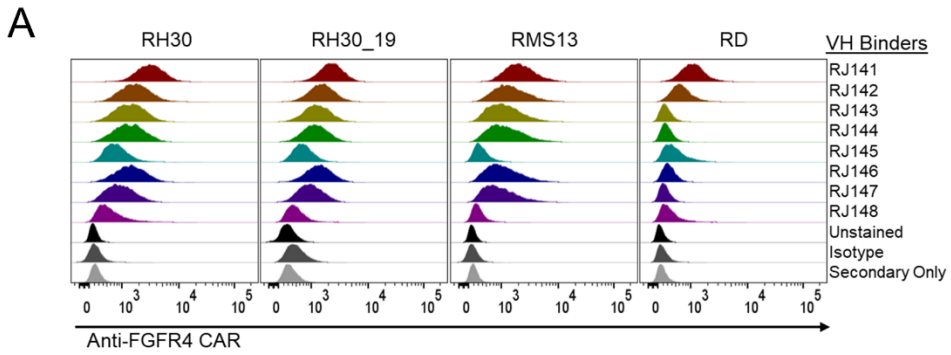
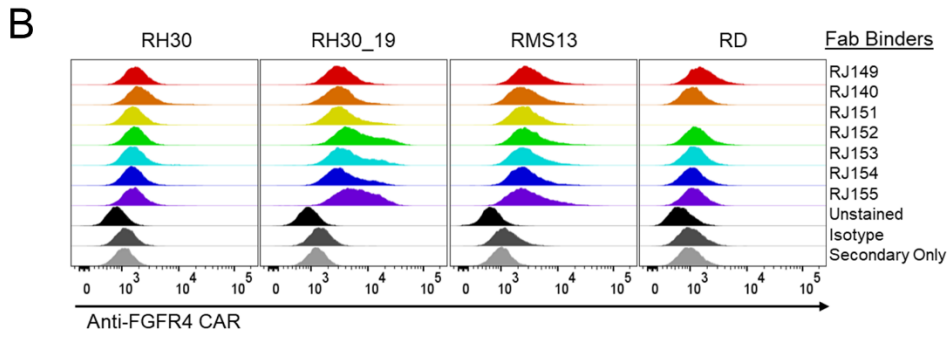
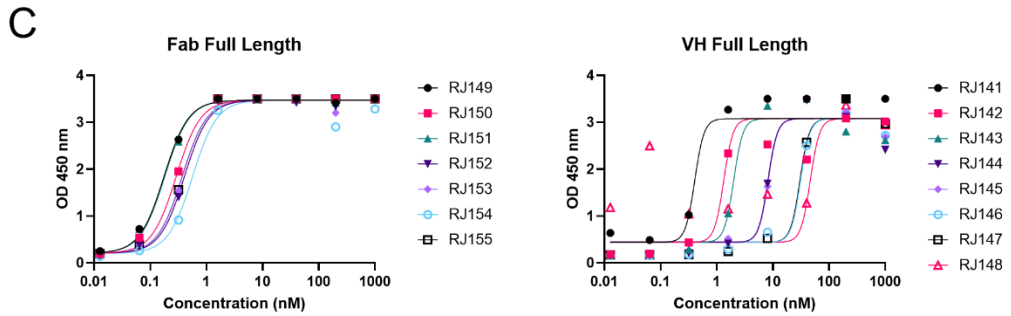
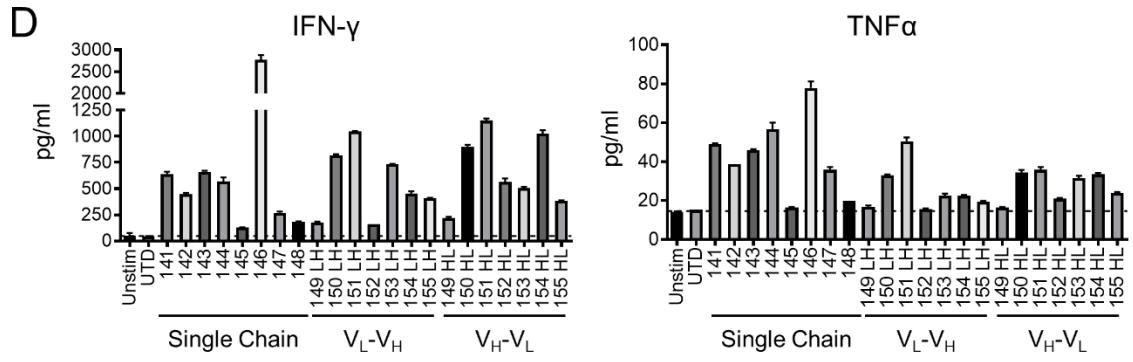
Supplementary Figures









S1. New anti-FGFR4 binder profiles for RMS cell lines. VH-only binders (**A**, RJ141 through RJ148) or Fab binders (**B**, RJ149 through RJ155) in purified VH/Fab-Fc-FLAG format recognize RMS cell lines RH30, RH30_19, RMS13, and RD. Unstained cells, isotype control, and secondary (anti-FLAG-PE) only samples were included as negative controls. **C**. ELISA targeting FGFR4 full-length extracellular domain with either Fab binders (left panel) or VH-only binders (right panel). **D.** IFN-γ and TNFα cytokines released during 20-hour co-incubation with FGFR4-expressing RH30_19 target cells were quantified using LegendPlex bead-based cytokine assay. Controls include unstimulated T cells (Unstim) and T cells activated for transduction but not exposed to LV (UTD).

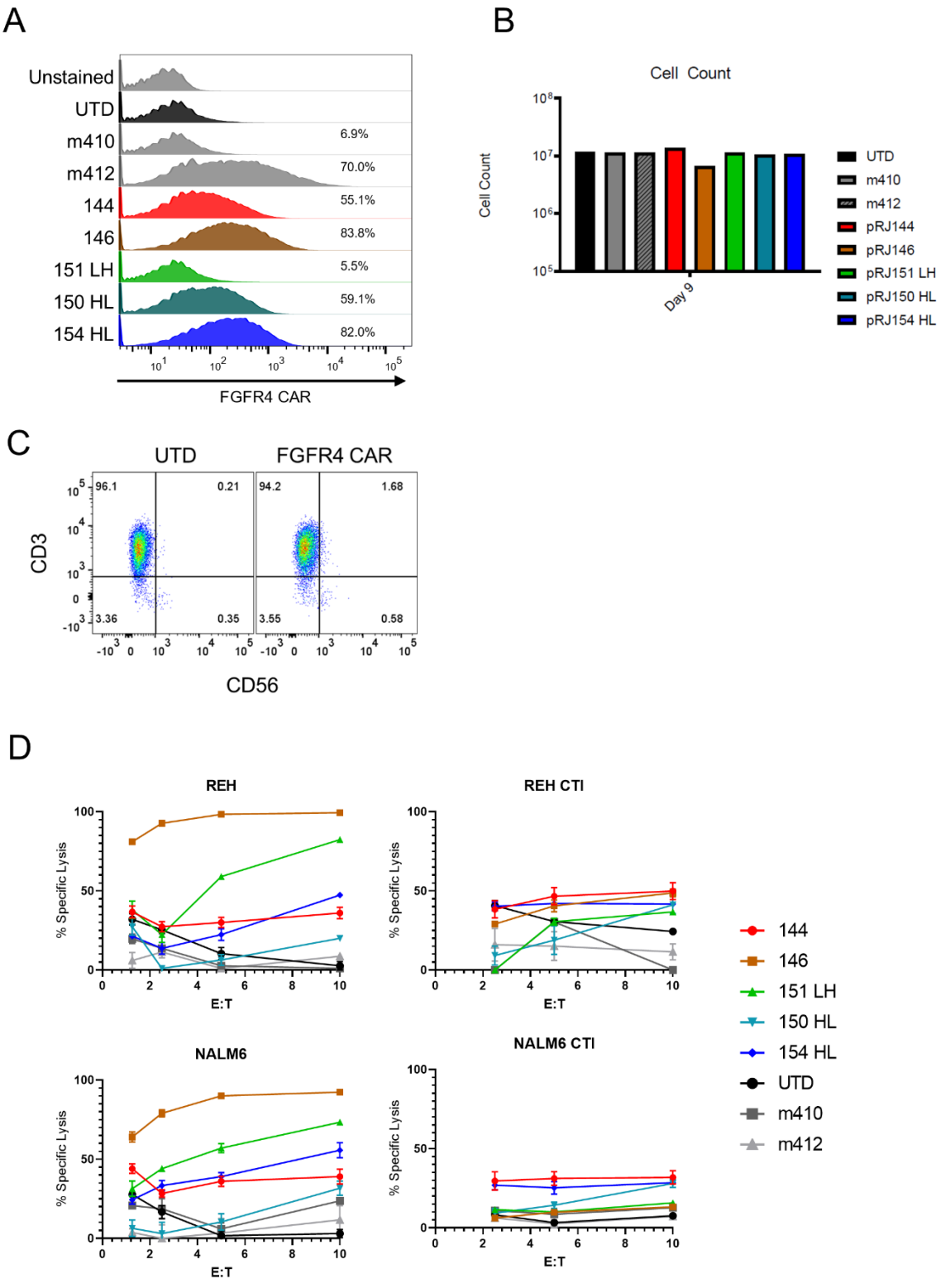


Figure S2. Screening top candidate anti-FGFR4 binders. A. Expression of anti-FGFR4 CAR was detected on the cell surface with biotinylated recombinant FGFR4-Fc

and streptavidin-PE. Unstained and untransduced (UTD) were used as negative comparison. m410 and m412 are previously tested anti-FGFR4 CARs and used for comparison to the new binders. **B.** Cell proliferation during in vitro expansion. Groups were started at 2x10^6 cells and final counts at day 9 post-activation are plotted. **C**. In vitro staining for CD56 to identify NK cell proportion of final CAR-T cell product. **D.** Cytotoxicity of candidate anti-FGFR4 CARs against non-FGFR4 expressing cells to determine non-specific killing. REH-ffLuc and NALM6-ffLuc with and without cold target inhibition (CTI), where unlabeled K562 were added at a 1:30 excess.

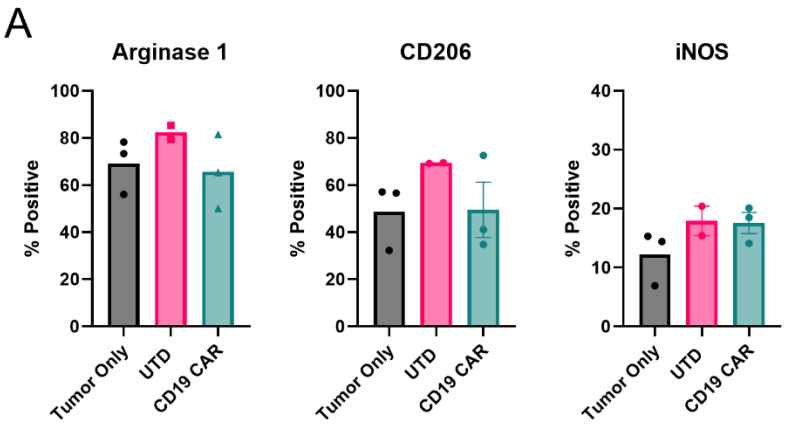


Figure S3. Phenotyping the TME for immunosuppressive cells. Mice were injected with $1x10^6$ RH30_19 tumor cells intramuscularly, followed by $7x10^6$ CD19 CAR-T cells on day 3. Mice were euthanized on day 21 and tumors processed and stained for flow cytometry analysis. **A.** Tumor associated macrophages (CD11b+, F4/80+) were stained for Arginase 1, CD206, and iNOS and graphed as percent positive of total macrophages for each marker.

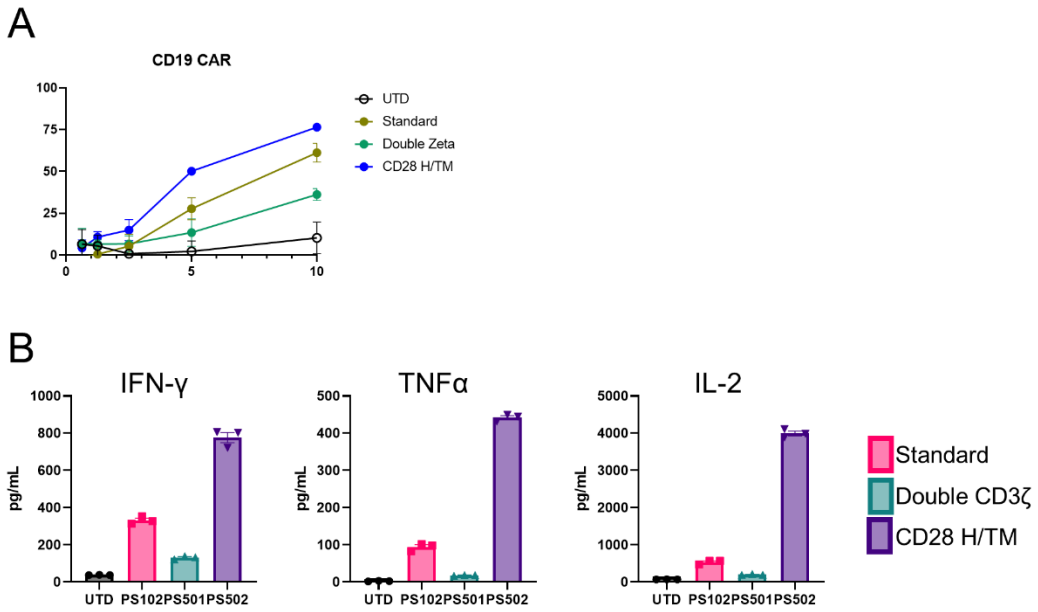


Figure S4. Tuning anti-CD19 CARs for low-density antigens. A. Cytotoxicity of anti-CD19 CAR-T cells against RD-ffLuc cell line after 5 hours of co-culture at indicated E:T ratios. **B.** Cytokine production by anti-CD19 CAR after 20 hours of co-culture with RH30_19 target cells.

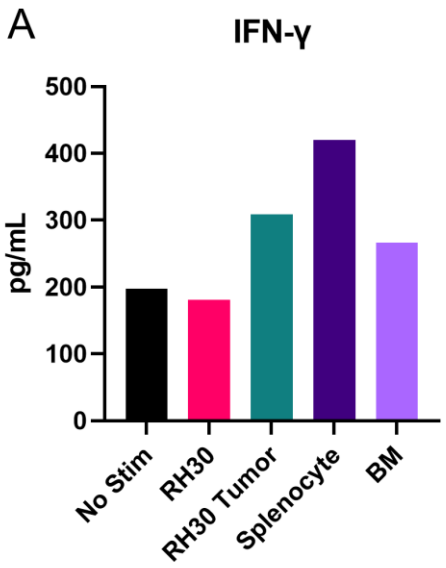


Figure S5. Untransduced (UTD) T cells produce IFN-y when co-incubated with mouse cells. A. Day 9 post-activation UTD T cells were co-incubated with mouse tissue-derived cells at a 1:2 ratio for 20 hours. Supernatant was assessed for IFN-y concentration by ELISA.

A

Symbol	Non-stimulated			IFNγ Stimulated		
	NS.1	NS.2	NS.3	IFN.1	IFN.2	IFN.3
IL32	10285	8134	8785	8047	6569	8413
CFH	2546	3466	3214	5824	6183	6415
GPI	5347	6566	6629	5423	5165	5380
MIF	2663	6607	6939	2815	5460	5862
TGFB1	1499	2058	2117	1255	1441	1546
IDO1	6	16	13	2099	933	993
CXCR4	2937	1965	2091	1310	981	1072
CCL24	1694	1484	1574	949	791	827
IL15	375	648	591	674	760	758
CSF1	134	122	117	836	657	627
LIF	424	569	530	612	653	614
CCL2	94	55	68	448	188	182

Low High

B

Symbol	Non-stimulated			IFNγ Stimulated		
	NS.1	NS.2	NS.3	IFN.1	IFN.2	IFN.3
ITGB1	13000	15465	14731	11449	11923	11465
CD99	12886	14718	14719	11065	11514	11271
IFITM1	21	64	51	6223	6931	7082
CD9	6401	8022	8269	5111	5946	6292
CD81	5235	6532	6734	4662	5188	5480
TGFBR1	4161	5740	5772	3942	4092	4200
NCAM1	5034	6224	5941	3898	4240	4094
ITGA6	2902	2848	2836	4344	3903	3711
BCAP31	3072	5427	5239	3323	4366	4168
CD276	3735	5446	5366	3225	3947	3967
NOTCH2	4307	4961	4650	3401	3807	3724
CD59	3966	5048	4946	3294	3839	3720
CD44	2753	3171	3268	3598	3008	2988
IFNAR2	2719	2789	2678	2974	2771	2671
CD46	2616	3272	3080	2571	2836	2759
TFRC	2634	3562	3509	1961	2498	2469
CD164	2294	2731	2569	1792	2036	2103
CD58	2195	2719	2505	1854	1960	1987
IL21R	1300	2343	2273	1100	1725	1639
IL4R	1579	2024	1999	1343	1476	1532
CD82	1454	1675	1822	1551	1360	1335
FCGRT	1219	1757	1905	1066	1472	1426
ITGAE	1410	1742	1674	1252	1249	1224
NOTCH1	1172	1458	1498	1106	1181	1242
TMEM173	902	1265	1243	1193	1109	1078
CXCR4	2937	1965	2091	1310	981	1072
CD97	845	1134	1140	1010	1085	1153
PDL1	73	48	41	604	394	418

Low High

C

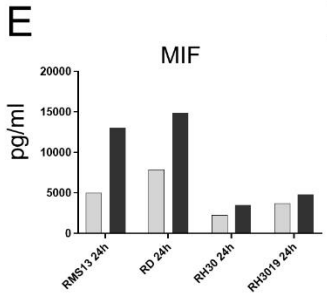
Symbol	Tumor Only			Tumor + UTD T Cells		
	mouse1	mouse 3	mouse 9	mouse 2	mouse 4	mouse 8
IL32	1152	1743	1642	3301	4608	2611
MIF	1524	882	1719	4396	2134	2727
GPI	984	746	677	2618	1281	1851
TGFB1	420	225	233	950	392	518
CFH	266	201	270	817	530	456
IL15	80	134	101	310	203	211
CCL2	11	7	23	20	14	374
CCL24	122	52	15	168	81	45
LIF	52	24	39	63	41	73
IL8	16	8	13	46	10	51

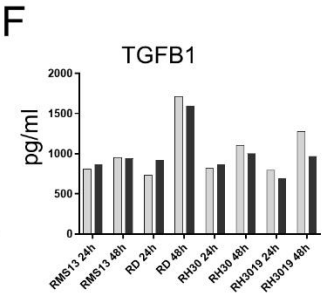
Low High

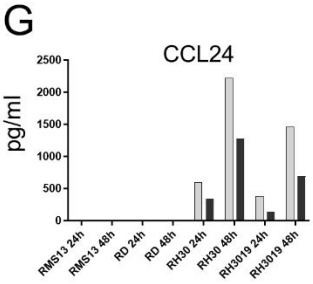
D

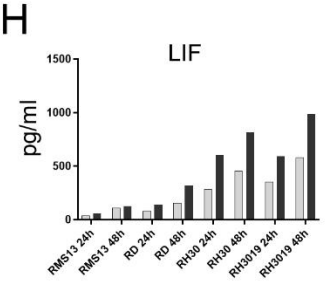
Symbol	Tumor Only			Tumor + UTD T Cells		
	mouse1	mouse 3	mouse 9	mouse 2	mouse 4	mouse 8
CD99	2653	1794	2426	5008	3342	3051
ITGB1	1208	737	934	3969	1778	1693
CD9	988	675	790	2794	1507	1606
TGFBR1	830	924	669	2474	1744	1544
CD81	834	584	705	2403	1368	1388
CD59	630	550	426	1587	1022	1097
CD276	641	535	381	1796	815	886
TFRC	449	327	335	1852	842	667
CXCR4	580	271	335	1807	636	858
NCAM1	576	312	527	1556	786	807
IL4R	370	372	234	1172	740	594
FCGRT	443	298	288	1162	508	619
CD46	232	232	222	1007	602	583
CD58	297	190	350	805	480	422
IFNAR2	308	191	312	633	416	506
CD164/MGC 24	96	248	100	539	472	378
CD82	223	186	128	612	345	334
ITGA6	262	125	238	639	292	290
ITGAE	105	140	113	389	272	250
CD44	357	115	138	376	257	272
IL13RA1	100	86	150	394	197	221
TGFBR2	138	134	123	330	225	211
CD83	108	89	111	230	131	217
ITGA5	109	62	32	244	123	117
IL21R	39	22	25	60	46	33
THY1	24	15	11	22	9	31
CD274	6	10	3	16	13	17

Low High









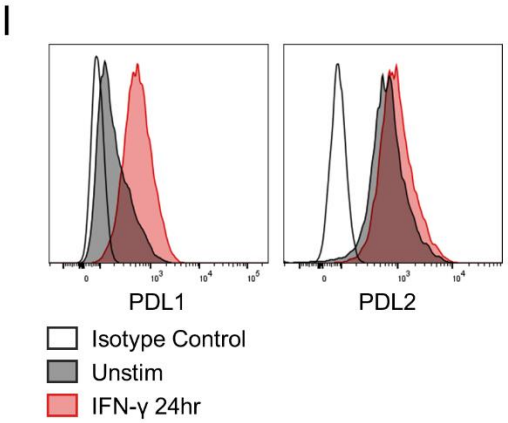


Figure S6. Expression analysis of soluble factors and surface receptors in RMS tumors. A, B. RH30_19 cells were treated with IFN-γ or left untreated for 24 hours in culture. RNA was isolated for Nanostring nCounter gene expression using the Human Immunology V2 Panel. Expression of soluble factors **(A)** or surface markers **(B)** were ranked by highest expression under IFN-γ treatment. Data from three independent experiments is shown in each column, and order by strength of expression in the IFN-γ treated samples. **C, D.** RH30_19 tumors were injected I.M. in NSG mice followed by untransduced T cell injection on day 3. Tumors were excised and processed into single cell suspensions. RNA was extracted and analyzed using a Nanostring nCounter with the Human Immunology V2 Panel. **C.** Highest expressed soluble factors were ranked by expression level in UTD T cell treatment. **D.** Surface receptor expression is ranked by highest expression with UTD T cell treatment. Each column is a replicate (N=3 mice). Ranks are based on the average of three tumor samples. **E-H.** Expression of mRNA for soluble factors was confirmed with ELISA for MIF **(E)**, TGFB1 **(F)**, CCL24 **(G)**, and LIF **(H)** in RH30_19, RH30 parental line, RD, and RMS13 cell lines. **(I)** PDL1 (CD274) and PDL2 (CD273) expression was assessed by flow cytometry on RH30_19 with and without 24 hour IFN-γ treatment. (isotype control, White; unstimulated RH30_19 cells, Black; 24 hour IFN-γ treatment, Red).

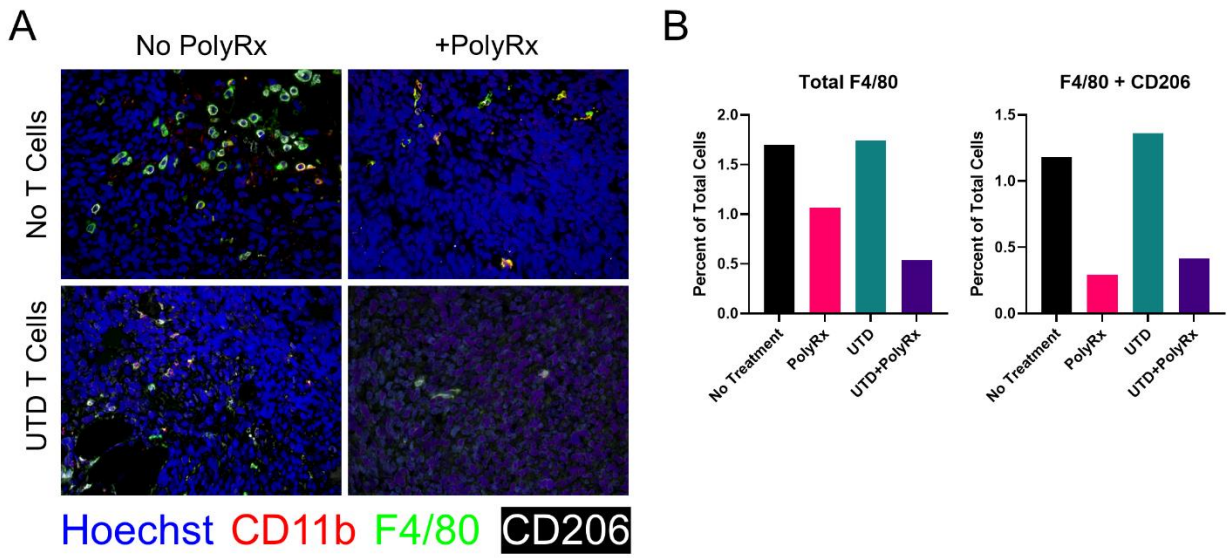


Figure S7. PolyRx treatment reduces immunosuppressive cells in the TME. Mice received No Treatment (no T cells or PolyRx), PolyRx only, UTD T Cells only, or UTD T Cells with PolyRx. Tumors were excised when mice reach endpoint and processed for IHC. **A, B.** Sections were stained for Hoechst (blue) CD11b (red), F4/80 (green) and CD206 (white) to quantify macrophages within the TME. 10 images from each tumor were used for quantification. Representative images are shown in **A,** and the total F4/80 positive cells or F4/80 and CD206 double positive cells as a percent of total cells are graphed in **B.**

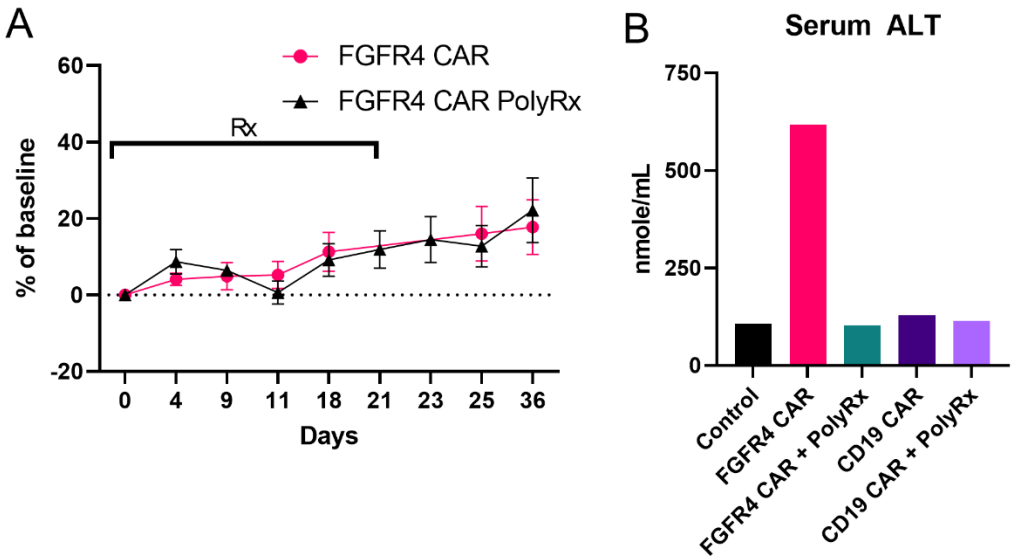


Figure S8. PolyRx does not cause overt toxicity in mice. A. Mice receiving either FGFR4 CAR or FGFR4 CAR with PolyRx were weighed throughout treatment and weights were plotted as percent of starting weight (baseline). N=5 animals per group **B.** Serum was collect from available mice at euthanization and analyzed for liver ALT using an ALT Activity Assay. FGFR4 CAR-T treated mice showed ALT increase over untreated control mice, but not with CD19 CAR-T cells or any PolyRx treated groups. N=2-3 per group.

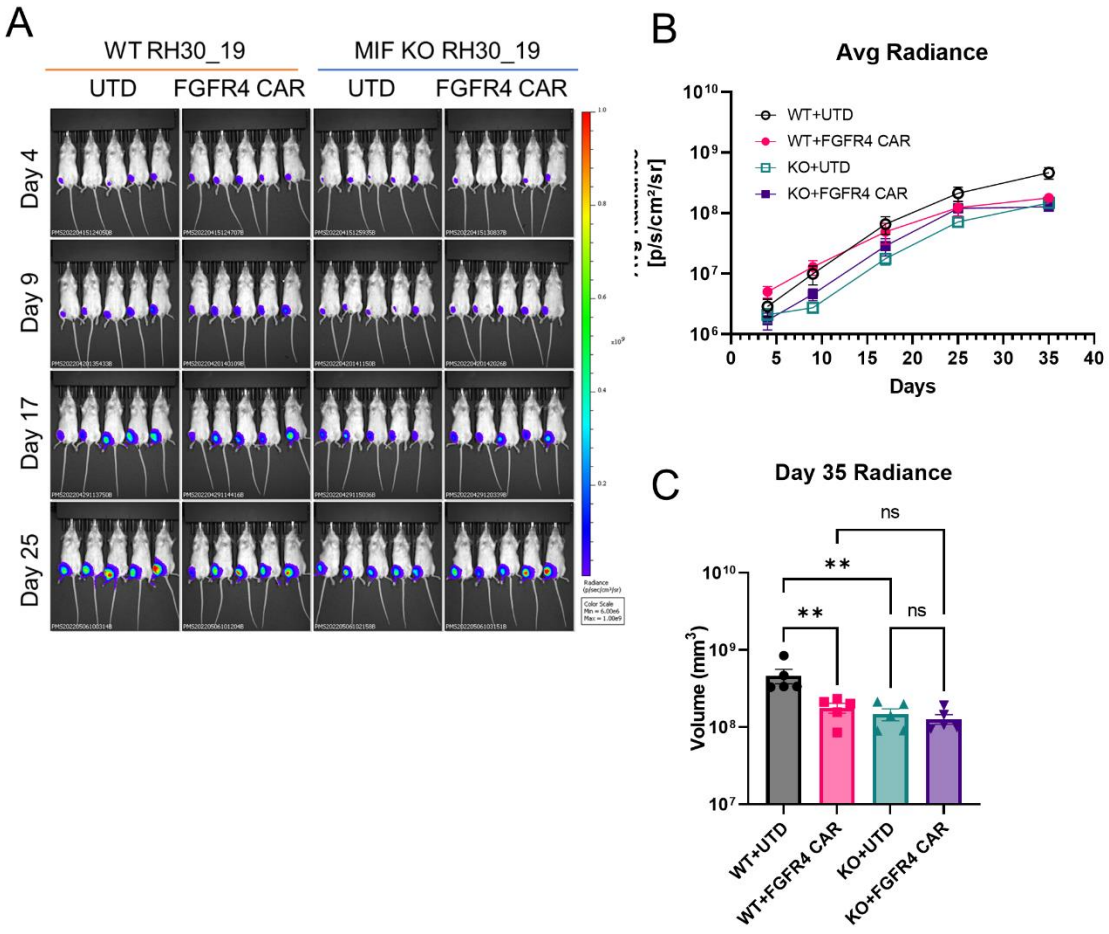


Figure S9. MIF KO slow RH30_19 tumor growth. NSG mice were injected intramuscularly with 1x10^6 RH30_19 WT or MIF KO tumors cells. On day 3, 1x10^7 UTD T cells or FGFR4 CAR-T were injected intravenously. Mice were followed with IVIS imaging weekly until tumor burden-defined endpoint (**A)**. IVIS measurements were graphed as average radiance (**B**). Day 35 IVIS average radiance was analyzed by one-way ANOVA (**C**). N=5 animals per group.

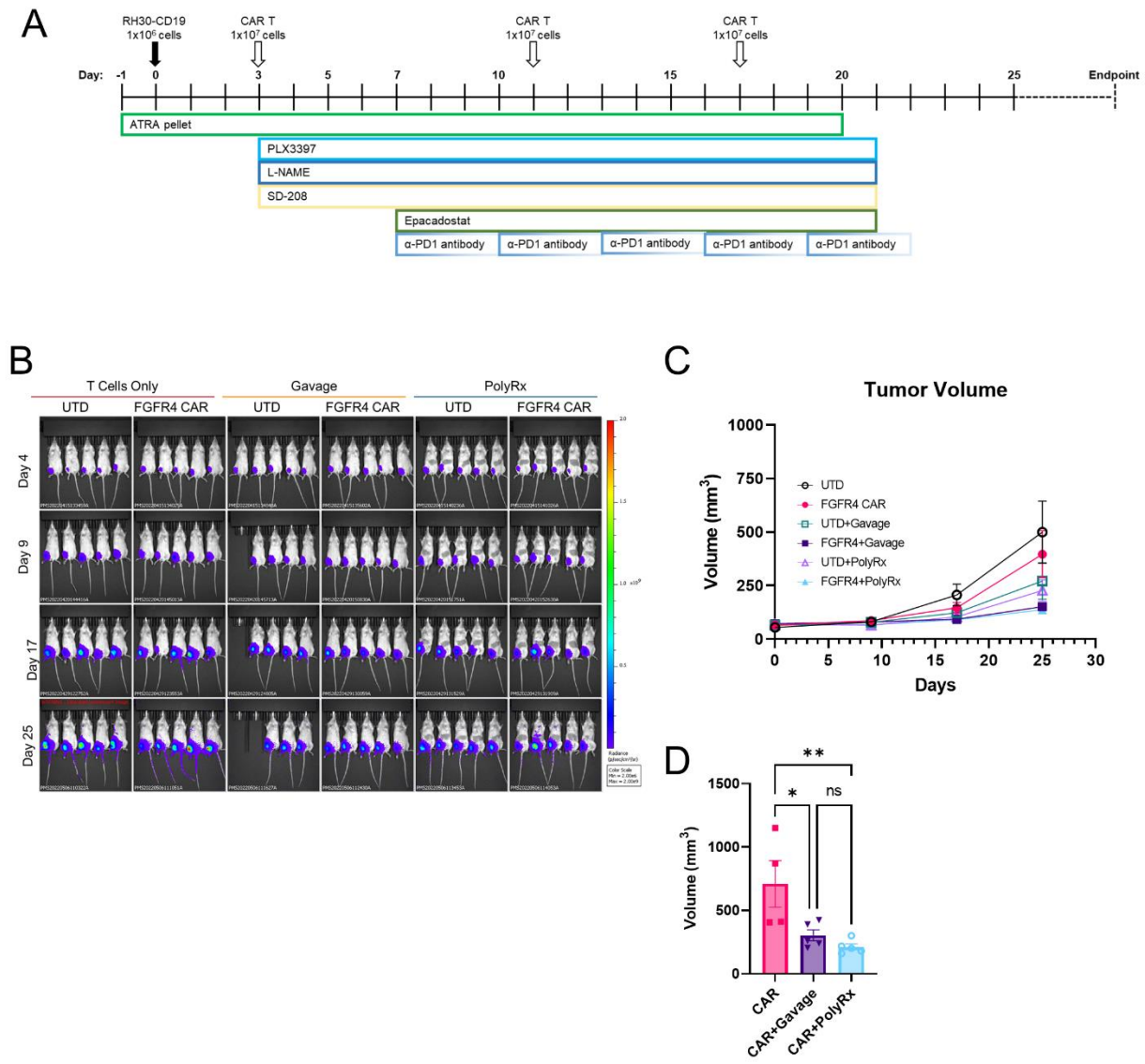


Figure S10. Reduced PolyRx achieves tumor control in RH30_19 tumors. **A**. Schematic of experimental plan. Mice were inoculated with WT RH30_19 tumors and treated with full PolyRx or a reduced PolyRx consisting of Epacadostat, Pexidartinib, and SD-208, "Gavage Only" treatment along with either UTD T cells or FGFR4 CAR-T cells. **B**. Mice were imaged by IVIS weekly. **C**. Tumor volume was also measured weekly by digital caliper and plotted. **D**. Day 25 tumor volumes were graphed and compared using one-way ANOVA.

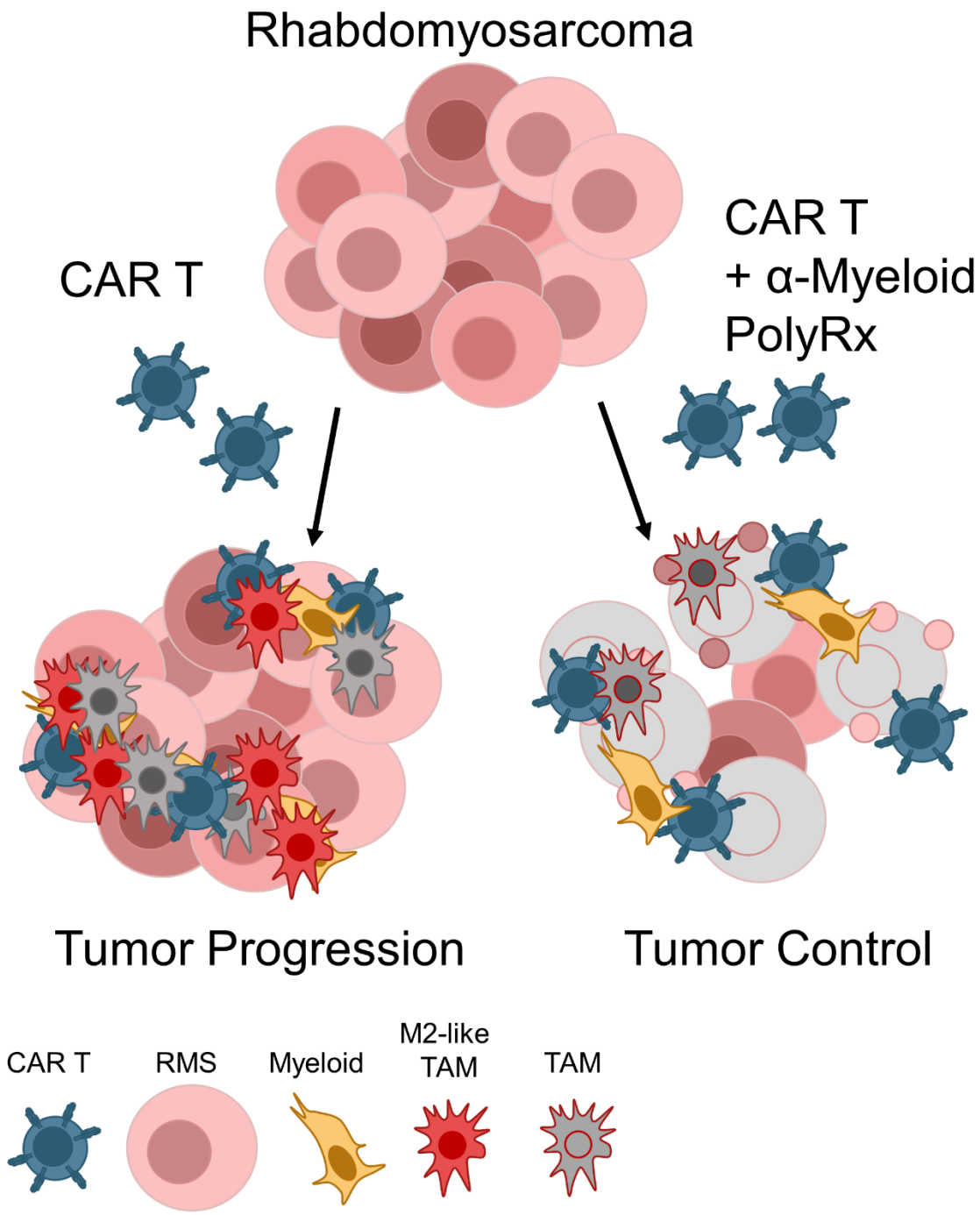


Figure S11. Illustration of RMS tumors with CAR-T cells alone or CAR-T cells with anti-myeloid PolyRx. RMS tumors in NSG mice respond to T cells (CAR T Cells) by producing a stroma containing suppressive myeloid populations (TAM, M2-like TAM, MDSC). Using anti-myeloid PolyRx disrupts this immunosuppression and allows CAR T cells to control the tumor.