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Supplementary Materials for

PD-1– and CTLA-4–Based Inhibitory Chimeric Antigen Receptors (iCARs) Divert Off-Target Immunotherapy Responses

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Other Supplementary Material for this manuscript includes the following:
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Supplementary Materials:

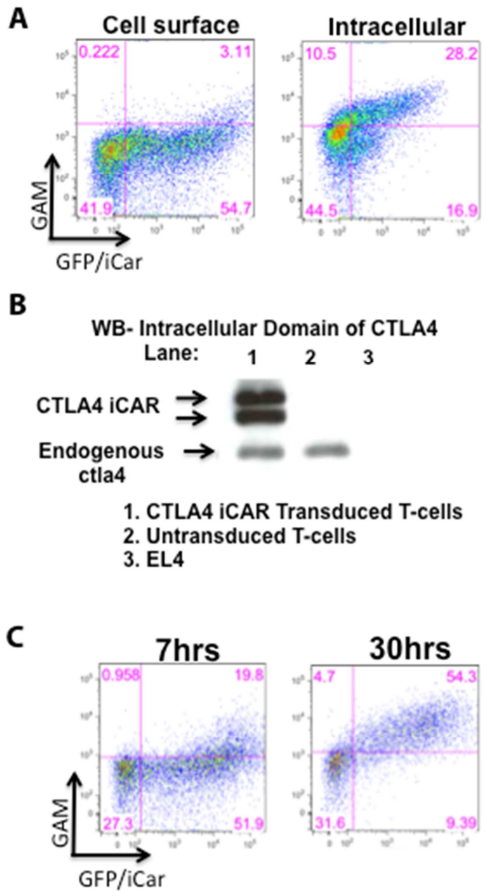


Fig. S1. CTLA-4 iCAR cell surface expression is increased after T cell activation.

(A) Cell surface and intracellular expression of CTLA4 iCAR-P on transduced primary human T cells (B) Western blot analysis using an antibody specific for the intracellular domain of CTLA4 on untransduced (2) and CTLA4 iCAR-P transduced (1) primary human T cells. Murine EL4 cells (3) served as negative control. (C) 1928z/CTLA4 iCAR T cells were activated using 3T3-19 AAPCs and analyzed for cell surface CTLA4 iCAR expression at 7hrs and 30hrs post activation (n=3).

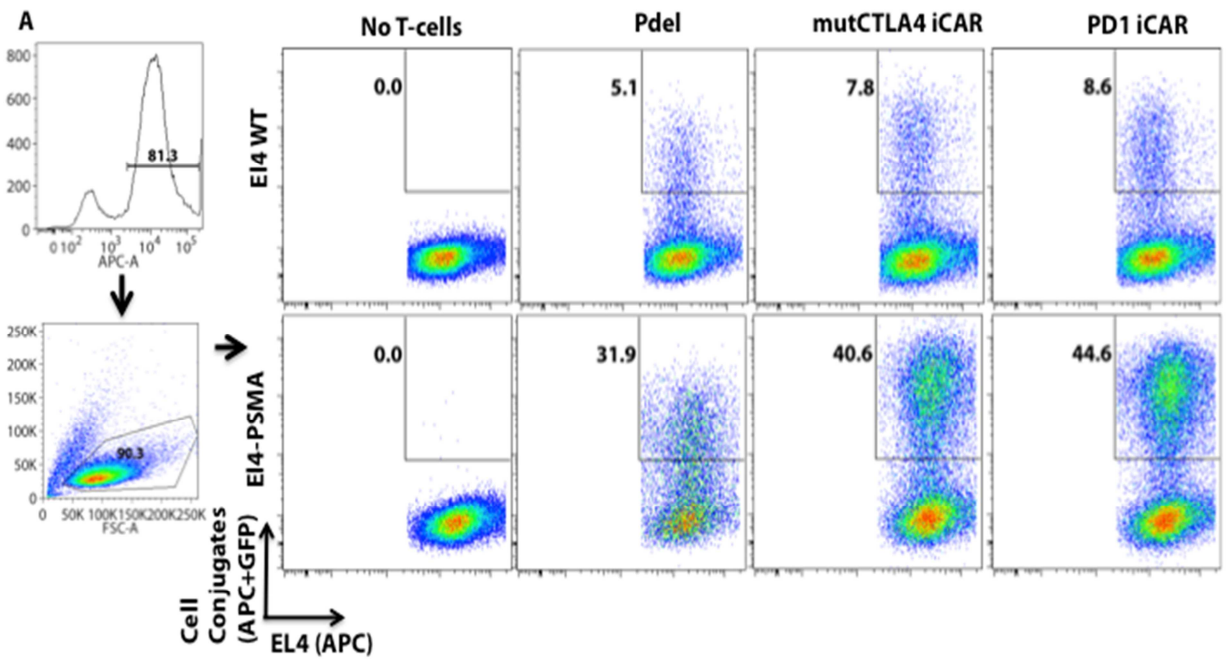


Fig. S2. iCAR-P bind to PSMA expressing cells. (**A**) EL4-wt or EL4- PSMA cells, labeled with the lipophilic DiD dye, were incubated with iCAR/GFP expressing T cells in a cellular conjugation assay. Conjugates are detected by flow cytometry as DiD/GFP double positive events.

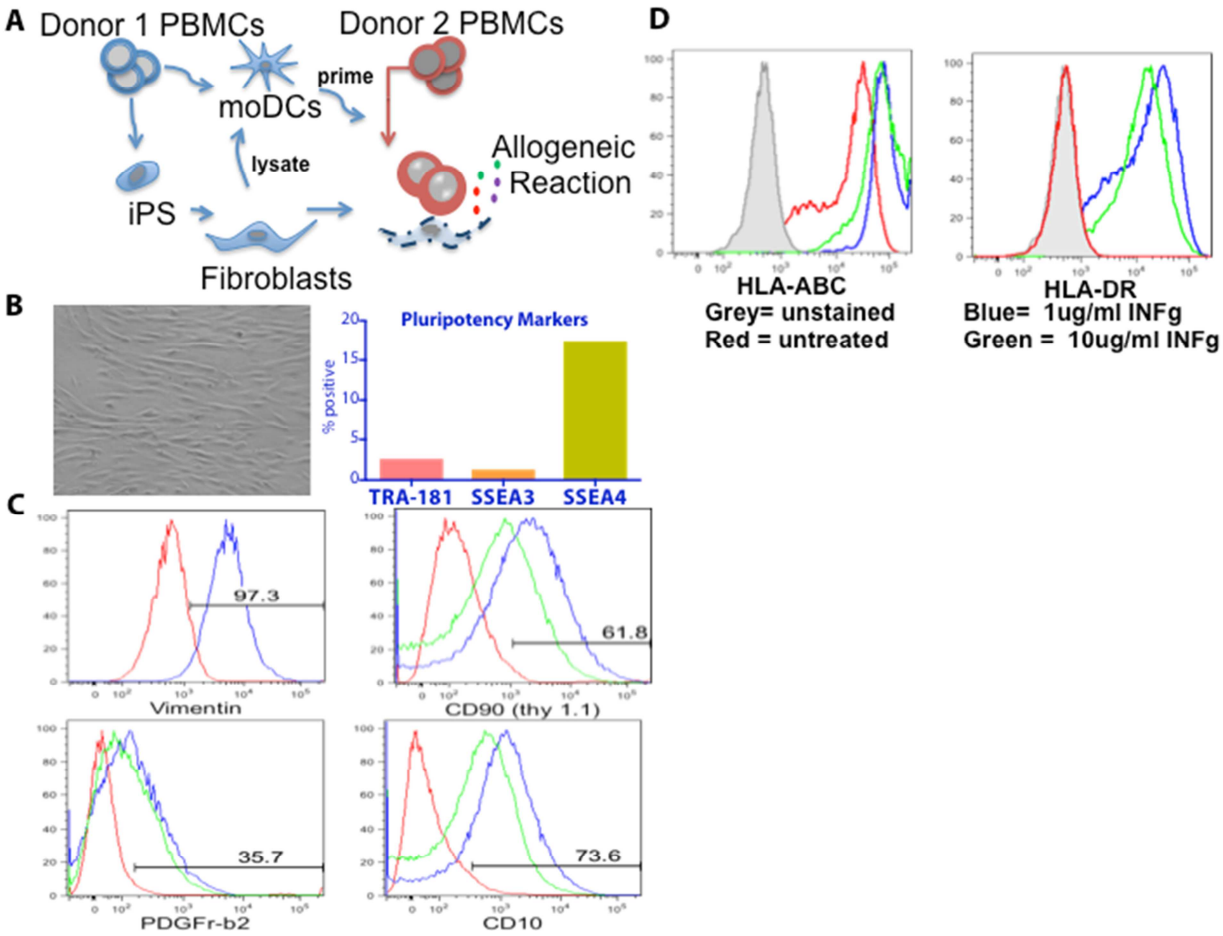


Fig. S3. Allogeneic reactivity model using iPS-derived fibroblasts and isogenic moDCs. (**A**) Induced pluripotent stem (iPS) cells were generated from Donor 1 PBMCs and used to derive fibroblasts. Donor 1 PBMCs were also used to derive moDCs, which were pulsed with fibroblast lysates and could prime an allogeneic reaction from a second donor's PBMCs. (**B**) Microscopy picture showing the morphology of teratoma-derived iPS-fibroblasts grown in culture. (**C**) iPS-fib, lacked expression of pluripotency markers, displayed fibroblast morphology, and stained positive for several fibroblast cell surface markers including CD90, PDGFr-b2, and CD10. (RED=isotype control; BLUE and GREEN are two independent isolated lines) (**D**) iPS-fib basally stained positive for HLA class I, but not class II, and rapidly upregulated expression of both upon recombinant INF gamma treatment.

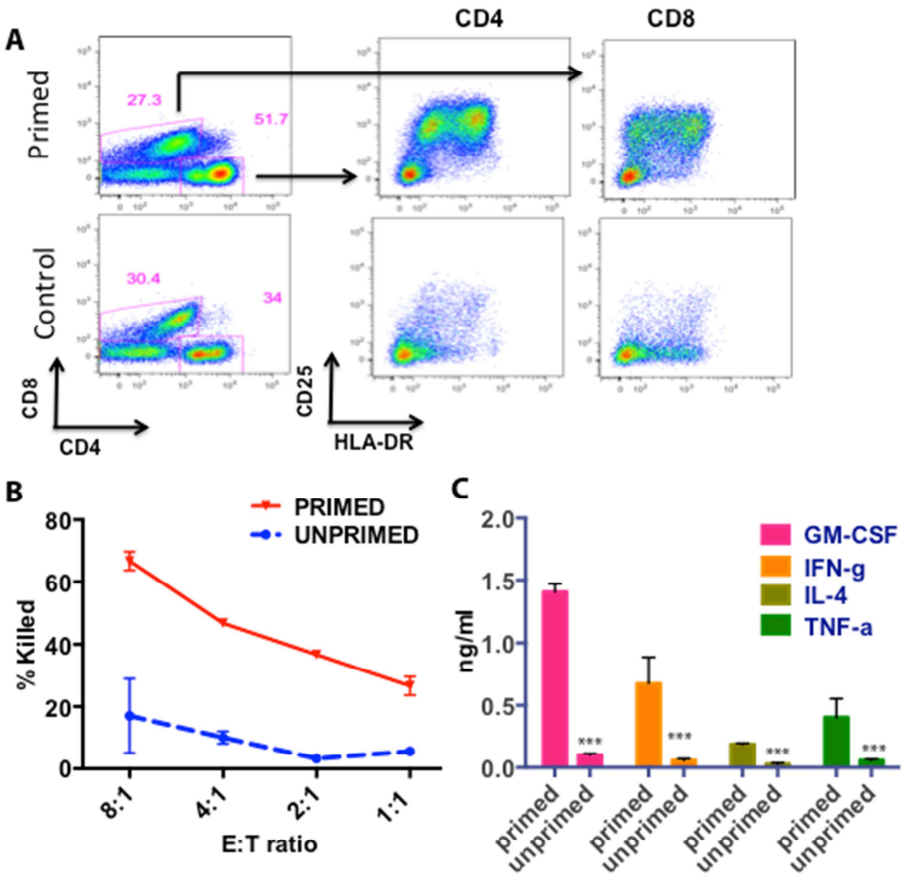


Fig. S4. Potent reactivity of iCAR-transduced primary human T cells against allogeneic iPS-derived fibroblasts. (**A**) iCAR transduced T cells from one donor were primed with moDCs pulsed with iPS-fib lysates of a different donor, and 6 days later stained for the activation markers CD25 and HLA-DR. (**B**) Twice primed iCAR transduced T cells were incubated for 18hrs with iPS-fib-luc and killing was quantified using the Bright-Glo assay system (n=3 for each condition). (**C**) Cytokines were measured at 18hrs in the cell culture supernatant from (B) at 4:1 E:T ratio. Error bars represent +/- SEM. Statistical comparison was carried out within each condition (ie T□T). ***p<0.001 by Student's *t* test.

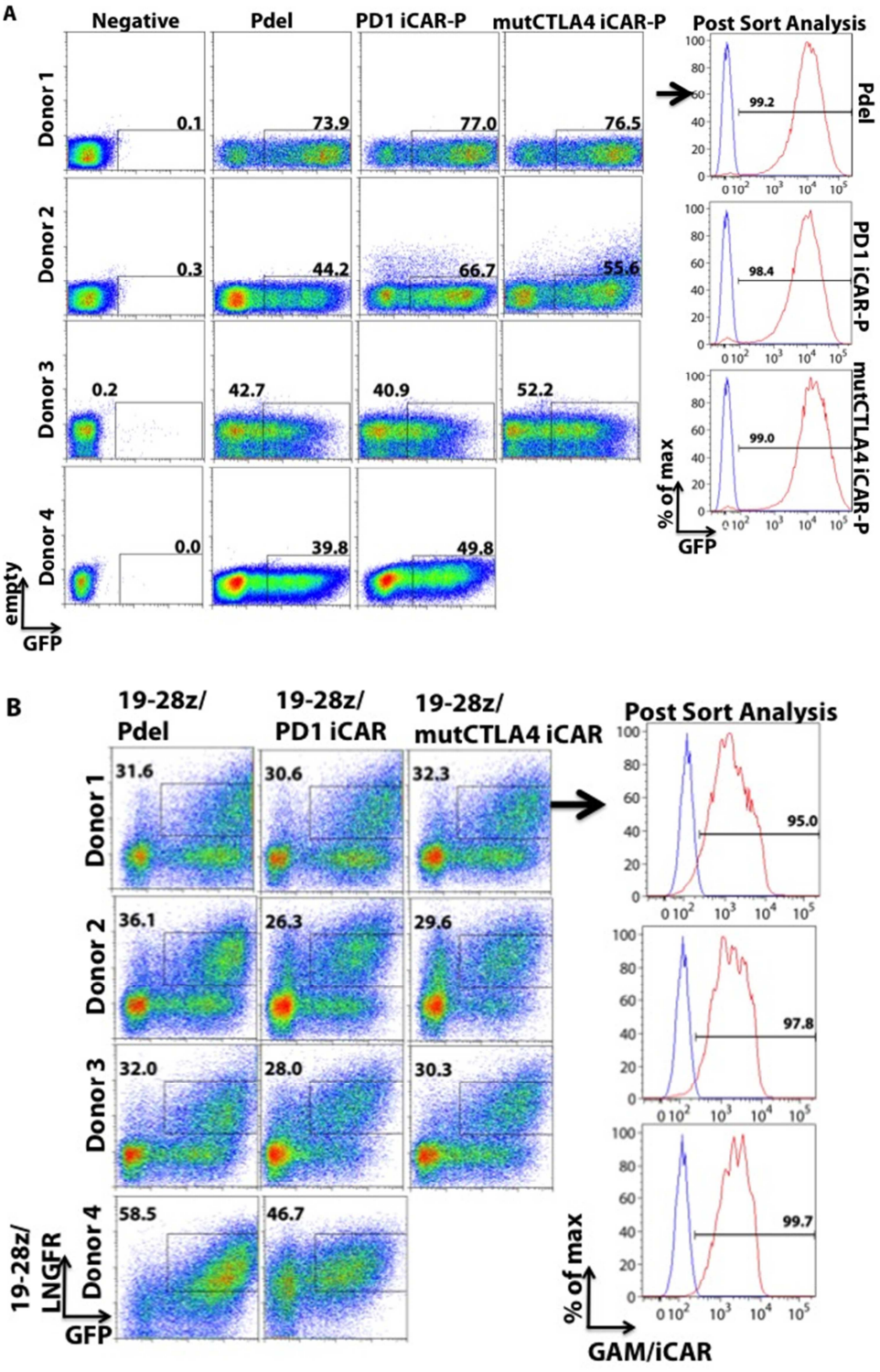
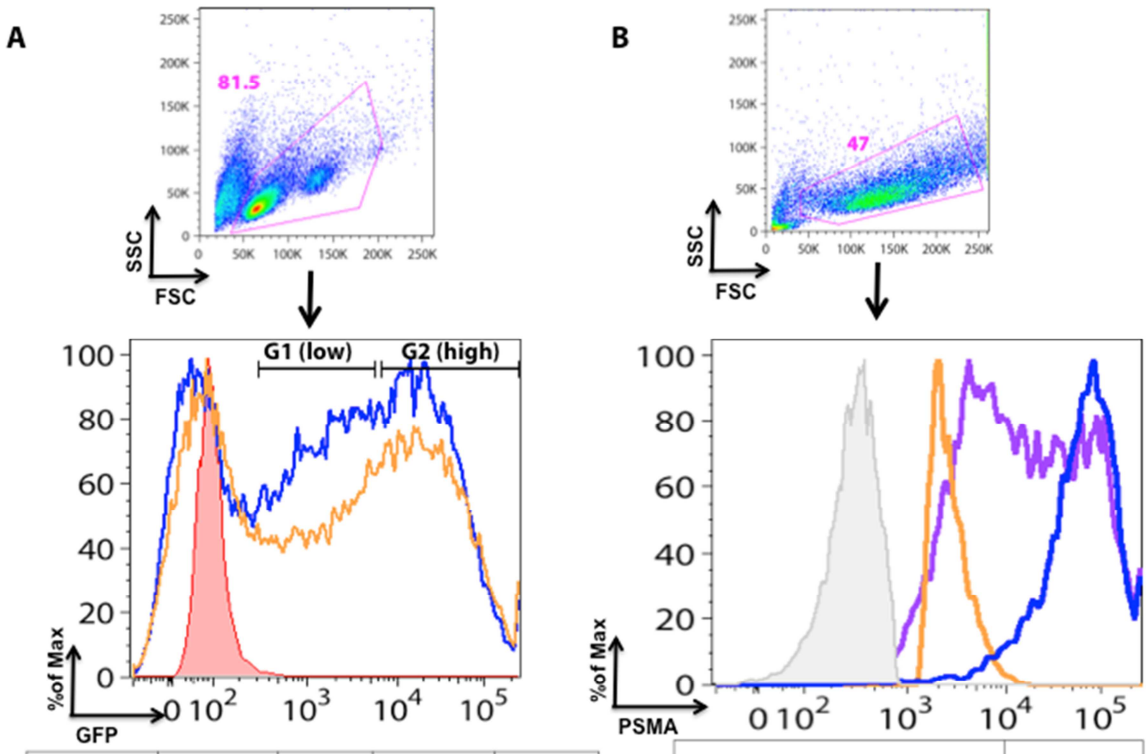


Fig. S5. Transduction and sorting strategy of iCAR or 19-28z/iCAR T cells. (A)

PD1-iCAR(GFP) or Pdel (GFP) transduced T cells were sorted for transgene expression

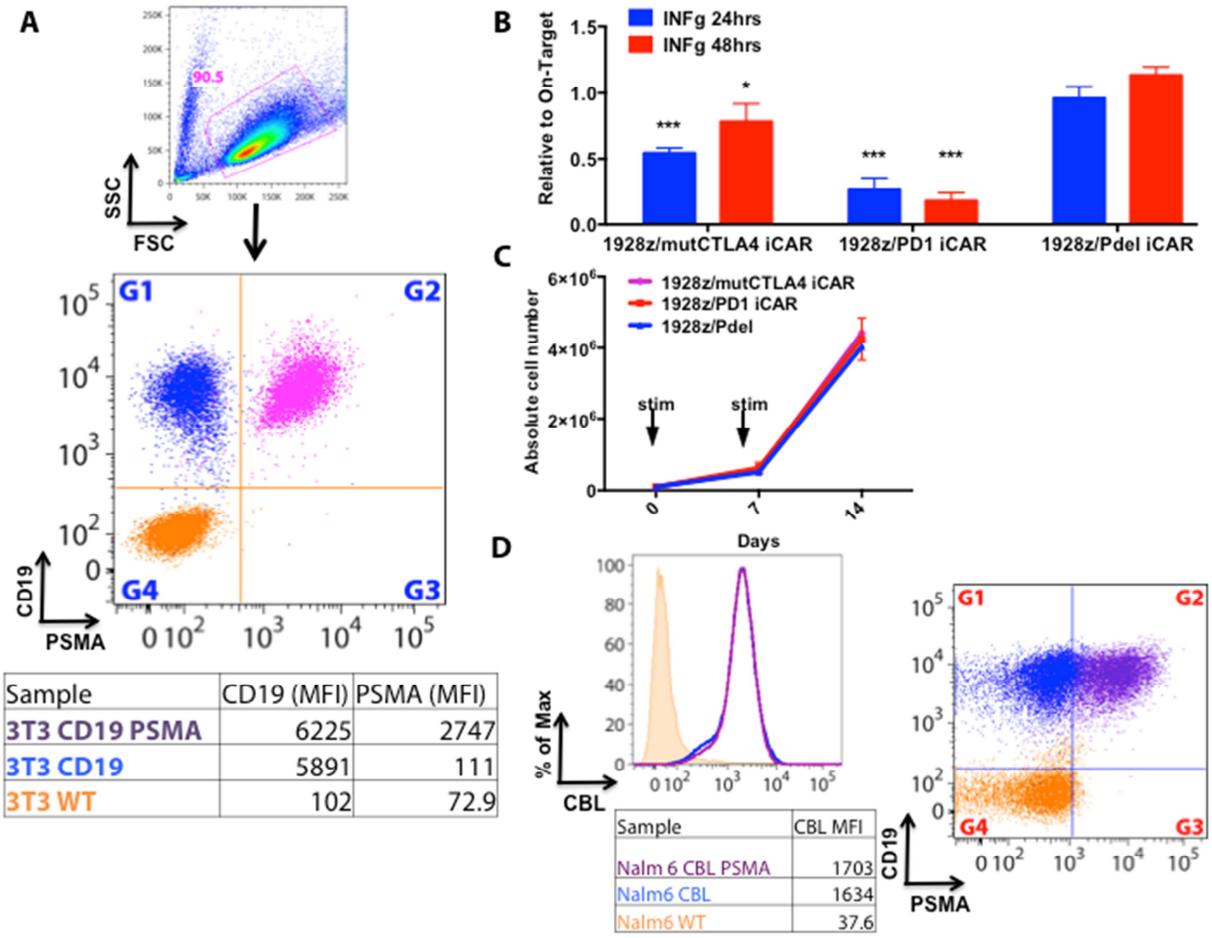
based on GFP expression level. Each donor represents a separate experiment. Post sort analysis was carried out to confirm purity. (B) 19-28z (LNGFR)/iCAR(GFP) or 19-28z/Pdel/GFP transduced T cells were sorted for transgene expression based on GFP expression and LNGFR level. Each donor represents a separate experiment. Post sort analysis was carried out to confirm purity and iCAR expression.



	G1 (frequency)	G1 (MFI)	G2 (frequency)	G2 (MFI)
PD1-iCAR-P	33.7	1403	34.8	25881
Pdel	27.8	1406	36.7	28487

Sample	PSMA (MFI)
iPS-fib PSMA (high level)	53404
iPS-fib PSMA (low level)	2627
iPS-fib PSMA (sort bulk +)	14622
iPS-fib wt	291

Fig. S6. Sorting strategy of low/high iCAR–expressing T cells and PSMA-expressing iPS fibroblasts. (A) PD1-iCAR/GFP or Pdel/GFP transduced T cells were sorted for low or high transgene expression based on GFP expression level. (B) iPS-derived fibroblasts (iPS-fib) were transduced to express PSMA and sorted using an anti-PSMA antibody (iPS-fib-PSMA sort bulk+). These cells were used for experiments in Fig. 2 and 4. A second separate sort was used to purify low or high surface PSMA expressing iPS-fib using an anti-PSMA antibody and these cells were used in experiments in Fig. 3.



Sample	CD19 (MFI)	PSMA (MFI)
3T3 CD19 PSMA	6225	2747
3T3 CD19	5891	111
3T3 WT	102	72.9

Sample	CBL MFI
Nalm 6 CBL PSMA	1703
Nalm6 CBL	1634
Nalm6 WT	37.6

Fig. S7. iCARs inhibit 19-28z–driven human T cell cytokine release and proliferation. (**A**) Representative INFγ cytokine analysis of supernatants at 24h and 48h post seeding of dual sorted 1928z/Pdel or iCAR transduced human T cells on CD19 (target) or CD19/PSMA (off-target) positive AAPCs. Data represented as a ratio of off-target/target values and are pooled from three independent experiments. (n=6 wells per condition). Error bars represent +/- SEM. (**B**) Absolute counts of double positive 1928z/Pdel or iCAR T cells stimulated on day 0 and 7 with CD19+ (target) AAPCs.

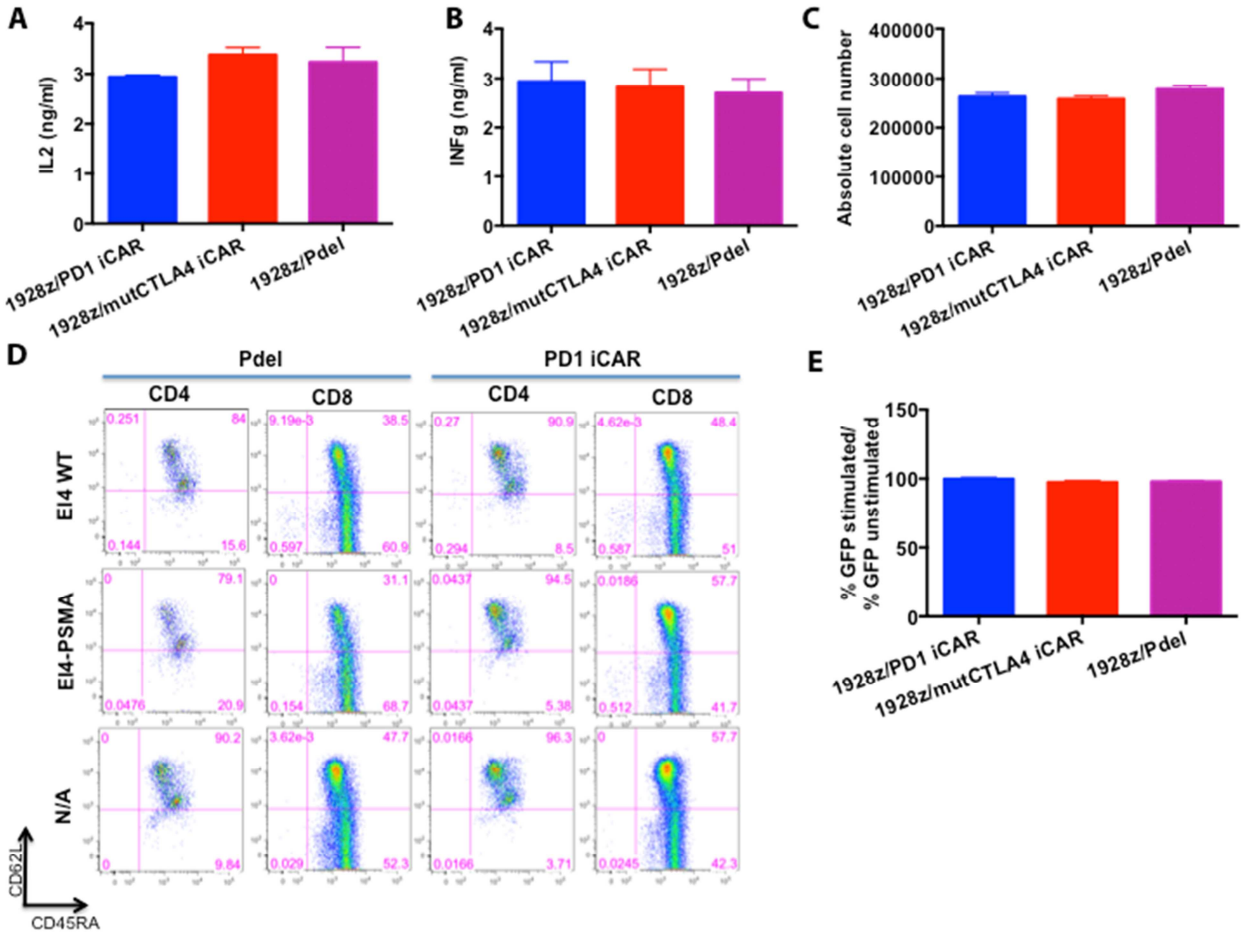


Fig. S8. Basal expression of iCARs does not affect function of primary human T cells. (A,B) Seven days post transduction with 1928z/iCAR, T cells were activated with CD3/CD28 beads and IL-2/INFy levels were assessed after 24hrs (**C**). At eight days after bead activation, absolute T cell expansion was quantified using CountBright beads (**D**) and the change in the percent of GFP positive cells in each iCAR group was normalized relative to unstimulated cells. (**E**)1928z/ iCAR T cells were co-cultured for five days with irradiated EL4-WT or EL4-PSMA cells and immunophenotyped using flow cytometry.

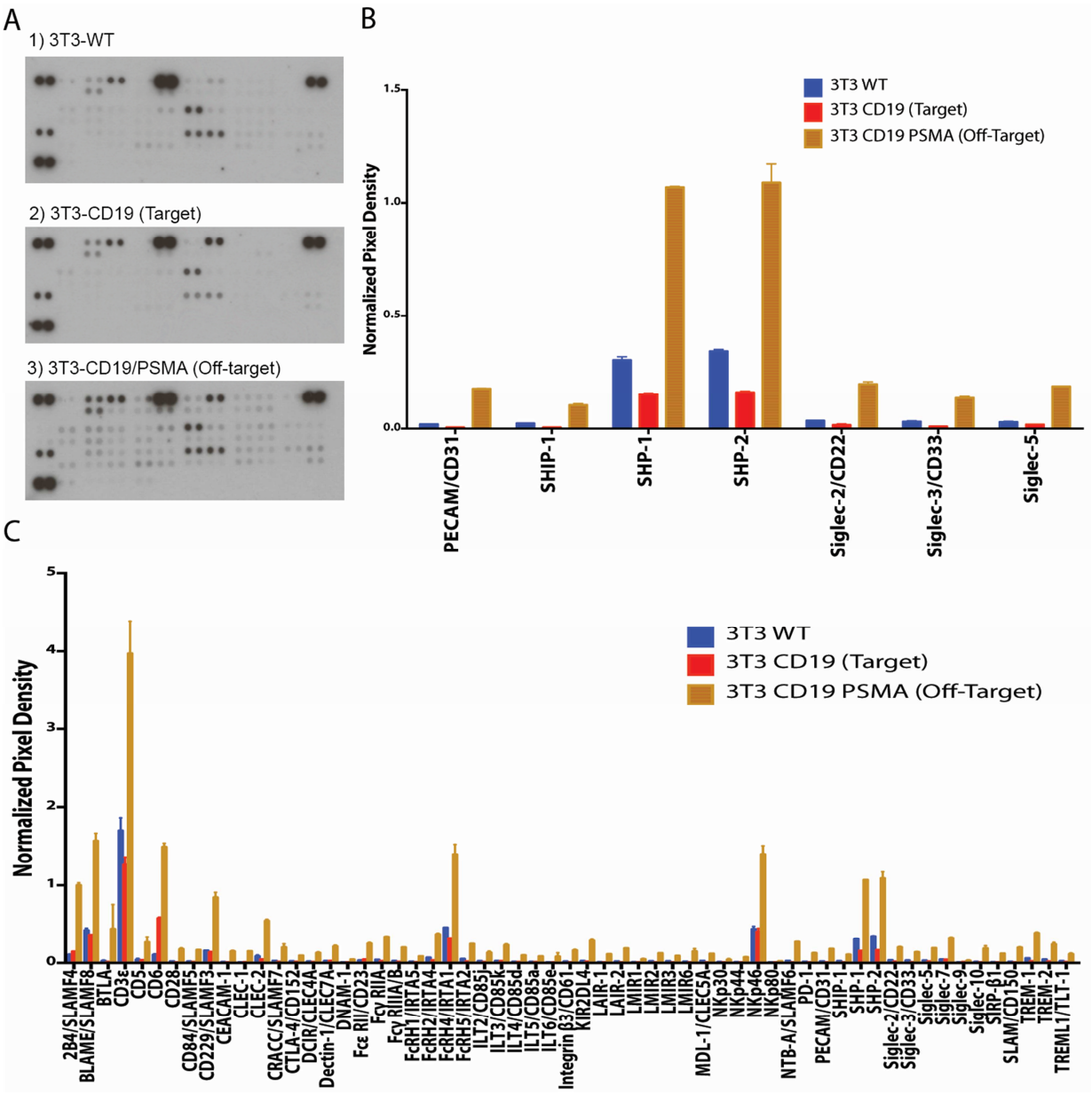


Fig. S9. Signaling and biochemical pathway characterization of the PD-1 iCAR. 19-28z/PD-1 iCAR cells were exposed to AAPCs expressing no antigen (WT), CD19 (Target), or CD19 and PSMA (Off-Target) at an E:T ratio of 4:1 for 60min. (**A)** Human Phospho-Immunoreceptor Array incubated with 100 µg of lysate from 19-28z/PD-1 iCAR T cells and respective AAPCs. All blots were detected using chemiluminescence on the same X-ray film to standardize exposure levels. (**B,C)** Quantification of arrays in

(A) using scanned X-ray film images analyzed using image analysis software. All pixel density is normalized on each array using internal pY controls. **(B)** SHP1 and SHP2 phosphorylation states on target, off-target, or control AAPCs. **(C)** Quantification of phosphorylation levels of 59 ITAM/ITIM-associated immunoreceptors.

Movies S1: iCAR- and CAR-expressing T cells discern targets in vitro. (A, B) 19-28z/Pdel **(A)** or 19-28z/PD1 iCAR-P **(B)** T cells were incubated with a 1:1 mix of target (GFP+) and off- target (mCherry+) AAPCs and time-lapse microscopy was used to visualize real-time killing of each population for 38 hours.

A

Raw BLI (Flux-photons)	mutCTLA4 iCAR			PD1 iCAR			Pdel		
4:1	1.5E+07	1.4E+07	1.4E+07	1.5E+07	1.5E+07	1.5E+07	1.6E+07	1.6E+07	1.6E+07
2:1	1.2E+07	9.9E+06	1.1E+07	1.2E+07	1.0E+07	1.1E+07	1.2E+07	1.2E+07	1.2E+07
1:1	8.2E+06	8.2E+06	8.2E+06	8.3E+06	8.5E+06	8.4E+06	7.9E+06	9.0E+06	8.4E+06
No T cells	2.4E+07	1.2E+07	1.9E+07						

B

pg/ml	mutCTLA4 iCAR			PD1 iCAR			Pdel		
GM-CSF	647.2	801.3	765.5	703.8	897.3	783.4	714.8	818.8	774.9
INFγ	621.2	552.2	534.4	533	595	553.2	581	622	618.8
TNF-a	379	422	400.1	388	379	375.5	394	643	523.2

C

Raw BLI (Flux-photons)	mutCTLA4 iCAR			PD1 iCAR			Pdel		
16:1	3.87E+01	4.28E+01	4.07E+01	1.67E+01	2.09E+01	1.85E+01	4.88E+01	4.74E+01	4.70E+01
8:1	3.09E+01	3.30E+01	3.23E+01	9.96E+00	1.19E+01	1.10E+01	4.56E+01	4.45E+01	4.50E+01
4:1	2.30E+01	2.62E+01	2.40E+01	3.27E+00	3.06E+00	3.21E+00	4.24E+01	4.28E+01	4.25E+01
2:1	2.11E+01	1.98E+01	2.02E+01	5.27E-01	3.48E+00	1.90E+00	3.80E+01	4.21E+01	3.99E+01
1:1	2.78E+01	1.81E+01	2.25E+01	5.01E+00	2.00E+00	3.53E+00	4.11E+01	4.15E+01	4.12E+01
No T cells	3.92E+07	3.85E+07	3.76E+07						

D

PD1 vs Pdel	Significant?	P value	PD1	Pdel	Difference	SE of difference	t ratio	df
16:1	*	2.72372E-05	35.47	90.58	-55.12	2.55	21.59	4
8:1	*	7.47524E-07	20.80	85.47	-64.67	1.22	53.20	4
4:1	*	9.99669E-10	6.03	80.83	-74.80	0.27	278.33	4
2:1	*	1.2563E-05	3.73	75.87	-72.13	2.75	26.23	4
1:1	*	1.74306E-06	6.67	78.23	-71.57	1.66	43.03	4

CTLA4 vs Pdel	Significant?	P value	CTLA4	Pdel	Difference	SE of difference	t ratio	df
16:1	*	0.00581413	77.33	90.58	-13.25	2.47	5.37	4
8:1	*	5.02912E-05	60.86	85.47	-24.61	1.33	18.50	4
4:1	*	4.23971E-05	46.33	80.83	-34.50	1.79	19.31	4
2:1	*	9.05225E-05	38.67	75.87	-37.20	2.33	15.94	4
1:1	*	0.0027265	43.27	78.23	-34.97	5.30	6.60	4

E

GM-CSF

RAW(pg/ml)	Pdel			Avg Pdel	mutCTLA4 iCAR			PD1 iCAR		
16:1	564.94	573.44	591.49	**576.62**	549.74	670.09	596.70	181.79	245.95	209.53
8:1	339.22	378.48	320.44	**346.05**	277.03	217.73	243.96	58.42	42.98	48.67
4:1	179.60	136.10	190.34	**168.68**	74.20	75.28	75.93	26.04	25.31	25.69
2:1	63.26	71.91	73.08	**69.42**	26.72	26.80	26.34	9.54	8.97	9.06
1:1	20.49	23.10	16.49	**20.03**	6.15	6.92	6.59	2.49	3.38	2.91

%PDEL	mutCTLA4 iCAR			PD1 iCAR		
16:1	95.34	116.21	103.48	31.53	42.65	36.34
8:1	80.05	62.92	70.50	16.88	12.42	14.06
4:1	43.99	44.63	45.02	15.44	15.00	15.23
2:1	38.49	38.61	37.94	13.74	12.92	13.06
1:1	30.72	34.57	32.89	12.42	16.88	14.55

Table S1. Raw data and statistical significance testing for Fig. 2. (**A**) Killing of the iPS-fibroblasts was quantified using the Bright-Glo assay system for Pdel, PD-1, or mutCTLA-4 iCAR-P transduced T cells. (**B**) Cytokine secretion in cell culture supernatants from (A) at 4:1 E:T ratio was assessed at 18hrs. (**C**) Pdel or iCAR positive T

cells were incubated for 24hrs with off-target iPS-fib expressing PSMA (iPS-fib-PSMA) and luciferase signal was quantified. (**D**) Posthoc analysis for (C) was carried out using multiple t-tests corrected with the Holm-Sidak method. (**E**) Cytokine secretion measured at 24hrs in cell culture supernatants from (C). Raw values for GM-CSF are presented. E:T ratio, effector: target ratio.

A

Normalized flux	Low Pdel			High Pdel			High P-PD1			Low P-PD1		
16:1	96.76	96.88	96.99	97.41	96.9	97.2	54.7	59.9	57.8	96.65	93.5	95
8:1	87.3	92.68	89.71	97.66	97.02	97.03	24.7	30.7	27.3	83.6	82.4	83.8
4:1	83.2	84.2	83.81	96.65	95.1	95.44	21.5	25.8	23.48	74.3	74.2	74.8
2:1	77.6	80.1	78	91.4	92.68	92.87	18.3	15.3	16.2	64.3	66.8	65.1
1:1	71.1	68	69.1	89.4	85.7	87.37	13.7	17.5	15.1	48.6	57.5	53.4

Low PD1 vs Low Pdel								
	Significant?	P value	Low PD1	Low Pdel	Difference	SE of difference	t ratio	df
16:1		0.12	95.05	96.88	-1.83	0.91	2.00	4.00
8:1	*	0.01	83.27	89.90	-6.63	1.62	4.10	4.00
4:1	*	0.00	74.43	83.74	-9.30	0.35	26.96	4.00
2:1	*	0.00	65.40	78.57	-13.17	1.07	12.31	4.00
1:1	*	0.00	53.17	69.40	-16.23	2.73	5.95	4.00
High PD1 vs High Pdel								
	Significant?	P value	High PD1	High Pdel	Difference	SE of difference	t ratio	df
16:1	*	0.00	57.47	97.17	-39.70	1.52	26.16	4.00
8:1	*	0.00	27.57	97.24	-69.67	1.75	39.81	4.00
4:1	*	0.00	23.59	95.73	-72.14	1.33	54.29	4.00
2:1	*	0.00	16.60	92.32	-75.72	1.00	75.60	4.00
1:1	*	0.00	15.43	87.49	-72.06	1.54	46.75	4.00

B

Normalized flux	PSMA low			PSMA high		
8:1	74.3	73	73.8	61.3	68.6	70.7
4:1	64.3	64.1	64.7	21.5	31.6	25.54
2:1	57.5	57	57.6	13.8	19.9	16.1
1:1	48.6	49.4	49.3	20.7	9.7	15.9

PSAM low vs PSMA High								
	Significant?	P value	PSMA low	PSMA high	Difference	SE of difference	t ratio	df
8:1		0.08	73.70	66.87	6.83	2.87	2.38	4.00
4:1	*	0.00	64.37	26.21	38.15	2.94	12.98	4.00
2:1	*	0.00	57.37	16.60	40.77	1.79	22.80	4.00
1:1	*	0.00	49.10	15.43	33.67	3.19	10.54	4.00

Table S2. Raw data and statistical significance testing for Fig. 3. (**A**) Killing of iPS-fib-PSMA relative to untreated cells was assessed by using the Bright-Glo assay system for sorted high and low Pdel or PD1 iCAR-P transduced alloreactive T cells. Posthoc analysis was carried out using multiple t-tests corrected with the Holm-Sidak method. (**B**) PD1 iCAR-P transduced alloreactive T- cells killing of iPS-fib-PSMA sorted for high or low levels of PSMA expression was quantified using the Bright-Glo assay system. Posthoc analysis was carried out using multiple t-tests corrected with the Holm-Sidak method.

A

Raw BLI (Flux-photons)															
Days	No T Cells					PD1 iCAR					Pdel				
3.00	7.78E+07	1.43E+07	1.40E+07	1.48E+07	1.44E+07	1.46E+07	1.69E+07	1.42E+07	4.44E+07	1.15E+07	2.74E+07	8.59E+05	5.46E+07	5.81E+06	2.32E+07
5.00	4.09E+07	1.91E+07	1.04E+07	4.73E+07	2.78E+07	8.26E+06	9.87E+06	3.95E+07	3.93E+07	2.58E+07	6.17E+06	4.41E+06	2.09E+06	2.05E+06	4.09E+06
8.00	2.22E+07	2.01E+07	1.50E+07	2.82E+07	2.60E+07	3.28E+07	1.47E+07	1.66E+07	3.29E+07	2.17E+07	5.28E+06	4.76E+06	5.43E+06	2.59E+06	3.19E+06
34.00	4.62E+07	2.55E+07	1.34E+07	5.53E+07	3.36E+07	5.63E+07	3.42E+07	2.84E+07	5.85E+07	3.31E+07	2.01E+06	6.37E+06	1.32E+06	1.25E+06	2.19E+06
48.00	4.39E+07	1.96E+07	3.81E+07	3.65E+07	2.70E+07	7.50E+07	2.56E+07	3.94E+06	5.23E+07	3.17E+07	2.83E+06	7.10E+05	1.44E+06	1.86E+06	2.06E+06

B

No T cells vs Pdel								
	Significant?	P value	No Tcells	Pdel	Difference	SE of difference	t ratio	df
3.00E+00		7.75E-01	2.71E+07	2.24E+07	4.69E+06	1.58E+07	2.96E-01	8.00E+00
5.00E+00	*	5.97E-03	2.91E+07	3.76E+06	2.53E+07	6.83E+06	3.71E+00	8.00E+00
8.00E+00	*	6.39E-05	2.23E+07	4.25E+06	1.81E+07	2.38E+06	7.59E+00	8.00E+00
3.40E+01	*	2.58E-03	3.48E+07	2.63E+06	3.22E+07	7.46E+06	4.31E+00	8.00E+00
4.80E+01	*	9.12E-05	3.30E+07	1.78E+06	3.12E+07	4.33E+06	7.21E+00	8.00E+00

No T cells vs PD1								
	Significant?	P value	No Tcells	PD1	Difference	SE of difference	t ratio	df
3.00E+00		6.45E-01	2.71E+07	2.03E+07	6.74E+06	1.41E+07	4.79E-01	8.00E+00
5.00E+00		6.48E-01	2.91E+07	2.45E+07	4.55E+06	9.61E+06	4.74E-01	8.00E+00
8.00E+00		7.58E-01	2.23E+07	2.37E+07	-1.44E+06	4.52E+06	3.18E-01	8.00E+00
3.40E+01		4.75E-01	3.48E+07	4.21E+07	-7.30E+06	9.74E+06	7.49E-01	8.00E+00
4.80E+01		7.25E-01	3.30E+07	3.77E+07	-4.69E+06	1.28E+07	3.65E-01	8.00E+00

Table S3. Raw data and statistical significance testing for Fig. 4. (A) Bioluminescent imaging (BLI) of iPS-fib-PSMA before and at selected time points after T cell infusion. (B) Posthoc analysis was carried out using multiple t-tests corrected with the Holm-Sidak method.

A

Relative to On-Target	1928z/Pdel				1928z/PD1 iCAR				1928z/mutCTLA4 iCAR	
INFg	1.05	0.93	0.87	0.26	0.35	0.18	0.58		0.51	0.54
IL2	0.85	0.94	0.89	0.14	0.10	0.11	0.40		0.38	0.42
TNFa	1.05	1.02	1.00	0.24	0.29	0.28	0.44		0.45	0.43
IL10	0.92	0.98	0.97	0.31	0.27	0.27	0.59		0.54	0.65
GMCSF	0.95	1.04	1.01	0.26	0.23	0.26	0.63		0.62	0.69

	Significant ?	P value	1928z/pdel	1928z/pd1	Difference	SE of difference	t ratio	df
INFg	*	0.00	0.95	0.26	0.69	0.07	9.51	4.00
IL2	*	0.00	0.89	0.12	0.78	0.03	27.09	4.00
TNFa	*	0.00	1.02	0.27	0.75	0.02	35.73	4.00
IL10	*	0.00	0.96	0.28	0.67	0.02	29.46	4.00
GMCSF	*	0.00	1.00	0.25	0.75	0.03	26.52	4.00

	Significant?	P value	1928z/pdel	1928z/ mutCTLA4	Difference	SE of difference	t ratio	df
INFg	*	0.00	0.95	0.54	0.41	0.06	7.18	4.00
IL2	*	0.00	0.89	0.40	0.49	0.03	17.32	4.00
TNFa	*	0.00	1.02	0.44	0.58	0.02	37.31	4.00
IL10	*	0.00	0.96	0.59	0.36	0.04	9.87	4.00
GMCSF	*	0.00	1.00	0.65	0.35	0.03	10.30	4.00

B

Cell counts	Significant?	P value	1928z/pdel	1928z/pd1	Difference	SE of difference	t ratio	df
0								
7	*	0.00226118	464667	246000	218667	31497.8	6.94229	4
14	*	0.000187776	4490000	840333	3649667	275554	13.2448	4

C

Normalized mCherry signal	1928z/mutCTLA4 iCAR				1928z/PD1 iCAR				1928z/Pdel				1928z			
2.00	0.98	1.23	1.19	1.21	0.96	0.98	0.90	1.02	1.00	1.02	1.02	1.06	1.03	1.11	1.02	0.98
18.00	0.88	1.43	1.09	1.21	1.27	1.58	1.00	1.02	0.38	0.42	0.41	0.27	0.43	0.69	0.40	0.26
38.00	0.73	0.54	0.62	0.78	0.79	1.09	0.75	1.20	0.08	0.08	0.06	0.06	0.09	0.09	0.09	0.10
120.00	0.12	0.27	0.11	0.06	1.66	1.58	1.00	1.26	0.04	0.05	0.05	0.03	0.07	0.09	0.07	0.07

	Significant ?	P value	1928z/pdel	1928z/pd1	Difference	SE of difference	t ratio	df
2.00		1.06E-01	1.02	0.97	-0.06	0.03	1.90	6.00
18.00	*	9.54E-04	0.37	1.22	0.85	0.14	6.01	6.00
38.00	*	2.11E-04	0.07	0.96	0.89	0.11	7.95	6.00
120.00	*	1.32E-04	0.04	1.38	1.33	0.15	8.64	6.00

	Significant ?	P value	1928z/pdel	1928z/mutCTLA4	Difference	SE of difference	t ratio	df
2.00		7.44E-02	1.02	1.15	-0.13	0.06	2.16	6.00
18.00	*	6.04E-04	0.37	1.15	-0.78	0.12	6.55	6.00
38.00	*	3.79E-05	0.07	0.67	-0.60	0.06	10.77	6.00
120.00		8.37E-02	0.04	0.14	-0.10	0.05	2.07	6.00

Table S4. Raw data and statistical significance testing for Fig. 5. (**A**) Luminex multiplex cytokine analysis of culture supernatant at 24h, data are represented as a ratio of off-target/target values and pooled from three independent experiments (n=6 wells per condition). Posthoc analysis was carried out using multiple t-tests corrected with the

Holm-Sidak method. (**B**) Posthoc analysis was carried out using multiple t-tests corrected with the Holm-Sidak method for Fig. 5B comparing the proliferation of 19-28z/Pdel and 19-28z/PD1 iCAR. **(C)** Quantification of mCherry signal against CD19 targets or CD19-PSMA off-target cells, as described in Methods. Posthoc analysis was carried out using multiple t-tests corrected with the Holm-Sidak method.