	0.003
O14683	TP53I11	S 14	1.881	0.013
Q7Z591	AKNA	S 1228	1.853	0.005
Q8N228	SCML4	S 277	1.841	0.000
Q13469	NFATC2	S 53	1.841	0.020
O60504	SORBS3	T 529	1.820	0.043
Q8IVT5	KSR1	S 406	1.818	0.001
Q8NB49	ATP11C	S 1126	1.814	0.029
P52333	JAK3	S 17	1.811	0.037
Q9NWQ8	PAG1	Y 317	1.803	0.020
Q8N3A8	PARP8	S 323	1.801	0.007
Q13342	SP140	S 277	1.800	0.004
Q9HCH5	SYTL2	S 562	1.793	0.026
Q8NF91	SYNE1	T 8360	1.792	0.000
Q9UQ35	SRRM2	S 1562	1.776	0.006
Q9UQ35	SRRM2	S 1561	1.761	0.017
Q9HCH5	SYTL2	S 488	1.759	0.029
Q03111	MLLT1	S 267	1.755	0.000
Q9UIK4	DAPK2	S 299	1.750	0.035
Q9NQ75	CASS4	S 305	1.750	0.001
Q12802	AKAP13	S 2500	1.731	0.023
P21580	TNFAIP3	S 573	1.729	0.034

Protein Accession Number	Gene Symbol	Phospho sites	FC	p values
P21580	TNFAIP3	S 575	1.729	0.034
Q6IBW4	NCAPH2	S 282	1.699	0.012
Q7Z591	AKNA	S 1387	1.691	0.030
Q8WVC0	LEO1	S 154	1.682	0.005
Q99698	LYST	S 2105	1.673	0.014
Q07955	SRSF1	Y 189	1.661	0.049
Q6IBW4	NCAPH2	S 284	1.652	0.043
Q13469	NFATC2	S 73	1.644	0.032
P07910	HNRNPC	S 241	1.636	0.003
O00161	SNAP23	S 20	1.632	0.017
Q7Z6I6	ARHGAP30	S 709	1.625	0.027
P46100	ATRX	S 974	1.619	0.003
P46100	ATRX	T 977	1.619	0.003
Q8N103	TAGAP	S 400	1.606	0.010
O14745	SLC9A3R1	S 280	1.605	0.004
Q14005	IL16	S 974	1.604	0.026
P17026	ZNF22	S 49	1.602	0.009
Q9Y2D9	ZNF652	S 57	1.602	0.017
Q9H1C0	LPAR5	S 320	1.599	0.021
Q9NWQ8	PAG1	S 50	1.592	0.009
Q96ST2	IWS1	S 54	1.583	0.001
Q53EL6	PDCD4	S 94	1.572	0.032
P49841	GSK3B	S 219	1.570	0.016
Q96JY6	PDLIM2	S 124	1.557	0.020
Q12802	AKAP13	S 2498	1.555	0.016
Q6PJF5	RHBDF2	S 385	1.554	0.004

Protein Accession Number	Gene Symbol	Phospho sites	FC	p values
Q8IY18	SMC5	S 35	1.544	0.012
Q8N2U9	SLC66A2	S 110	1.536	0.012
Q7Z591	AKNA	S 770	1.532	0.013
Q96T58	SPEN	T 1619	1.531	0.013
Q92522	H1-10	S 33	1.529	0.006
Q96T58	SPEN	S 725	1.526	0.011
P53999	SUB1	S 19	1.520	0.004
Q5T1R4	HIVEP3	S 496	1.517	0.003
P04150	NR3C1	S 134	1.514	0.009
Q9Y4H4	GPSM3	S 35	1.513	0.000
Q9Y3Q8	TSC22D4	S 62	1.510	0.013
Q7Z4S6	KIF21A	S 1239	1.506	0.003
Q07352	ZFP36L1	S 54	1.506	0.002
P29350	PTPN6	S 582	1.477	0.038
P68431	H3C13	S 29	0.666	0.003
Q6JBY9	RCSD1	S 82	0.665	0.003
P04075	ALDOA	S 39	0.664	0.001
Q09666	AHNAK	S 5400	0.664	0.028
Q6PKG0	LARP1	T 526	0.664	0.000
P54578	USP14	S 394	0.663	0.004
O60841	EIF5B	S 135	0.662	0.001
O14737	PDCD5	S 119	0.662	0.001
P22234	PAICS	S 107	0.661	0.007
Q92890	UFD1	S 299	0.661	0.007
P84101	SERF2	S 21	0.660	0.005
Q9NS75	CYSLTR2	S 102	0.660	0.037

Protein Accession Number	Gene Symbol	Phospho sites	FC	p values
P36578	RPL4	S 295	0.660	0.011
P78371	CCT2	S 260	0.660	0.032
Q01826	SATB1	S 47	0.659	0.043
P33241	LSP1	S 212	0.659	0.043
P24941	CDK2	Y 15	0.659	0.001
P14618	PKM	S 37	0.657	0.003
Q7Z6Z7	HUWE1	S 3555	0.656	0.028
P21796	VDAC1	S 104	0.656	0.000
P06748	NPM1	S 227	0.655	0.014
Q9Y5U5	TNFRSF18	S 217	0.654	0.017
P06748	NPM1	T 199	0.653	0.035
Q15149	PLEC	S 4382	0.653	0.010
Q6ZSZ5	ARHGEF18	S 155	0.652	0.015
A0FGR8	ESYT2	S 691	0.652	0.021
A0FGR8	ESYT2	S 693	0.652	0.021
Q8WU79	SMAP2	S 219	0.652	0.002
P52701	MSH6	S 309	0.651	0.006
Q9NX55	HYPK	S 38	0.650	0.037
Q99700	ATXN2	T 741	0.650	0.012
Q09666	AHNAK	S 4986	0.650	0.031
O14578	CIT	S 1971	0.649	0.008
Q09666	AHNAK	S 561	0.647	0.012
Q15149	PLEC	S 720	0.644	0.014
P11388	TOP2A	S 1247	0.643	0.004
O43516	WIPF1	S 276	0.643	0.001
Q15555	MAPRE2	S 223	0.642	0.011

Protein Accession Number	Gene Symbol	Phospho sites	FC	p values
Q8WYL5	SSH1	S 576	0.642	0.004
Q9ULW0	TPX2	S 186	0.640	0.003
Q8IU68	TMC8	S 683	0.640	0.020
Q8NFN8	GPR156	S 212	0.639	0.030
Q8NFN8	GPR156	T 211	0.639	0.030
Q9UPT6	MAPK8IP3	S 585	0.638	0.025
Q01850	CDR2	S 145	0.638	0.043
P14317	HCLS1	S 149	0.638	0.037
Q01082	SPTBN1	S 2165	0.635	0.020
P24941	CDK2 CDK3	T 14	0.634	0.003
Q01082	SPTBN1	S 2169	0.630	0.032
Q9UI08	EVL	T 343	0.630	0.002
Q14155	ARHGEF7	S 249	0.630	0.006
Q8IWZ3	ANKHD1	S 1679	0.630	0.003
P02794	FTH1	S 179	0.630	0.007
Q9UQ80	PA2G4	S 363	0.626	0.001
P21333	FLNA	S 2152	0.626	0.002
Q16566	CAMK4	S 360	0.625	0.006
Q9BRZ2	TRIM56	T 442	0.625	0.001
P14317	HCLS1	S 299	0.625	0.013
Q5SW79	CEP170	T 969	0.625	0.040
Q9H1E3	NUCKS1	S 181	0.624	0.010
P17096	HMGA1	T 39	0.624	0.011
Q14242	SELPLG	S 389	0.623	0.019
O43768	ENSA	S 43	0.621	0.000
P16949	STMN1	S 38	0.620	0.012

Protein Accession Number	Gene Symbol	Phospho sites	FC	p values
P16150	SPN	T 341	0.618	0.040
Q6ZVF9	GPRIN3	S 583	0.616	0.005
Q6ZVF9	GPRIN3	T 581	0.616	0.005
P02545	LMNA	S 423	0.615	0.002
Q8N556	AFAP1	S 548	0.613	0.038
Q8TAA9	VANGL1	S 42	0.613	0.039
P78559	MAP1A	S 1069	0.611	0.013
P68363	TUBA1A	S 48	0.611	0.035
P19174	PLCG1	S 1248	0.610	0.001
Q9Y3Z3	SAMHD1	S 23	0.610	0.044
Q05655	PRKCD	S 304	0.610	0.010
P53985	SLC16A1	S 213	0.608	0.001
Q01082	SPTBN1	S 2164	0.608	0.007
P06733	ENO1	S 419	0.605	0.011
Q6P6C2	ALKBH5	S 371	0.605	0.017
Q5T4S7	UBR4	S 457	0.604	0.002
O94804	STK10	S 514	0.604	0.002
Q9H1E3	NUCKS1	T 179	0.603	0.002
P27708	CAD	S 1859	0.602	0.010
O75475	PSIP1	T 115	0.599	0.017
Q14147	DHX34	S 825	0.596	0.039
Q14147	DHX34	T 831	0.596	0.039
O43491	EPB41L2	S 598	0.596	0.017
O14617	AP3D1	S 688	0.596	0.042
Q16637	SMN1	S 31	0.595	0.004
Q14CB8	ARHGAP19	S 422	0.593	0.000

Protein Accession Number	Gene Symbol	Phospho sites	FC	p values
Q9Y2V2	CARHSP1	S 52	0.591	0.003
Q6ZVF9	GPRIN3	S 734	0.590	0.000
Q6ZVF9	GPRIN3	S 736	0.590	0.000
Q01433	AMPD2	S 168	0.588	0.025
P27361	MAPK3	T 202	0.587	0.007
P27361	MAPK3	Y 204	0.587	0.007
P41252	IARS1	S 1049	0.583	0.025
Q8IY81	FTSJ3	S 347	0.583	0.024
P23588	EIF4B	S 283	0.579	0.047
Q6JBY9	RCSD1	S 83	0.578	0.008
P46013	MKI67	S 1071	0.573	0.013
Q9UQC2	GAB2	S 405	0.571	0.016
P56192	MARS1	S 825	0.570	0.003
P24941	CDK2	Y 15	0.569	0.009
Q16637	SMN1 SMN2	T 25	0.568	0.024
Q00536	CDK16	S 153	0.567	0.032
P52701	MSH6	S 830	0.566	0.008
Q8NCD3	HJURP	S 557	0.565	0.004
P06748	NPM1	S 260	0.565	0.002
P78559	MAP1A	T 2105	0.564	0.004
Q6ZVF9	GPRIN3	S 740	0.564	0.006
O75534	CSDE1	S 514	0.564	0.012
P14316	IRF2	S 339	0.563	0.001
P51684	CCR6	S 347	0.562	0.048
O15021	MAST4	S 1467	0.561	0.015
P26583	HMGB2	S 181	0.561	0.019

Protein Accession Number	Gene Symbol	Phospho sites	FC	p values
Q9Y570	PPME1	S 243	0.560	0.013
P21333	FLNA	S 1459	0.558	0.006
Q13177	PAK2	S 141	0.558	0.016
Q13303	KCNAB2	S 14	0.557	0.006
Q9NQW6	ANLN	S 295	0.556	0.027
P09104	ENO2	T 265	0.556	0.001
P11388	TOP2A	S 1106	0.556	0.035
Q684P5	RAP1GAP2	S 558	0.554	0.035
P49006	MARCKSL1	S 104	0.552	0.014
Q53QZ3	ARHGAP15	S 64	0.550	0.002
Q9BYV9	BACH2	S 159	0.546	0.043
P53985	SLC16A1	S 467	0.541	0.014
Q15149	PLEC	S 1721	0.539	0.045
Q5SW79	CEP170	S 838	0.537	0.003
Q9H019	MTFR1L	S 238	0.536	0.005
P42229	STAT5A	Y 694	0.535	0.006
P56192	MARS1	T 824	0.535	0.001
O94851	MICAL2	S 515	0.530	0.007
O95425	SVIL	S 245	0.530	0.000
P61978	HNRNPK	S 214	0.529	0.000
Q9UI08	EVL	S 369	0.528	0.000
Q6JBY9	RCSD1	S 105	0.525	0.006
Q08495	DMTN	S 85	0.522	0.034
Q08495	DMTN	S 87	0.522	0.034
P11166	SLC2A1	S 473	0.521	0.006
Q9H3M7	TXNIP	S 361	0.520	0.036

Protein Accession Number	Gene Symbol	Phospho sites	FC	p values
Q9Y6Q6	TNFRSF11A	S 580	0.519	0.026
Q9Y4F9	RIPOR2	S 37	0.519	0.031
P06733	ENO1	Y 44	0.519	0.034
P30050	RPL12	S 38	0.518	0.001
P05771	PRKCB	T 642	0.517	0.040
P22314	UBA1	S 835	0.516	0.003
Q9NSI8	SAMSN1	S 74	0.515	0.006
Q5SW79	CEP170	S 971	0.515	0.008
Q13415	ORC1	S 311	0.513	0.000
Q96BY6	DOCK10	S 12	0.507	0.017
P60174	TPI1	S 21	0.504	0.000
O75569	PRKRA	S 18	0.502	0.000
P16949	STMN1	S 25	0.499	0.000
P62753	RPS6	S 247	0.499	0.022
Q6JBY9	RCSD1	S 108	0.495	0.006
Q96TA1	NIBAN2	S 665	0.491	0.004
Q9Y3Z3	SAMHD1	T 592	0.490	0.000
P49795	RGS19	S 65	0.490	0.001
O43491	EPB41L2	S 87	0.486	0.004
P62753	RPS6	S 240	0.485	0.000
Q9Y5K6	CD2AP	S 224	0.483	0.001
Q9UI08	EVL	S 283	0.483	0.021
P27708	CAD	S 1407	0.482	0.029
Q8IY18	SMC5	S 131	0.477	0.007
Q8IY18	SMC5	T 137	0.477	0.007
A0JNW5	UHRF1BP1L	S 953	0.477	0.009

Protein Accession Number	Gene Symbol	Phospho sites	FC	p values
P62753	RPS6	S 235	0.474	0.004
P41236	PPP1R2	S 20	0.474	0.001
Q12965	MYO1E	S 1002	0.473	0.003
Q13177	PAK2	S 64	0.463	0.008
B0I1T2	MYO1G	S 1010	0.462	0.013
P62753	RPS6	T 241	0.460	0.003
Q9H3M7	TXNIP	T 349	0.454	0.018
Q9H3M7	TXNIP	T 348	0.450	0.006
P62857	RPS28	S 23	0.450	0.017
Q96TA1	NIBAN2	S 692	0.449	0.001
Q96TA1	NIBAN2	S 696	0.448	0.001
P48167	GLRB	S 411	0.441	0.040
P48167	GLRB	T 410	0.441	0.040
Q14242	SELPLG	S 358	0.441	0.002
P62753	RPS6	S 236	0.440	0.003
Q9UIQ6	LNPEP	S 80	0.428	0.000
Q86VZ1	P2RY8	S 324	0.422	0.003
P31350	RRM2	S 20	0.414	0.001
P35611	ADD1	S 726	0.410	0.003
P51692	STAT5B	Y 699	0.405	0.003
Q9H3M7	TXNIP	S 346	0.400	0.015
Q96TA1	NIBAN2	S 691	0.400	0.011
Q6ZVF9	GPRIN3	S 462	0.397	0.002
Q09666	AHNAK	S 5830	0.396	0.000
P33993	MCM7	Y 600	0.392	0.014
P05412	JUN	S 58	0.388	0.001

Protein Accession Number	Gene Symbol	Phospho sites	FC	p values
O00148	DDX39A	S 183	0.386	0.000
P22234	PAICS	S 27	0.385	0.008
P41236	PPP1R2	T 21	0.374	0.001
P13612	ITGA4	S 1021	0.366	0.000
Q9Y4F9	RIPOR2	S 21	0.364	0.000
P16949	STMN1	S 16	0.351	0.000
P00558	PGK1	S 203	0.342	0.000
P11171	EPB41	S 85	0.338	0.013
Q5SW79	CEP170	S 446	0.337	0.000
Q5SW79	CEP170	S 450	0.336	0.042
P78559	MAP1A	S 2629	0.331	0.002
Q13642	FHL1	S 98	0.327	0.000
P78559	MAP1A	S 2617	0.321	0.007
P09972	ALDOA	Y 204	0.316	0.009
P13598	ICAM2	T 259	0.302	0.001
Q9H400	LIME1	S 70	0.273	0.001
P78559	MAP1A	S 986	0.271	0.029
Q6UX15	LAYN	S 299	0.258	0.011
P01730	CD4	S 440	0.248	0.014
Q9UN19	DAPP1	Y 139	0.236	0.008
P01732	CD8A	S 229	0.212	0.001
P01732	CD8A	S 231	0.183	0.000

Table S1. Differentially phosphorylated proteins sites between activated γδ and αβ CAR T cells. S: Serine, T: Threonine, Y: Tyrosine.

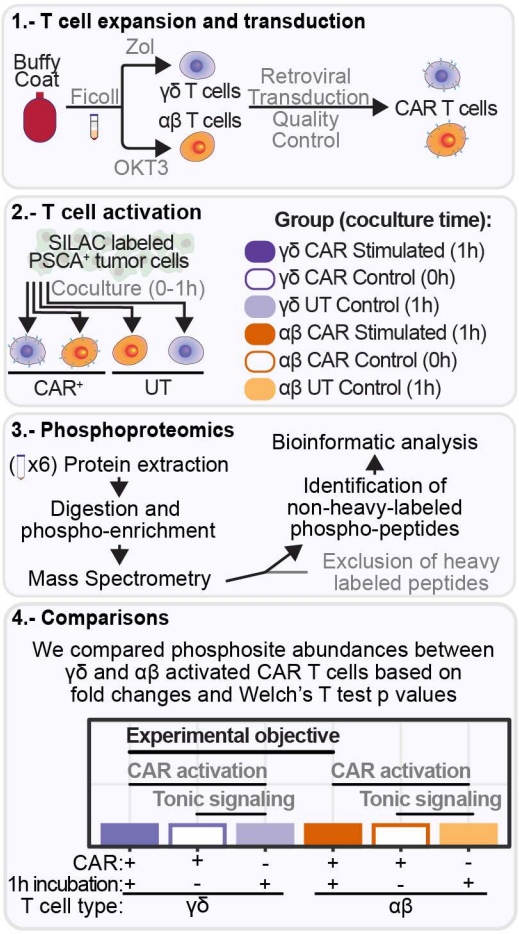

Figure S2. Phosphoproteomics approach to determine CAR-driven differential signaling. Diagram of the experimental design to analyze phosphorylation events triggered by CAR activation. Briefly, T cells were expanded from healthy donor PBMCs with either zoledronate (Zol) or OKT3. Once activated, T cells were transduced with a retroviral vector to express PSCA-8t28z CAR. Five days later, CAR expression was confirmed by flow cytometry. In parallel, PSCA-expressing tumor cells (C4-2B) were cultured with SILAC media until >95% of their proteome was labelled with $^{13}C_6$ $^{14}N_4$ L-arginine, and $^{13}C_6$ L-lysine. T cells and tumor cells are co-cultured for either 1h at 37°C or "0h" and pelleted down at 4°C. Pellets are washed with cold PBS and quickly frozen for further analysis. After protein extraction and digestion, phosphorylated peptides are enriched and analyzed by tandem mass spectrometry. Heavy isotope-labeled peptides (from tumor cells) were

filtered out, and unlabeled peptides were mapped to the proteome and quantified. Finally, differentially phosphorylated proteins were identified as those having a fold change greater than 1.5 and a p-value (Welch's t-test) of less than 0.05.

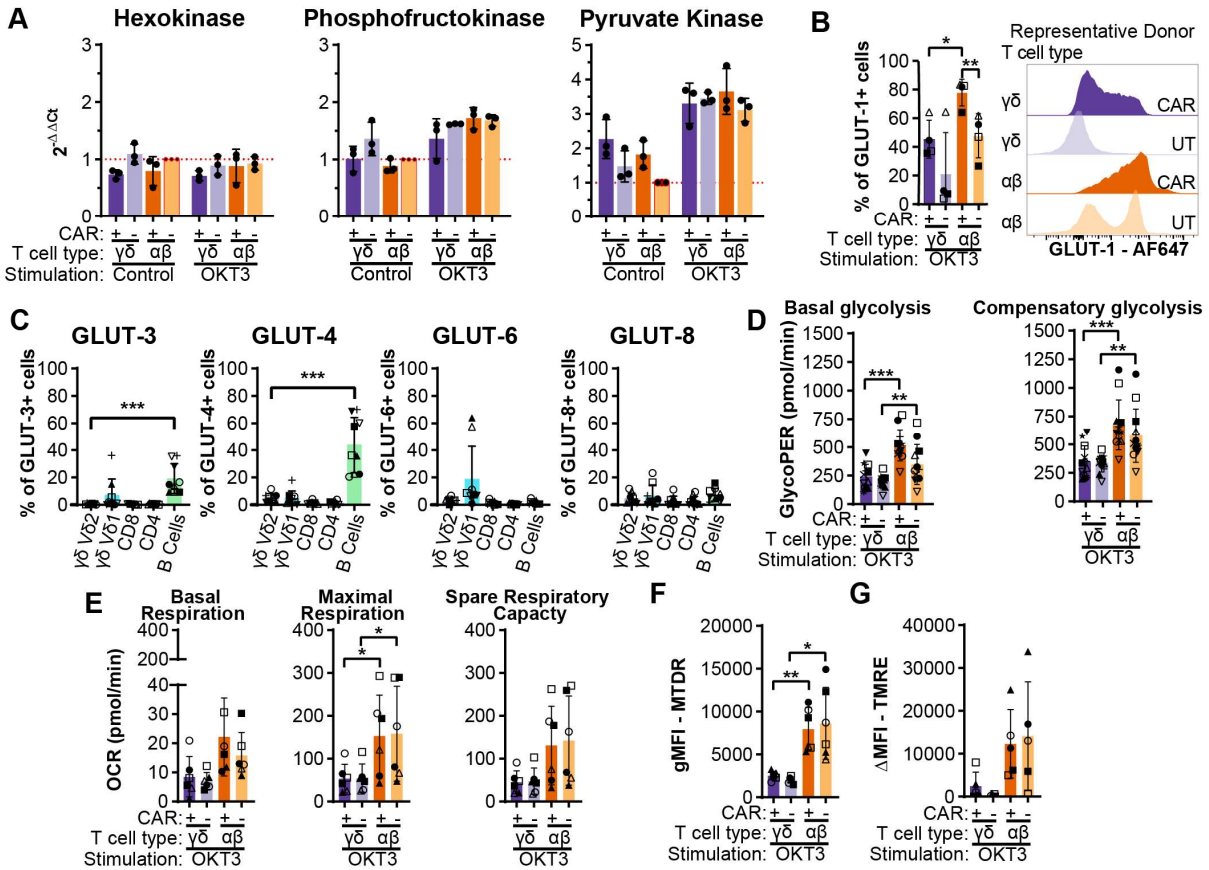

Figure S3. Metabolic differences between γδ and αβ CAR T cells. (A) Bar chart of the relative abundance (normalized by αβ unstimulated UTs, represented by the dotted red line) of glycolytic enzymes in unstimulated and 24h-OKT3 stimulated T cells (mean ±SD; n=3). **(B)** Bar chart of surface expression (mean ±SD; n=8) of glucose receptor GLUT-1 in 24h-OKT3 stimulated T cells. **(C)** Bar chart of surface expression (mean ±SD; n=9) of alternative glucose receptors GLUT-3, GLUT-4, GLUT-6, and GLUT-8 in non-cultured PBMCs. **(D)** Bar charts representing the Glycolytic Proton Efflux Rate (GlycoPER) as an indirect measurement of basal glycolysis and compensatory glycolysis levels of 48h-OKT-3 stimulated T cells (mean ±SD; n=12). **(E)** Bar charts of the OCR as indirect measurement of basal respiration, maximal respiration and spare respiratory capacity (SRC) of 48h-OKT-3 stimulated T cells (mean ±SD; n=6). **(F)** Bar chart of MitoTracker™

DeepRed (MTDR) geometric mean fluorescence intensity (gMFI) on 24h-OKT-3 stimulated T cells (mean ±SD; n=8). **(G)** Bar chart of the delta Tetramethylrhodamine (TMRE) MFI as an indirect measurement of the mitochondrial membrane potential of 24h-OKT-3 stimulated T cells (mean ±SD; n=5). The data points in (B) were collected from lymphocytes/singlets/live/CD3+/ either Vδ2+ or αβTCR+ gates; in (C) were collected from lymphocytes/singlets/live/CD45+/either CD19+, CD3+/αβTCR+, CD3+/ Vδ1+, or CD3+/ Vδ2+ gates. The symbols from each bar in (B-G), represent an independent healthy donor sample between and within experiments. Statistically significant differences in (B-G) are annotated as * for $p < 0.05$, ** for $p < 0.01$, *** for $p < 0.001$, and **** for $p < 0.0001$ (paired t-test).

Reagent name and specifications	CAT#	Company
PBS (pH7.4)	10-010-049	GIBCO
Ammonium-Chloride-Potassium (ACK) Lysing buffer	A10492-01	GIBCO
RPMI 1640 with GlutaMAX	61-870-127	GIBCO
DMEM	11965-118	GIBCO
Antibiotic-Antimycotic 100X	15240-062	GIBCO
MycoAlert, mycoplasma test	LT07-318	Lonza
D-luciferin-potassium salt	LUCK-1G	GoldBio
TCRγ/δ+ T Cell Isolation Kit, human	130-092-892	Miltenyi Biotec
Zoledronic Acid (Zol)	S1314	Selleckchem
Lymphocyte Separation Media (LSM)	850494	MP Biomedicals
RPMI 1640 for SILAC media	88365	ThermoFisher Scientific
IFN-γ monoclonal antibody 2G1	M700A	ThermoFisher Scientific
IFN-γ monoclonal biotinylated antibody B133.5	M701B	ThermoFisher Scientific
HRP-conjugated streptavidin	N100	ThermoFisher Scientific
3,3′,5,5′-Tetramethylbenzidine	34028	ThermoFisher Scientific
LIVE/DEAD™ Fixable Near-IR Dead Cell, for 633 or 635 nm	L10119	ThermoFisher Scientific
LIVE/DEAD™ Fixable Yellow Dead Cell, for 405 nm	L34968	ThermoFisher Scientific
CellTrace™ Violet Cell Proliferation kit	C34571	ThermoFisher Scientific
Lipofectamine® 2000	11668500	ThermoFisher Scientific
Biotin-Protein L	M00097	GenScript
Proleukin (IL-2)	NDC 76310-022-01	Prometheus Laboratories
13C6 14N4 L-arginine	608033	Sigma-Aldrich
13C6 L-lysine	643459	Sigma-Aldrich

Reagent name and specifications	CAT#	Company
Phosphatase inhibitors	P5726-1ML, P0044-1ML	Sigma-Aldrich
Bovine Serum Albumin (BSA)	A3803-100G	Sigma-Aldrich
GlucoseCy5	SML3233	Sigma-Aldrich
X-Vivo™ 15	04-418Q	Lonza
Human serum AB (HSAB)	100-512-100	Gemini-bioproducts
Heat-inactivated Fetal Bovine Serum (FBS)	A021011	Gemini-bioproducts
RetroNectin®	T100B	Takara Bio
Human PSCA Protein, ECD (Extracellular Domain), Fc-fusion	FCL2506	G&P Biosciences
Sea-Horse XF RPMI and supplements	103576-100	Agilent
Sea-Horse XF calibrant, plates and sensor cartridges (XFe96 FluxPak mini)	102601-100	Agilent
Sea-Horse Glycolytic Assay Rate kit	103710-100	Agilent
Sea-Horse T Cell Metabolic Profiling kit	103772-100	Agilent
2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-Deoxyglucose (2-NBDG)	N13195	Invitrogen
Mitotracker Deep Red™	M46753	Invitrogen
Pierce™ Protein L Magnetic Beads	88849	Invitrogen
Protease inhibitors	4693116001	Roche
Trans-Blot Turbo RTA Midi 0.2 µm Nitrocellulose Transfer Kit	1704271	Bio-Rad
4–20% Mini-PROTEAN® TGX™ Precast Protein Gels, 15-well	4561096	Bio-Rad
Precision Plus Protein™ ladder All Blue	1610373	Bio-Rad
TidyBlot Western Blot Detection Reagent	STAR209PA	Bio-Rad

Reagent name and specifications	CAT#	Company
Mitochondrial Membrane Potential Assay kit (II) (TMRE, CCCP and PBS)	13296	Cell Signaling Technology
G4S Linker (E7O2V) Rabbit Monoclonal Antibody (PE Conjugate)	38907	Cell Signaling Technology
p-Tyr-1000 beads	8803S	Cell Signaling Technology
IMAC magnetic beads	20432	Cell Signaling Technology
IMAC resin	20432	Cell Signaling Technology
Customized ELLA simple plex (granzyme B, IFN-γ, IL-2 and TNF-α) cartridge kits	NA	Biotechne® / Protein Simple
Nuclear Extract Kit	40010	ActiveMotif
TransAM ® AP-1 c-Fos	44096	ActiveMotif
TransAM ® JUND	43496	ActiveMotif
TransAM ® c-Jun	46096	ActiveMotif
E-Plates 96 PET	300600900	ACEA Biosciences
Anti-human CD45-BV785 (Clone HI30)	304048	BioLegend
Anti-human CD3-BV711 (clone OKT3)	317328	BioLegend
DAPI dye	422801	BioLegend
Anti-human CD4-PECy7 (clone RPA-T4)	317442	BioLegend
Anti-human CD19-BV605 (clone HIB-19)	302243	BioLegend
Anti-human CD314-BV785 (NKG2D, clone 1D11)	320829	BioLegend
Anti-human PD-1-AF700 (clone EH12.2H7)	329952	BioLegend
Anti-human CD28-PerCPCy5.5 (clone CD28.2)	302922	BioLegend
Anti-human CD27-BV605 (clone O323)	302830	BioLegend

Reagent name and specifications	CAT#	Company
Streptavidin-BV421	563259	BioLegend
Anti-human TCRVd2-PE (clone 123R3)	130-125-855	Miltenyi Biotec
Anti-human TCRVd2-APC (clone 123R3)	130-121-339	Miltenyi Biotec
Anti-human TCRVd1-FITC (clone REA173)	130-118-362	Miltenyi Biotec
Anti-human TCRVd1-VioGreen (clone REA173)	130-120-442	Miltenyi Biotec
Anti-human TCRα/β-APC (clone REA652)	130-113-535	Miltenyi Biotec
Anti-human TCR αβ-BV786 (clone T10B9.1A-31)	563825	BD biosciences
Anti-human CD3-BUV395 (clone SK7)	564001	BD biosciences
Anti-human CD8-BUV395 (clone RPA-T8)	563795	BD biosciences
Anti-human CD8-PE-Cy7 (clone RPA-T8)	557746	BD biosciences
Anti-human GLUT1-AF647 (clone 202915)	566580	BD biosciences
Anti-human TIM3-BV650 (clone 7D3)	565564	BD biosciences
Anti-human CD69-BV650 (clone FN50)	563835	BD biosciences
Anti-human CD25-BV711 (clone 2A3)	563159	BD biosciences
Anti-human phosphoY142 CD3z (clone K25-407.69)	558402	BD biosciences
Anti-human GLUT4-PECy7 (polyclonal)	NBP1-49533PECY7	Novus Biologicals
Anti-human GLUT6-AF405 (polyclonal)	NBP1-59891AF405	Novus Biologicals
Anti-human GLUT3-PECy7 (clone 202017)	FAB1415G-100UG	R&D Systems
Anti-human GLUT8-PE (polyclonal)	orb2574936	Biorbyt
Anti-human CD3z (clone F-3)	sc-166275	Santa Cruz
Anti-human phospho-Y83 CD3z (clone EP776(2)Y)	ab303153	Abcam
Anti-human phospho-Y72 CD3z (polyclonal)	SAB4200330	Millipore Sigma

Reagent name and specifications	CAT#	Company
Single-Cell Adaptive Immune Chip – (H) 8	ISOCODE-1001-8	Bruker Cellular Analysis

Table S2. Reagents.