OMTO, Volume *20*

Supplemental Information

Adoptive T cell immunotherapy for medullary thyroid carcinoma targeting GDNF family receptor alpha 4

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Supplemental Materials:

Table S1. Raw data (Ct values) corresponding to Figure 1b.

Cell / Tissue	Ct values (ACTB)					Ct values (GFRa4)					Mean Ct (ACTB)	Mean Ct (GFRa4)
TT	20.93	20.93	20.95	20.94	26.79	25.69	25.74	25.88	20.9375	26.025		
MZCRC-1	16.88	17.07	17.04	17.06	30.01	29.33	29.3	29.65	17.0125	29.5725		
K562	16.6	16.67	16.6	16.58	Not Detected				16.6125	Not Detected		
Fetal Brain	19.17	19.01	19.12	19.19	36.65	36.62	36.88	36.29	19.1225	36.61		
Whole Brain	18.38	18.3	18.37	18.35	34.5	34.98	34.63	34.53	18.35	34.66		
Cerebellum	19.16	19.14	19.11	19.14	40	39.2	39.61	40	19.1375	39.7025		
Spinal Cord	19.72	19.73	19.71	19.78	38.81	40	39.37	40	19.735	39.545		
Cerebral Cortex	18.32	18.38	18.37	18.39	35.82	34.55	34.73	34.25	18.365	34.8375		
Temporal Lobe	18.61	18.62	18.53	18.6	33.85	33.54	33.99	33.37	18.59	33.6875		
Hippocampus	18.73	18.83	18.77	18.9	34.66	34.81	35.51	35.21	18.8075	35.0475		
Parietal Lobe	19.12	18.98	19.03	19.01	35.68	35.75	36.34	36.22	19.035	35.9975		
Occipital Lobe	19.29	19.29	19.28	19.23	36.61	36.46	35.93	36.27	19.2725	36.3175		
Postcentral Gyrus	19.64	19.66	19.65	19.68	35.84	35.91	35.47	36.16	19.6575	35.845		
Pons	19.1	19.03	19.04	19.01	36.61	35.63	36.03	36.43	19.045	36.175		
Insula	19.78	19.77	19.75	19.8	34.94	35.25	34.91	35.21	19.775	35.0775		
Dura Matter	17.58	17.56	17.56	17.56	Not Detected				17.565	Not Detected		
Dorsal Root Ganglion	19.8	19.84	19.82	20.05	Not Detected				19.8775	Not Detected		
Thymus	17.83	17.81	17.79	17.87	38.04	36.45	36.79	35.91	17.825	36.7975		
Thyroid	19.96	19.92	19.95	20.01	31.67	31.89	31.61	31.76	19.96	31.7325		
Kidney	19.83	19.78	19.8	19.83	34.42	34.7	34.76	34.58	19.81	34.615		
Prostate	18.9	18.86	18.87	18.87	40	Not Detected	39.21	38.02	18.875	39.07666667		
Testis	19.03	19.07	18.99	18.99	31.1	31.07	31.34	31.31	19.02	31.205		
Uterus	18.13	18.19	18.2	18.13	39.29	Not Detected	40	38.25	18.1625	39.18		
Colon	18.08	18.15	18.18	18.13	38.71	40	Not Detected	40	18.135	39.57		
Small Intestine	18.58	18.41	18.58	18.58	38.22	40	38.74	40	18.5375	39.24		
Bone Marrow	19.07	19.15	19.14	19.2	Not Detected				19.14	Not Detected		
Lung	18.53	18.57	18.52	18.55	Not Detected				18.5425	Not Detected		
Adrenal	19.26	19.49	19.46	19.54	Not Detected				19.4375	Not Detected		
Fetal Liver	18.89	18.9	18.9	18.9	Not Detected				18.8975	Not Detected		
Liver	22.24	22.16	22.22	22.22	Not Detected				22.21	Not Detected		
Placenta	20.79	20.8	20.7	20.7	Not Detected				20.7475	Not Detected		
Salivary Gland	20.71	21.24	21.21	21.31	Not Detected				21.1175	Not Detected		
Skeletal Muscle	20.73	20.73	20.75	21.27	Not Detected				20.87	Not Detected		
Spleen	18.99	18.99	19.08	19.08	Not Detected				19.035	Not Detected		

Table S2. Summary of *GFRA4* mRNA expression analysis by in-situ hybridization. FFPE samples of the indicated tissues were analyzed by in situ hybridization (RNAscope) with probes targeting human or cynomolgus macaque GFRα4. Positive and negative control probes to PPIB and DapB were tested in parallel for each experimental run. Results of qualitative analysis is shown. N/A, not applicable; N/T, not tested

Tissue	**Human**	**Cynomolgus Macaque**
MTC	+	N/A
TT cell line	+	N/A
Thyroid	+	+
Parathyroid	-	-
Adrenal	-	-
Pituitary	-	-
Ovary	-	-
Thymus	-	-
Pancreas	-	N/T
Esophagus	-	N/T
Stomach	-	N/T
Liver	-	N/T
Small Bowel	-	N/T
Colon	-	N/T
Urinary Bladder	-	-
Testis	-	N/T
Cerebellum	-	-
Cerebral Cortex	-	-
Temporal lobe	-	
Frontal lobe	-	
Occipital lobe	-	
Insula	-	N/T
Pons	-	-
Hippocampus	-	N/T
Medulla	-	N/T
Spinal cord	-	-

Table S3. Summary of toxicity in P4-10 CAR T cell treated mice. NSG mice bearing TT cell tumors were treated with P4-10bbz CAR T cells and were euthanized between 2-3 weeks after T cell injection, and animals were subjected to necropsy including gross and microscopic evaluation.

* Compared to non-CAR and 19bbz CAR T cell treated mice.

Organs without lesion*	**Affected Organs**
Heart	Stomach
Skeletal muscle	Skin
Salivary glands	Oral cavity/upper esophagus (portion with squamous epithelium)
Lower esophagus	
Trachea	
Lung	
Liver	
Gall bladder	
Spleen	
Kidneys	
Adrenal glands	
Cecum	
Small intestine	
Large intestine	
Pancreas	
Mesentery	
Mesenteric lymph nodes	
Testicles, epididymis	
Vas deferens	
Prostate gland, ampulla	
Seminal vesicles	
Preputial glands	
Urinary bladder	
Bone marrow	
Brain	
Thyroid glands	

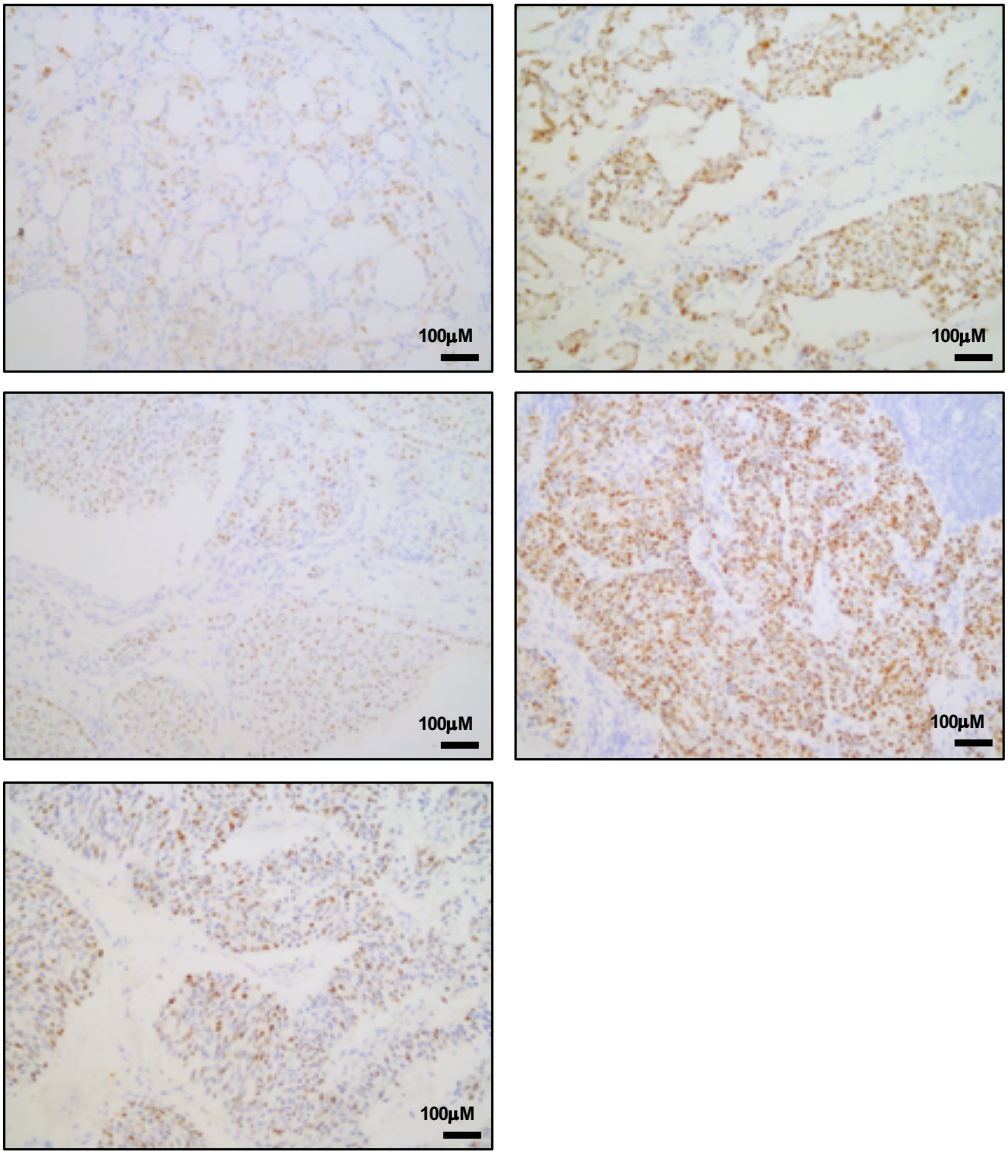

Figure S1: *GFRA4* mRNA in situ hybridization reveals expression in MTC. Five cases of MTC were tested by RNA in situ hybridization (RNAscope) using probes specific to *GFRA4*.

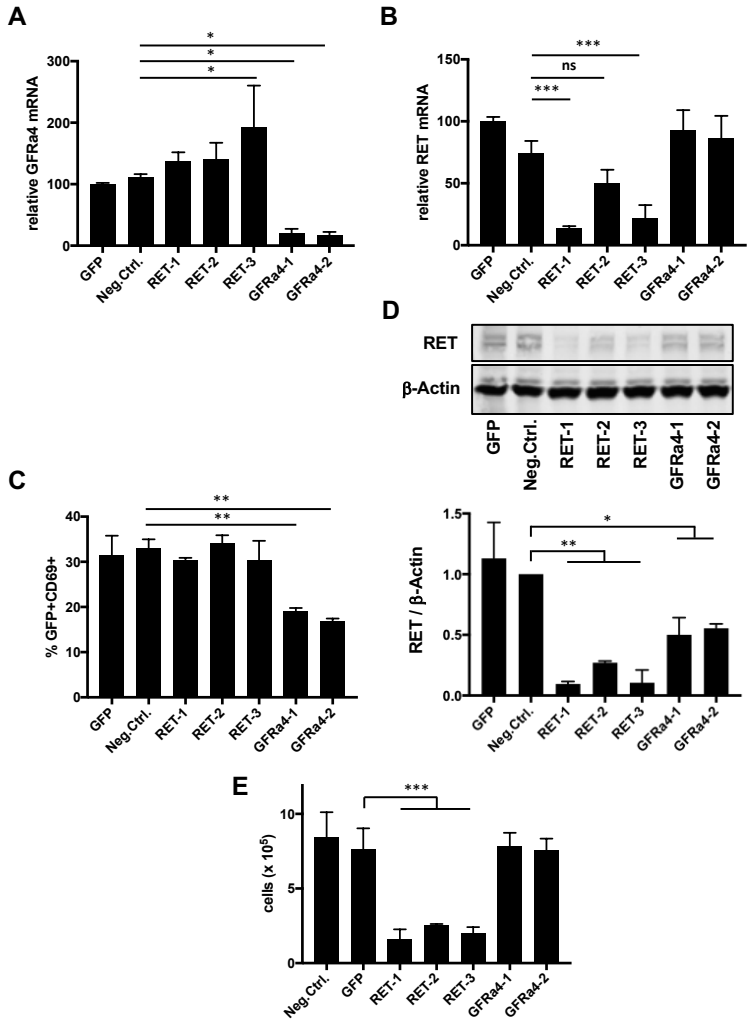

Figure S2: RET but not GFRα4 is required for TT cell proliferation. 2 x 10^5 TT cells expressing GFP were electroporated with siRNAs targeting GFP, RET, GFRα4 or a non-targeting siRNA (Neg. Ctrl.). 24-hours later, GFRα4 **(A)** and RET **(B)** mRNA was measured by qPCR. **(C)** GFRα4 protein level was functionally assessed by co-culture with NFAT-GFP reporter Jurkat cells expressing the GFRα4-specific CAR, P4-10bbz, and upregulation of GFP and CD69 was assessed as an indication of surface GFRα4 expression. **(D)** RET protein expression was determined by western blot. **(E)** Seven days after siRNA electroporation, cells were counted to determine survival/proliferation. Error bars indicate standard deviation. (* $p<0.05$,** $p<0.005$, *** $p<0.0005$, One-way ANOVA with Dunnett's multiple comparisons test).

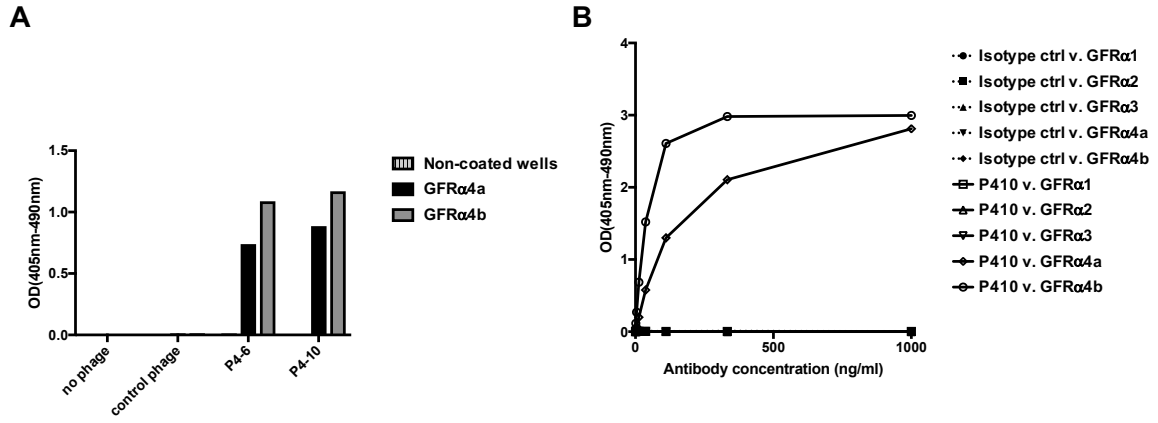

Figure S3: Binding specificities of P4-6 and P4-10 antibodies to GFRα proteins. (A) Phage ELISA of P4-6 and P4-10 rabbit/human chimeric Fab fragments binding to immobilized GFRα4a and GFRα4b proteins. Control phage is phage-displayed isotype control rabbit/human γλ Fab fragment; (B) ELISA of full-length rabbit/murine chimeric IgG binding to GFRα1, GFRα2, GFRα3, GFRα4a, and GFRα4b proteins.

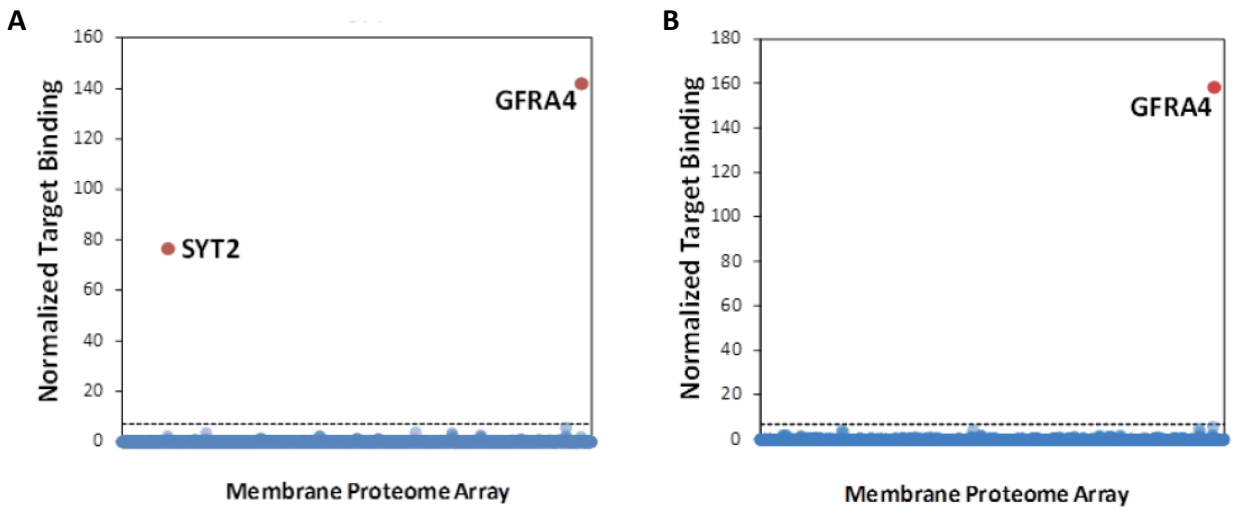

Figure S4. Identification of membrane protein binding targets for P4-6 and P4-10. A Membrane Proteome Array (MPA) was probed with P4-6 (A) and P4-10 (B) Fab proteins followed by detection using a fluorescently-labeled secondary antibody. Fluorescence readings from each experimental plate were validated using positive (construct expressing GFRα4) and negative (empty vector) controls. Binding values for each individual membrane protein target were normalized and transformed to give a single numerical value for binding of the antibody against each target protein (Normalized Target Binding). Non-specific fluorescence was determined to be any value below 3 standard deviations above noise (dotted line). Targets that showed increased antibody binding are displayed above the dotted line and denoted in red.

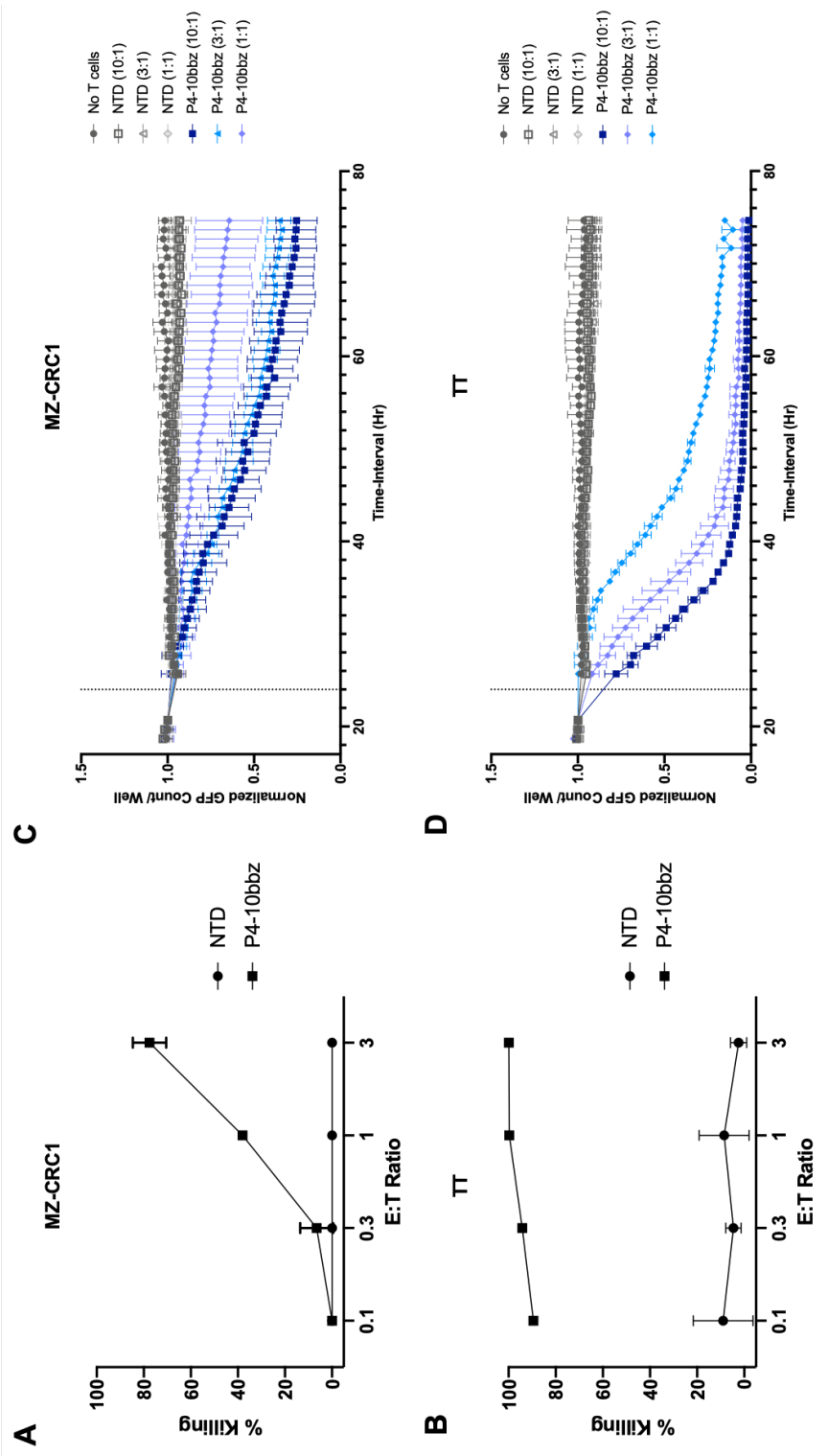

Figure S5. Lysis of MZ-CRC1 and TT cells by P4-10bbz CAR T cells. (A, B) Primary T cells expressing the P4-10bbz, or no CAR (NTD) were co-cultured with MZ-CRC1 or TT cells engineered to express click-beetle-green luciferase. After 96 hours of co-culture luciferin was

added to culture wells and the level of luminescence was measured to determine % lysis. Error bars represent one standard deviation. **(C, D)** MZ-CRC1 or TT cells engineered to express GFP were plated and GFP^+ cells were imaged over time using an xCelligence RTCA eSight instrument. 24 hours later (dotted line), primary T cells expressing the P4-10bbz, or no CAR (NTD) were added to the MZ-CRC1 and TT cells and GFP^+ cells were counted to determine lysis of target cells. Counts are normalized to values prior to addition of T cells. Error bars represent one standard deviation.

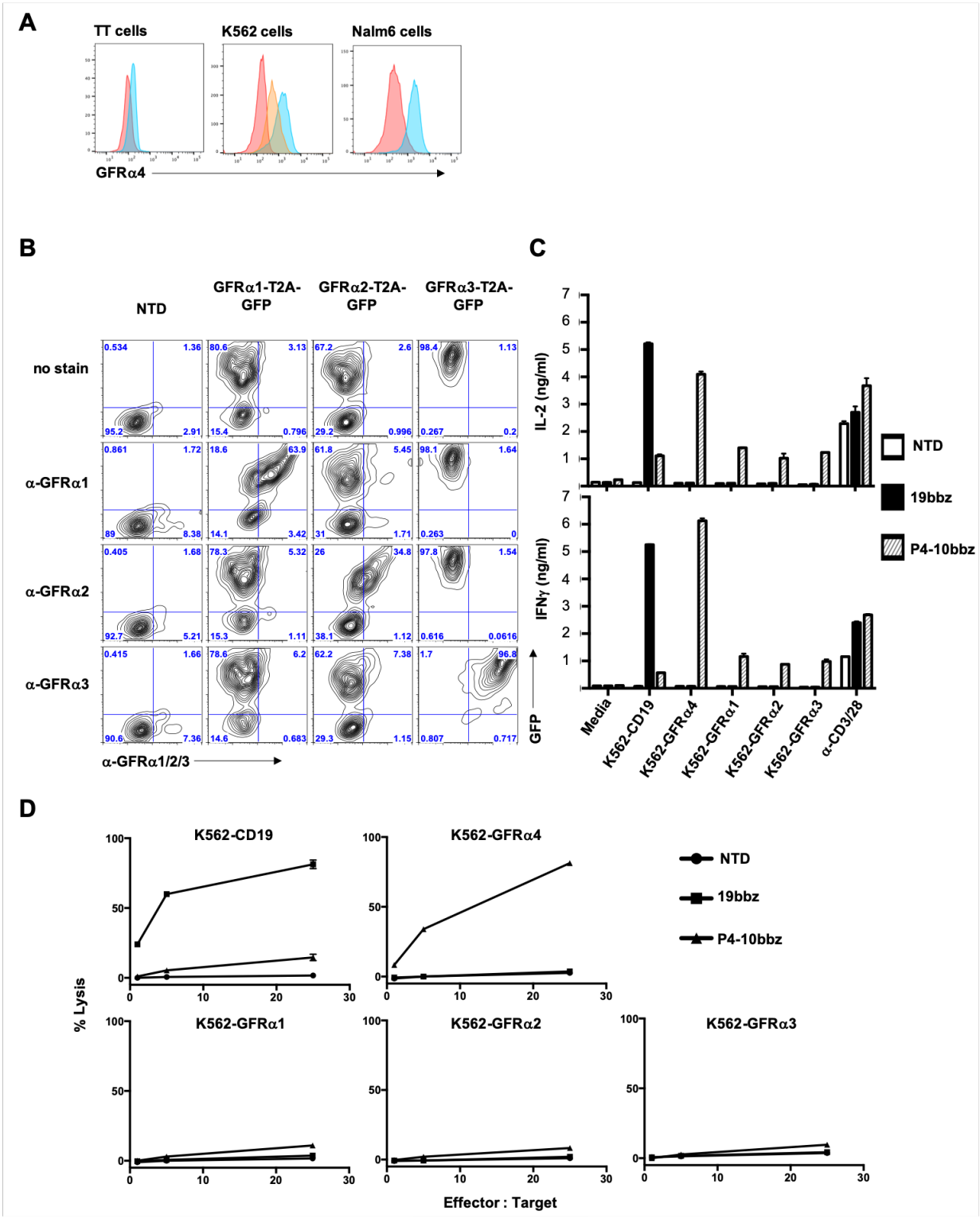

Figure S6. P4-10bbz targets K562 cells expressing GFRα4 but not GFRα1, GFRα2, or GFRα3. (A) TT cells were stained with P4-10 antibody (blue histogram, MFI=151) or was left unstained (MFI=89). P4-10 antibody staining was also performed on wild-type K562 cells (red

histogram, MFI=141), K562 engineered to express GFRα4a (orange histogram, MFI=515) or GFRα4b (blue histogram, MFI=1192) and on wild-type Nalm6 cells (red histogram, MFI=232) and Nalm6 engineered to express GFRα4b (blue histogram, MFI=1688), the latter used for experiments in Fig 4d. **(B)** GFRα1, 2, and 3 expression was measured on K562 cells engineered to express the indicated human GFR family member, each linked to GFP. **(C)** Culture supernatant IL-2 and IFNγ was measured following overnight co-culture of primary human T cells expressing not CAR (NTD), or the indicated CARs, with target K562 cells as in (A) or with anti-CD3/anti-CD28 coated beads. **(D)** Primary human T cells expressing no CAR (NTD) or the indicated CARs were co-cultured for approximately 18 hours with Cr51-loaded TT cells or K562 cells expressing the indicated antigens, at the indicated effector : target ratios. Following co-culture, supernatant was assessed for released radioactivity. % Lysis was calculated according to the formula: 100*((experimental – spontaneous)/(maximum – spontaneous), where spontaneous represents radioactivity released from cultures of target cells alone and maximum represents radioactivity release by target cells lysed with 5% SDS. Error bars represent 1 standard deviation of three technical replicates. Results in (A), (C), and (D) are representative of at least 2 independent experiments.

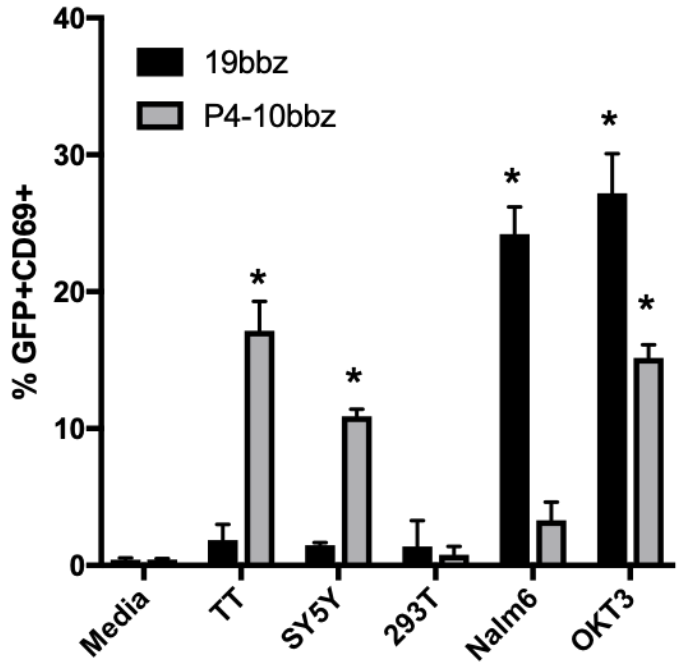

Figure S7. P4-10bbz is activated by MTC (TT) and a neuroblastoma cell line (SY5Y). NFAT-GFP reporter Jurkat cells expressing 19bbz or P4-10bbz were co-cultured overnight with the indicated stimuli, followed by assessment of $GFP^{+}CD69^{+}$ expression in the Jurkat cells by flow cytometry (* $p<0.05$, ANOVA with Dunnett's multiple comparisons test compared to media control for each CAR).

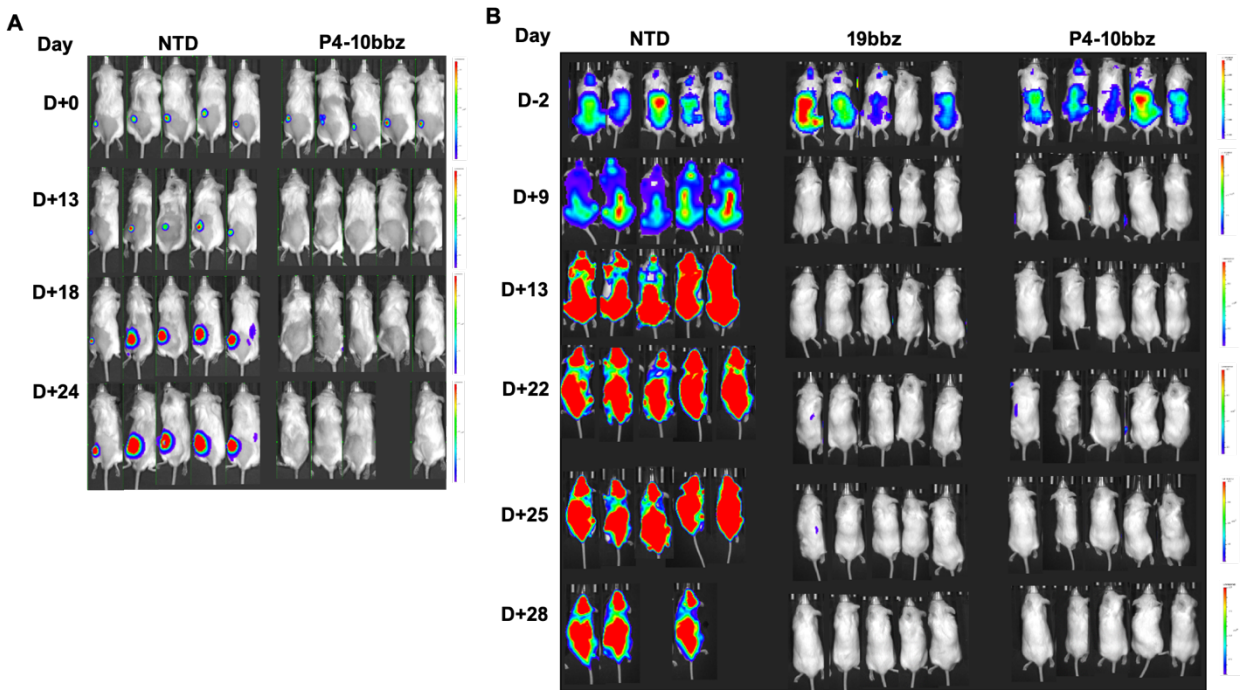

Figure S8. Bioluminescence images of mice from experiments shown in Figure 4a and 4d. Following TT cell engraftment **(A)** and Nalm6 **(B)** engraftment**,** T cells were injected on Day 0 and mice were imaged on indicated days.

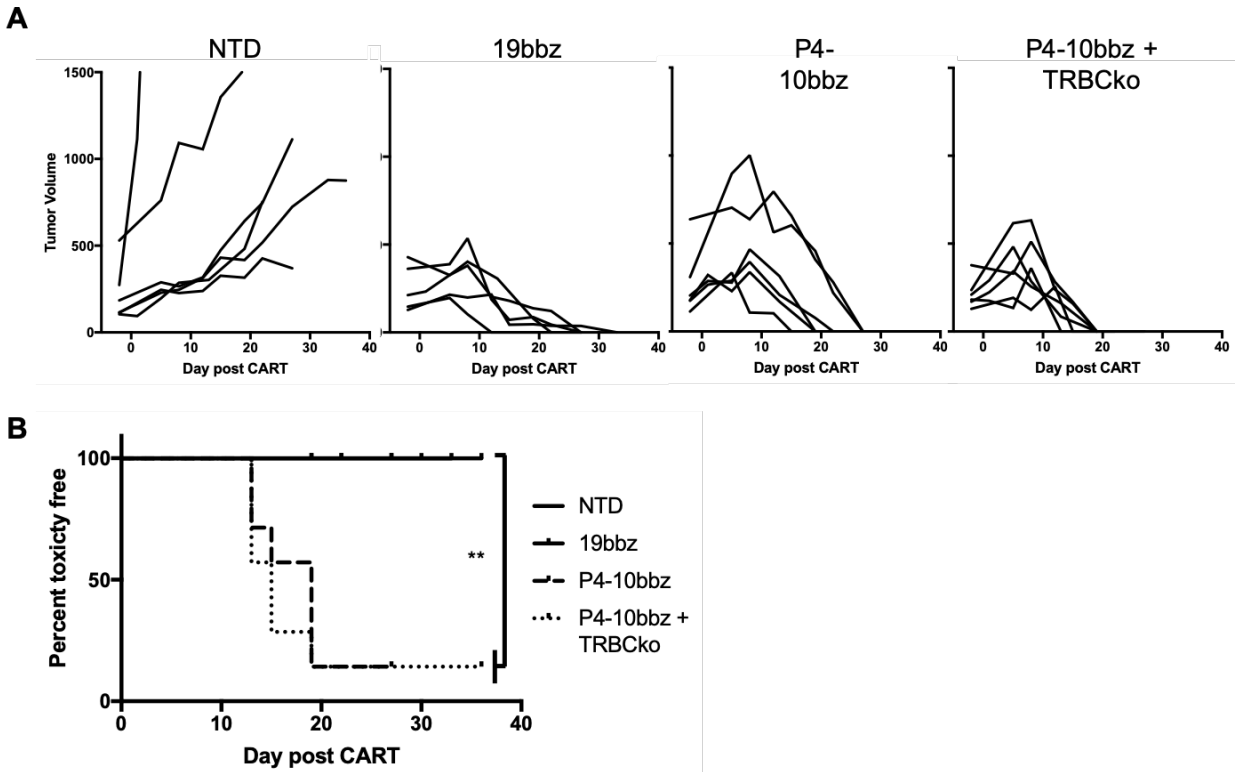

Figure S9. Skin toxicity is mediated by P4-10bbz CAR. NSG mice were injected with $5x10^6$ TT cells engineered to express CD19. When tumors reached approximately $100mm^3$, mice were injected intravenously with $1x10^7$ T cells expressing no CAR (NTD, n=6), 19bbz (n=5), P4-10bbz (n=6), or T cells expressing P4-10bbz in which the TRBC was disrupted by CRISPR-Cas9 (n=6). Tumor volumes were measured by caliper measurement (A) and incidence of skin toxicity was assessed by gross observation (B). Curves in (A) represent individual mice. * $p<0.005$ Log-rank test.

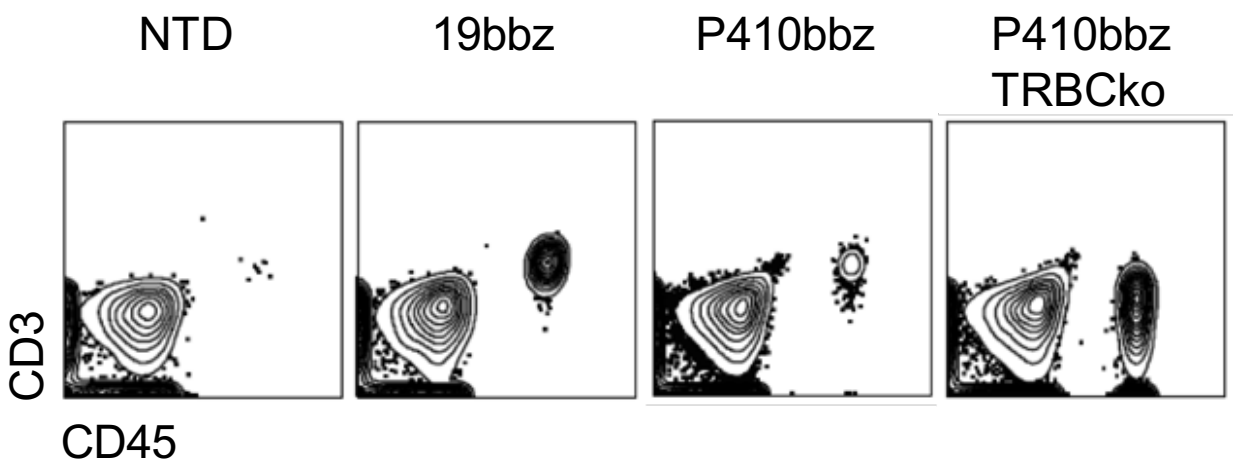

Figure S10. T cells in the blood of the TRBC-ko group remain surface CD3-neg. NSG mice, as shown in Figure S5, were injected with $5x10^6$ TT cells engineered to express CD19. When tumors reached approximately $100mm^3$, mice were injected intravenously with $1x10^7$ T cells expressing no CAR (NTD, n=6), 19bbz (n=5), P4-10bbz (n=6), or T cells expressing P4-10bbz in which the TRBC was disrupted by CRISPR-Cas9 (n=6). On Day 20 post T-cell injection, mice were bled, and samples were stained with antibodies against CD8, CD3, CD4, and CD45 followed by flow cytometric analysis. Representative plots from each group are shown.

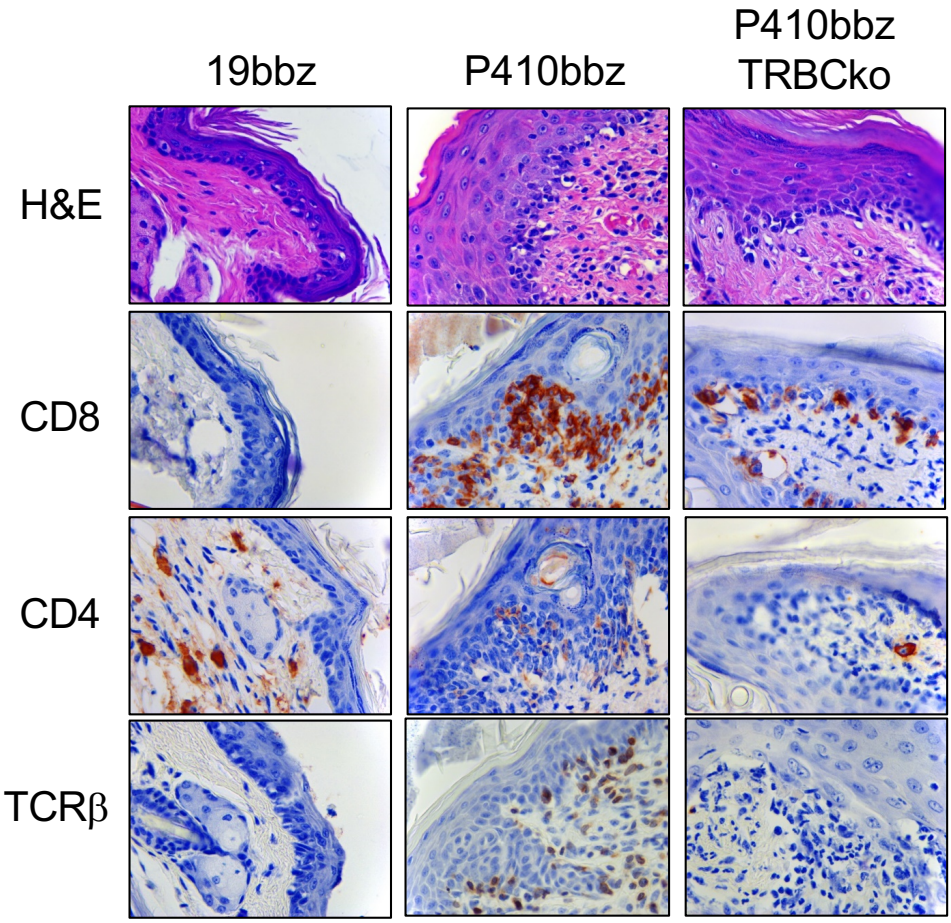

Figure S11. T cells in the skin of the TRBC-ko group remain TCR-β negative. NSG mice, as shown in Supplemental Figure 5, were injected with $5x10^6$ TT cells engineered to express CD19. When tumors reached approximately $100mm^3$, mice were injected intravenously with $1x10^7$ T cells expressing no CAR (NTD, n=6), 19bbz (n=5), P4-10bbz (n=6), or T cells expressing P4-10bbz in which the TRBC was disrupted by CRISPR-Cas9 (n=6). After mice were euthanized, skin was analyzed by hematoxylin and eosin staining and by immunohistochemistry with antibodies specific for human CD8, CD4, and TCR-β. Representative sections from the indicated groups are shown.

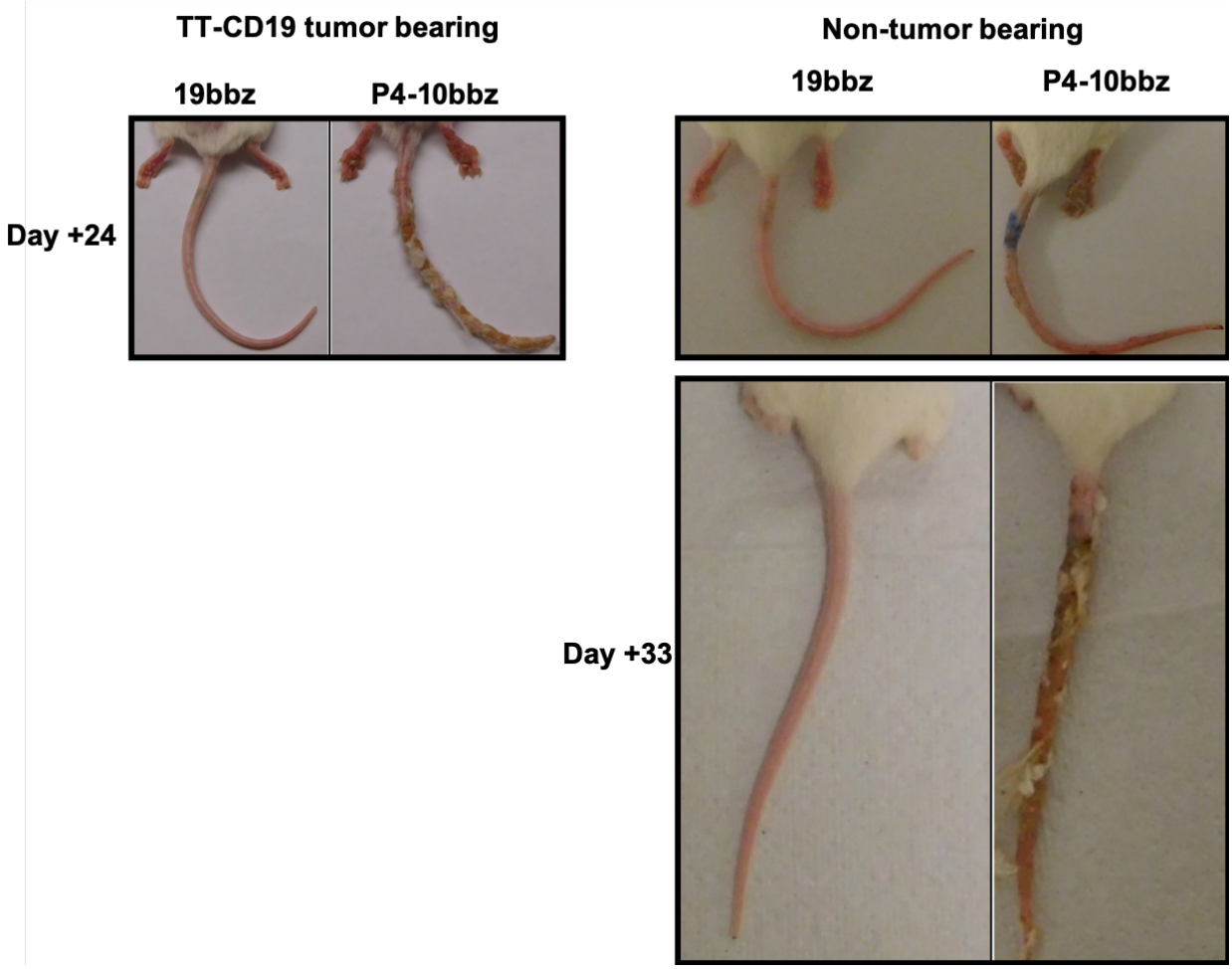

Figure S12. Skin toxicity resulting from P4-10bbz CAR is independent of tumor. NSG mice with and without subcutaneously implanted TT-CD19 tumor cells received T cells expressing 19bbz or P4-10bbz. By day 24, tumors were cleared in both 19bbz and P4-10bbz treated tumor bearing mice and P4-10bbz treated mice developed skin toxicity. In non-tumor bearing mice, P4-10bbz treated mice also developed skin toxicity but with slower kinetics.

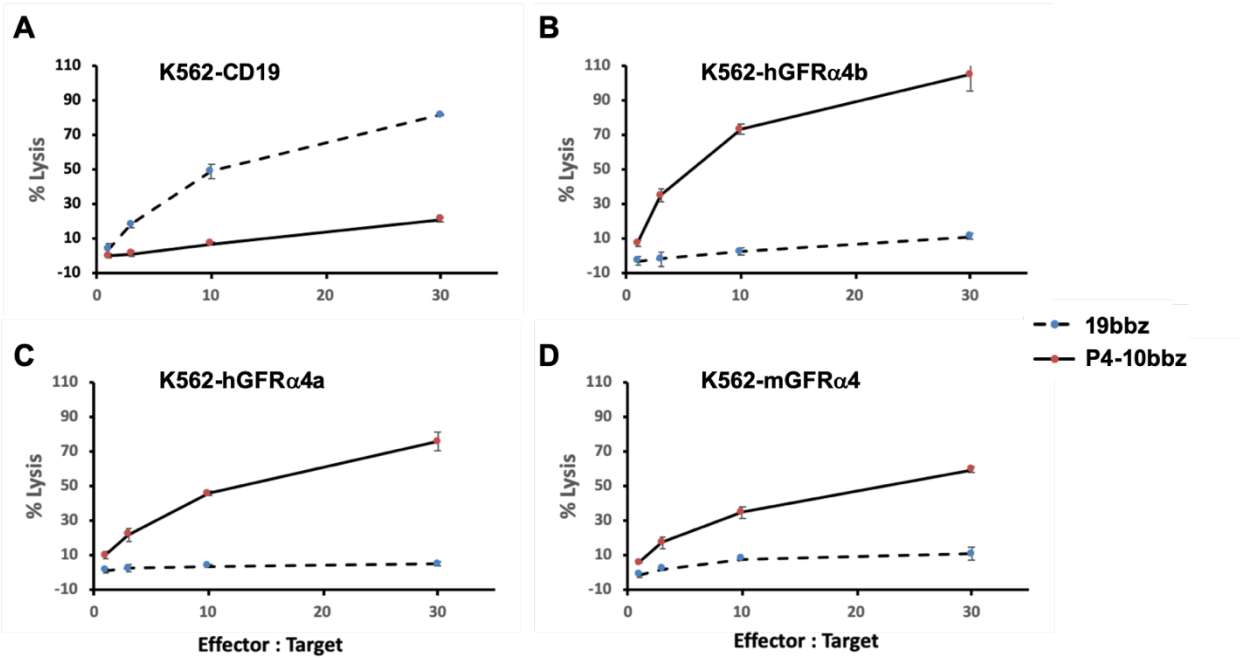

Figure S13. P4-10bbz CAR targets murine GFRα4 expressed on K562 cells. Primary T cells expressing the 19bbz or P4-10bbz CAR were co-cultured with ^{51}Cr-loaded K562 cells engineered to express human CD19 (A), human GFRα4b (B), human GFRα4a (C), or murine GFRα4 (D). After overnight co-culture at the indicated effector:target ratios, supernatants were harvested and released radioactivity was measured to determine % lysis. Error bars represent one standard deviation of technical triplicates.

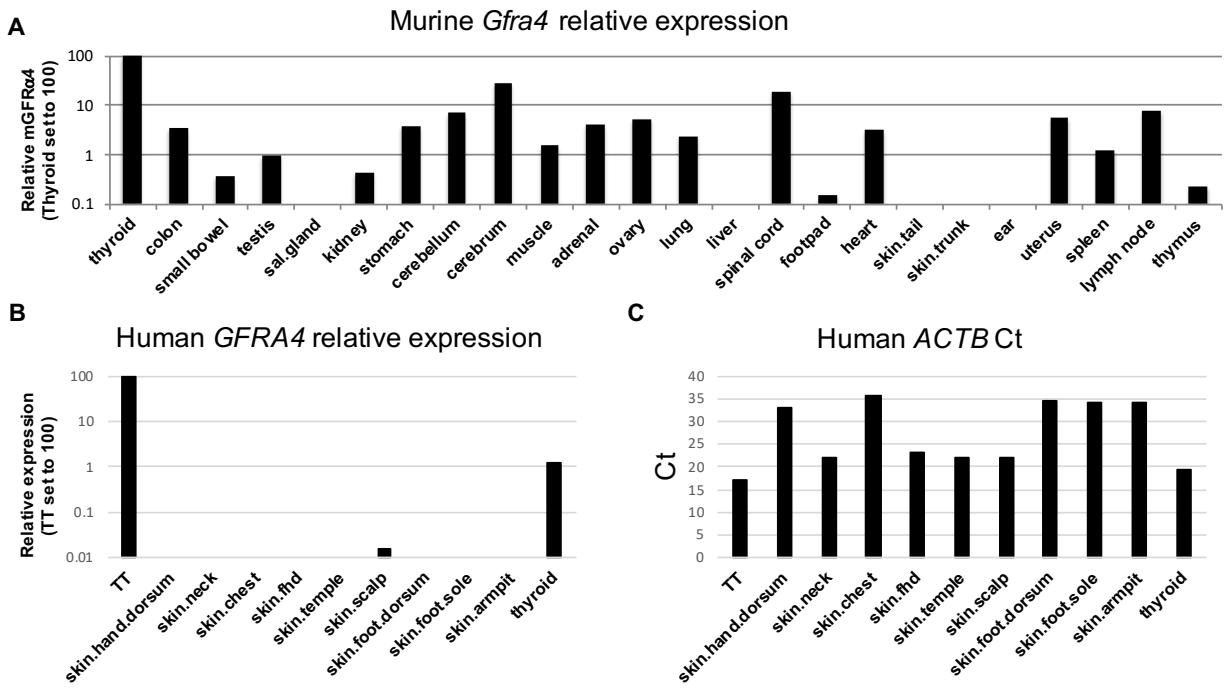

Figure S14. Murine *Gfra4* and human *GFRA4* mRNA are not detected in skin by qPCR. (A) Relative *Gfra4* transcript level measured by qPCR of cDNA created from various murine tissue samples. The signal from tissues is relative to thyroid which was set to 100. Transcript levels are normalized to β-actin as a housekeeping gene. (B) Relative *GFRA4* transcript level measured by qPCR of cDNA created from various human skin samples, human thyroid and TT cells. The signal from tissues is relative to TT cells which was set to 100. Transcript levels are normalized to β-actin as a housekeeping gene. (C) The Ct level of amplification of β-actin (ACTB) transcripts from samples shown in (B).

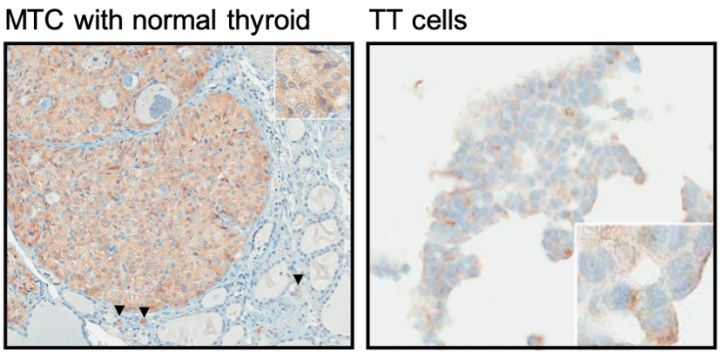

Figure S15. Immunohistochemistry using P4-10 antibody. A sample of MTC with adjacent normal thyroid (left) and TT cells (right) were stained with P4-10 antibody. Positively staining parafollicular cells are indicated with arrowheads.

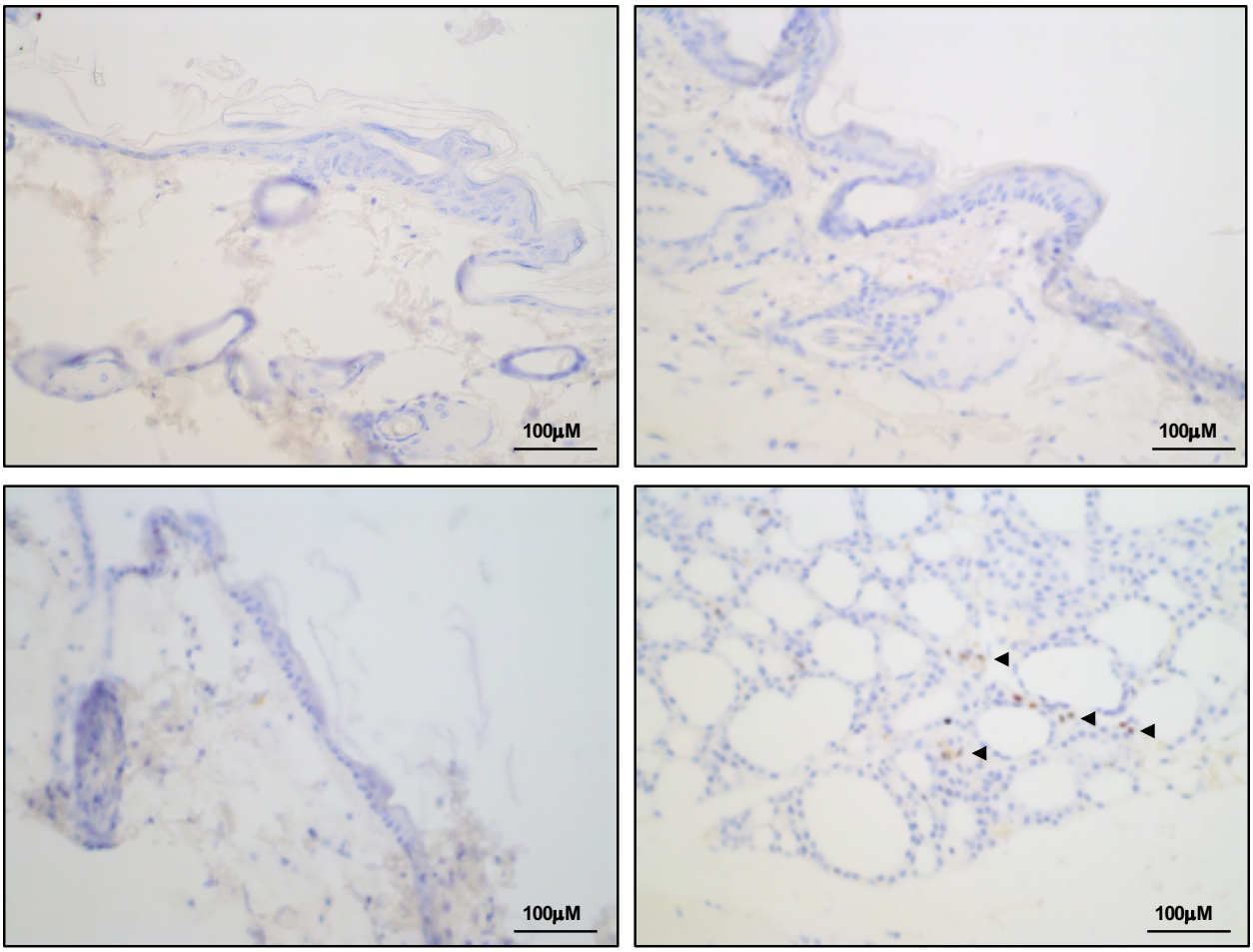

Figure S16. *Gfra4* mRNA is detected in murine thyroid but not skin. Murine ear, tail, and flank skin and murine thyroid (bottom right) were tested by RNA in situ hybridization (RNAscope) using probes specific for murine *Gfra4* mRNA. Arrowheads indicate areas with positively staining cells.

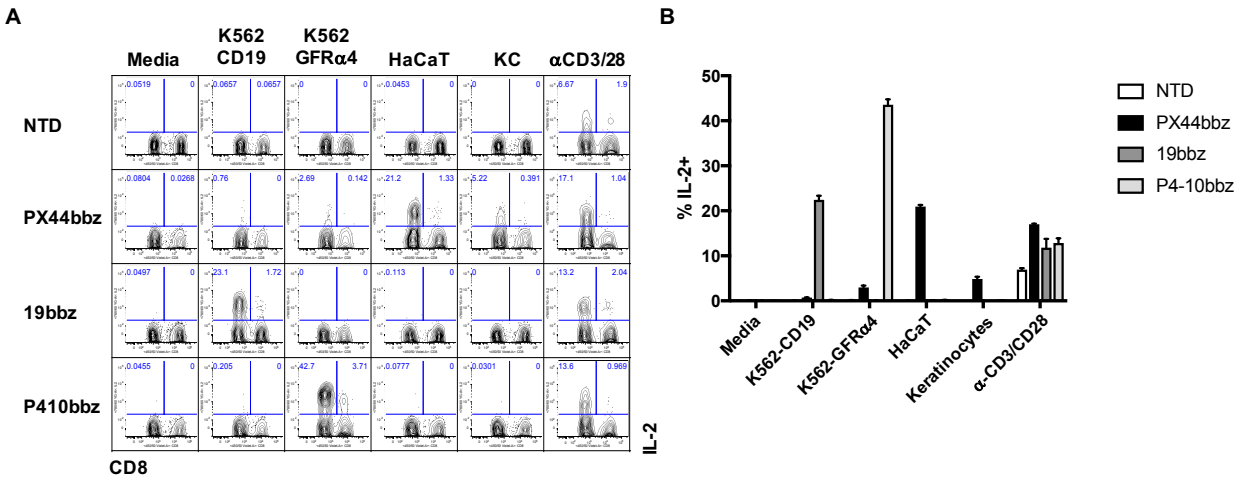

Figure S17. Primary P4-10 CAR T cells do not react to human keratinocytes. (A) Primary human T cells expressing no CAR (NTD), PX44bbz, 19bbz, or P4-10bbz were co-cultured for 6 h with the indicated target cells or with anti-CD3/anti-CD28 coated beads. Following co-culture, cells were fixed, permeabilized and stained for intracellular IL-2 and surface CD8 and analyzed by flow cytometry. Representative plots of replicates are shown. KC = primary human keratinocytes. **(B)** Percentage of IL-2^{+} T cells from (A) is shown for each condition. Error bars represent one standard deviation of technical replicates.

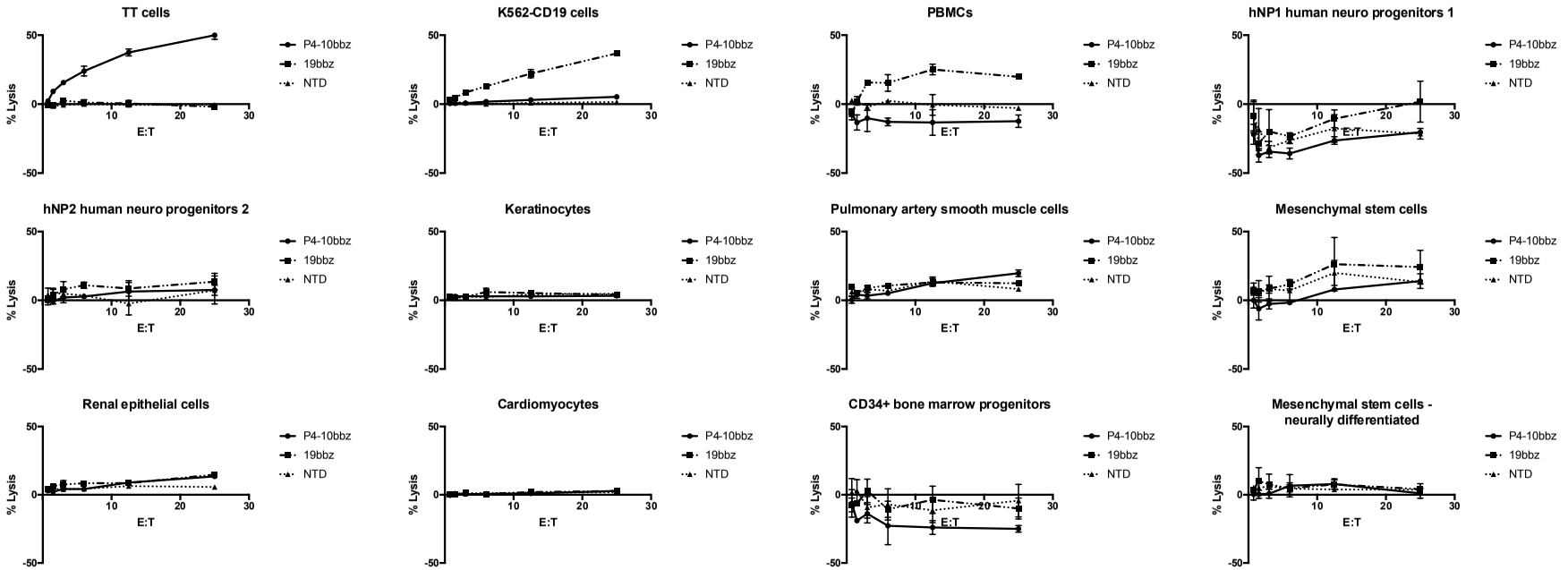

Figure S18. P4-10bbz CAR T cells specifically target TT cells but not a panel of human primary cells. Primary T cells expressing the 19bbz, P4-10bbz, or no CAR (NTD) were co-cultured with ^{51}Cr-loaded primary human cells, TT cells, and K562-CD19 cells at the indicated effector:target ratios. After 4 h of co-culture supernatants were harvested and released radioactivity was measured to determine % lysis. Error bars represent one standard deviation.

Heavy chains	V region germline gene	% Human homology	FR1	CDR1	FR2	CDR2	FR3	CDR3	FR4
P410	Rabbit IGHV1S34*01	54%	QSVKESEGGLFKPTDTLTLTCTVSGFSLS	RHALT	WVRQAPGNGLEWIG	AIDNAGTTYYASWAKS	RSTITRNTDLHTVTLKMTSLTASDTATYFCAR	VFYDINSGYYLDGMDL	WGPGTLVTVSS
CAR25	Human IGHV4-38-2*02	85%	QVQLQESGPGLVKPSETLSLTCAVSGYSIS	RHALT	WIRQPPGKGLEWIG	AIDNAGTTYYASWAKS	RVTISVDTSKNQFSLKLSSVTAADTAVYYCAR	VFYDINSGYYLDGMDL	WGPGTLVTVSS
CAR29	Human IGHV3-48*03	85%	EVQLVESGGGLVQPGGSLRLSCAASGFTFS	RHALT	WVRQAPGKGLEWVS	AIDNAGTTYYASWAKS	RFTISRDNAKNSLYLQMNSLRAEDTAVYYCAR	VFYDINSGYYLDGMDL	WGPGTLVTVSS
Light chains									
			FR1	**CDR1**	**FR2**	**CDR2**	**FR3**	**CDR3**	**FR4**
P410	Rabbit IGLV4S4*01	70%	QFVLTQSPSVSAALGASAKLTC	TLSSAHKTYTID	WYQQQQGEAPRYLMQ	VKSDGSYTKGT	GVPDRFSGSSSGADRYLIIPSVQADDEAGYVC	GADDNGGYV	FGGGTQLTVT
CAR25/CAR29	Human IGLV4-69*01	85%	QLVLTQSPSASASLGASVKLTC	TLSSAHKTYTID	WYQQQPEKGPRYLMQ	VKSDGSYTKGT	GVPDRFSGSSSGADRYLTISSLQSEDEADYYC	GADDNGGYV	FGGGTQLTVL

Figure S19: Sequence analysis of rabbit and humanized P4-10 variable regions. Heavy and light chain variable region gene assignments were determined using IMGT Domain-GapAlign tool (37).

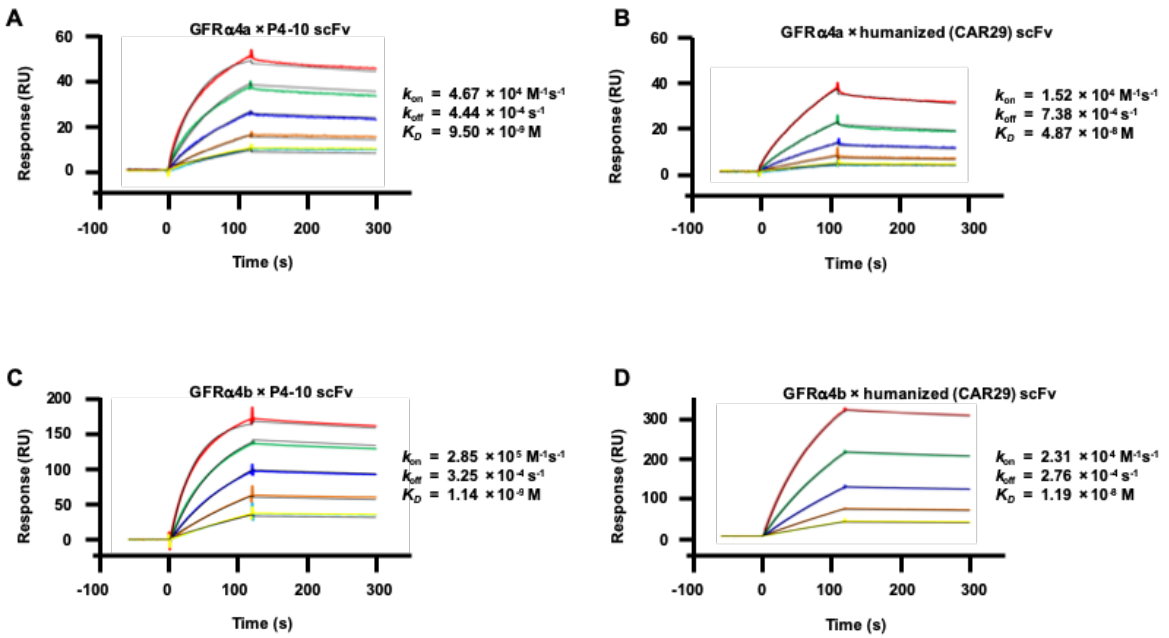

Figure S20. SPR analysis of the binding of scFv to GFRα4. huFc-GFRα4a (**A, B**) or huFc-GFRα4b (**C** and **D**) were captured via a mouse anti-human IgG C_{H2} monoclonal antibody immobilized on a sensor chip, followed by injection of rabbit scFv (**A** and **C**) or humanized scFv (**B** and **D**) at five concentrations, serially diluted by two-fold from the highest concentration (100 nM for **C** and 500 nM for **A**, **B**, and **D**) with a replicate of the lowest concentration to confirm regeneration of the sensor chip. Sensorgrams show the response (RU, resonance units) plotted against time (s, seconds). Colored lines are the experimental tracings obtained from the SPR experiments and black lines are the best global fits (1:1 Langmuir binding model) used to calculate the association (k_{on}) and dissociation (k_{off}) rate constants. The equilibrium dissociation constant (K_D) was calculated as k_{off}/k_{on}.

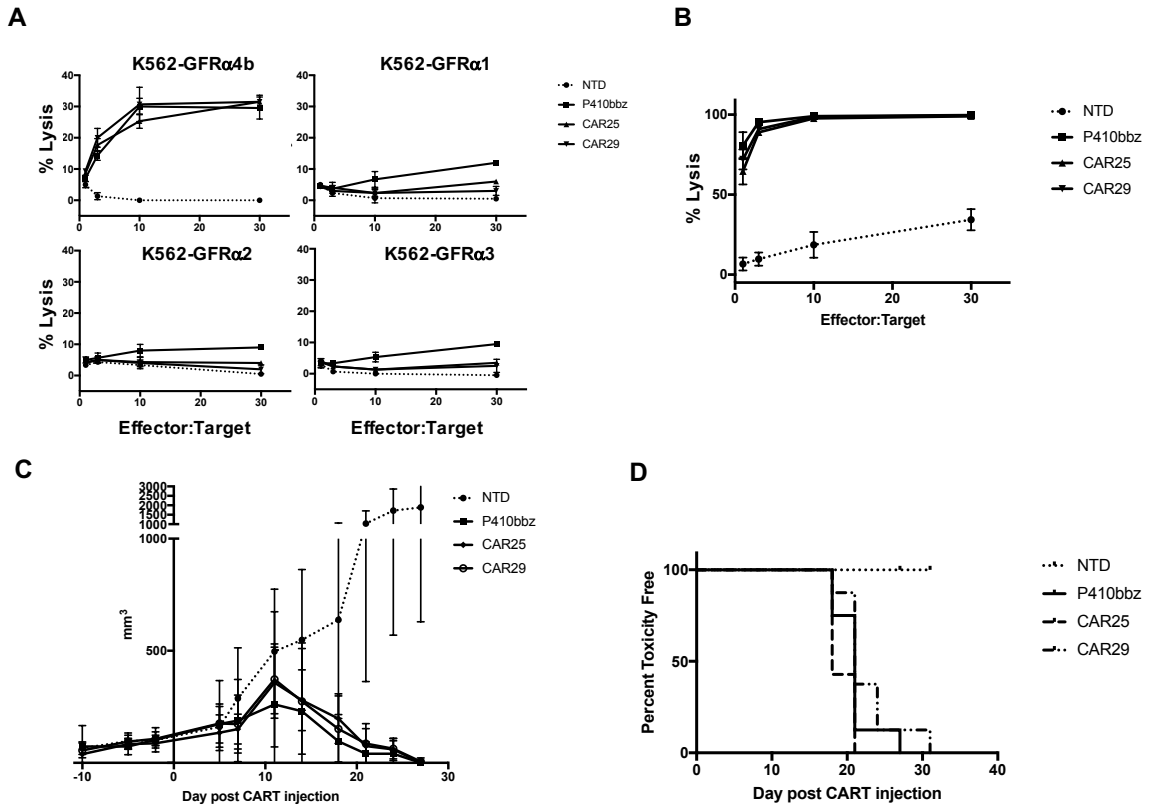

Figure S21. Humanized P4-10bbz CARs demonstrate equivalent efficacy *in vitro* and *in vivo*. (A, B) Human T cells expressing the indicated CARs or no CAR (NTD) were co-cultured overnight with ^{51}Cr-loaded K562 cells expressing GFRα1, GFRα2, GFRα3, or GFRα4b (A) or with ^{51}Cr-loaded TT cells (B) at the indicated effector to target ratios. Culture supernatants were harvested and assayed for radioactivity and % lysis was calculated according to the formula: 100*((experimental-spontaneous)/(maximum/spontaneous)). Error bars represent one standard deviation. (C) TT tumor volumes measured over time in NSG mice treated with 5 x 10^6 P4-10bbz (n=8 mice), CAR25 (n=7 mice), or CAR29 (n=8 mice) CAR T cells or an equivalent number of non-transduced cells (NTD, n=7 mice) 10 days after subcutaneous implantation of 5 x 10^6 wild-

type TT cells. Curves plot means of tumor volumes +/- one standard deviation for each experimental group.