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Supplemental Information

Checkpoint Blockade Reverses Anergy in IL-13Rα2 Humanized scFv-Based CAR T Cells to Treat Murine and Canine Gliomas

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Supplementary Materials

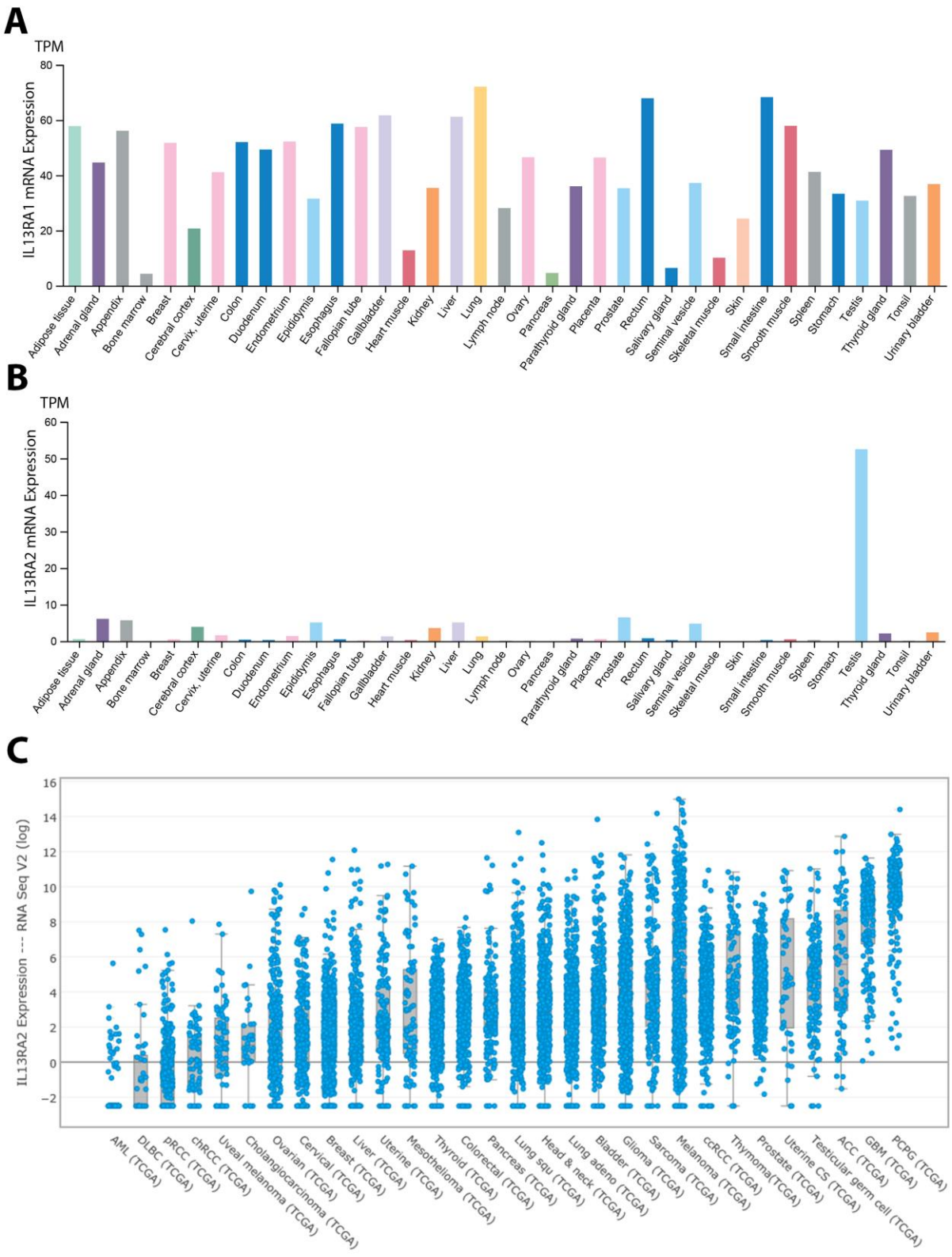
A
TPM
IL13RA1 mRNA Expression
0
20
40
60
80
Adipose tissue
Adrenal gland
Appendix
Bone marrow
Breast
Cerebral cortex
Cervix, uterine
Colon
Duodenum
Endometrium
Epididymis
Esophagus
Fallopian tube
Gallbladder
Heart muscle
Kidney
Liver
Lung
Lymph node
Ovary
Pancreas
Parathyroid gland
Placenta
Prostate
Rectum
Salivary gland
Seminal vesicle
Skeletal muscle
Skin
Small intestine
Smooth muscle
Spleen
Stomach
Testis
Thyroid gland
Tonsil
Urinary bladder
B
TPM
IL13RA2 mRNA Expression
0
10
20
30
40
50
60
C
IL13RA2 Expression --- RNA Seq V2 (log)
−2
0
2
4
6
8
10
12
14
16
AML (TCGA)
DLBC (TCGA)
pRCC (TCGA)
chRCC (TCGA)
Uveal melanoma (TCGA)
Cholangiocarcinoma (TCGA)
Ovarian (TCGA)
Cervical (TCGA)
Breast (TCGA)
Liver (TCGA)
Uterine (TCGA)
Mesothelioma (TCGA)
Thyroid (TCGA)
Colorectal (TCGA)
Pancreas (TCGA)
Lung squ (TCGA)
Head & neck (TCGA)
Lung adeno (TCGA)
Bladder (TCGA)
Glioma (TCGA)
Sarcoma (TCGA)
Melanoma (TCGA)
ccRCC (TCGA)
Thymoma(TCGA)
Prostate (TCGA)
Uterine CS (TCGA)
Testicular germ cell (TCGA)
ACC (TCGA)
GBM (TCGA)
PCPG (TCGA)

Fig. S1. IL13Rα1 and IL13Rα2 expression panel in the human normal or tumor tissues. (**A** and **B**) IL13Rα1 and IL13Rα2 expression in human normal tissues based on the Human Protein Atlas (HPA) (www.proteinatlas.org) RNA-seq data, which is reported as mean TPM (transcripts per million). (**C**) IL13Rα2 expression panel in the human tumors listed as the median of the expression based on the cancer genome atlas (TCGA) data available on cBioPortal.

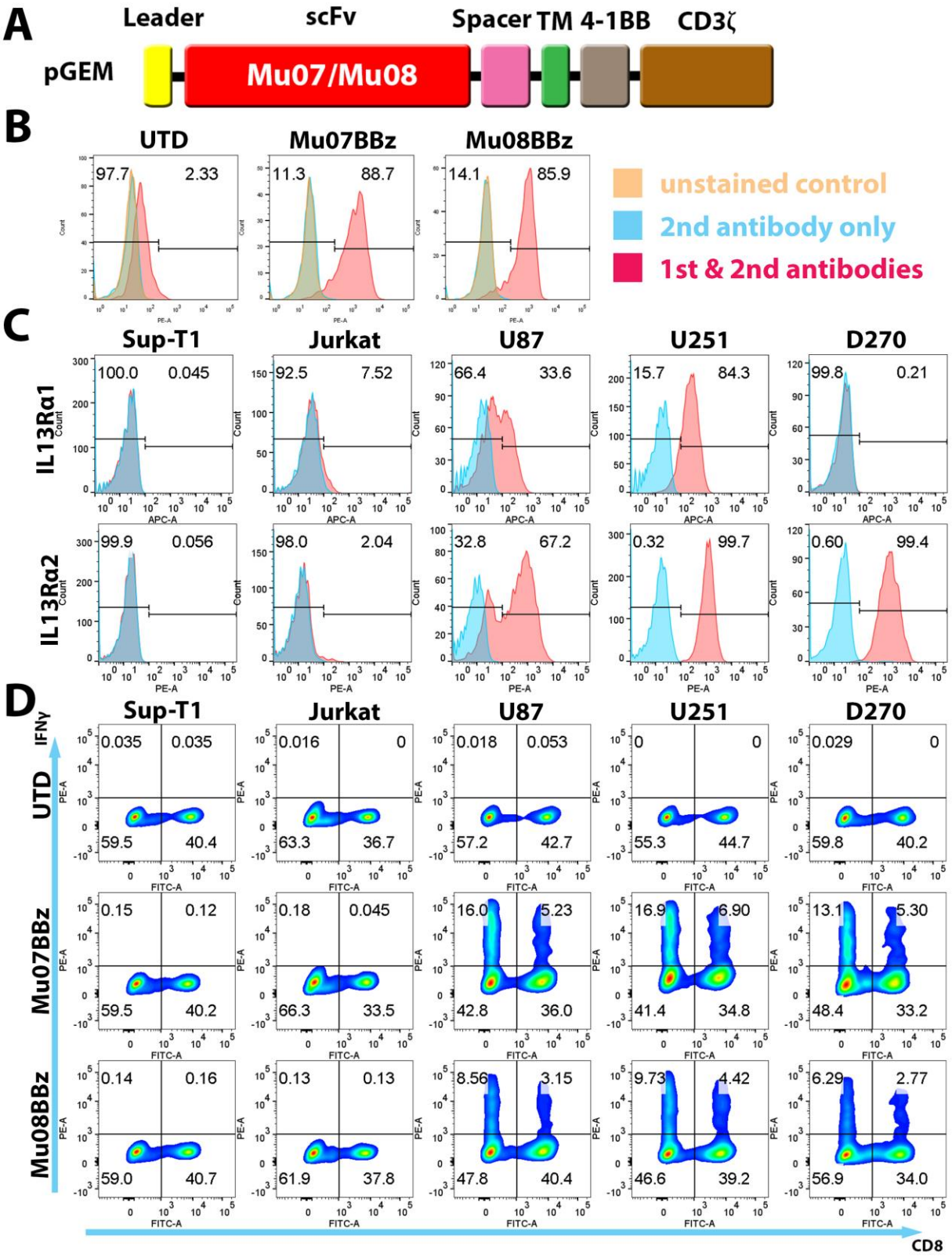
A
Leader
scFv
Spacer
TM
4-1BB
CD3ζ
pGEM
Mu07/Mu08
B
UTD
Mu07BBz
Mu08BBz
97.7
2.33
11.3
88.7
14.1
85.9
unstained control
2nd antibody only
1st & 2nd antibodies
C
Sup-T1
Jurkat
U87
U251
D270
IL13Rα1
IL13Rα2
100.0
0.045
92.5
7.52
66.4
33.6
15.7
84.3
99.8
0.21
99.9
0.056
98.0
2.04
32.8
67.2
0.32
99.7
0.60
99.4
D
IFNγ
UTD
Mu07BBz
Mu08BBz
CD8
0.035
0.035
59.5
40.4
0.016
0
63.3
36.7
0.018
0.053
57.2
42.7
0
0
55.3
44.7
0.029
0
59.8
40.2
0.15
0.12
59.5
40.2
0.18
0.045
66.3
33.5
16.0
5.23
42.8
36.0
16.9
6.90
41.4
34.8
13.1
5.30
48.4
33.2
0.14
0.16
59.0
40.7
0.13
0.13
61.9
37.8
8.56
3.15
47.8
40.4
9.73
4.42
46.6
39.2
6.29
2.77
56.9
34.0

Fig. S2. Murine scFv based IL13Rα2 targeting CAR T cells. (**A**) Vector maps of tested murine scFv based anti-IL13Rα2 CAR design. (**B**) Expression of murine scFv (07 and 08) based IL13Rα2 targeting CAR constructs on electroporated human-T cells. (**C**) IL13Rα1 and IL13Rα2 expression analysis on the human tumor cell lines (Sup-T1, Jurkat, U87, U251 and D270); the isotype antibodies depicted in blue. (**D**) Flow-based intracellular cytokine (IFNγ) staining of the murine scFv based IL13Rα2 CAR T cells (Mu07BBz and Mu08BBz) co-cultured with human tumor cell lines in (C) controlled with un-transduced T cells (UTD). Human CD8 was stained to distinguish the CD4 positive and CD8 positive subgroups of T cells along the x axis.

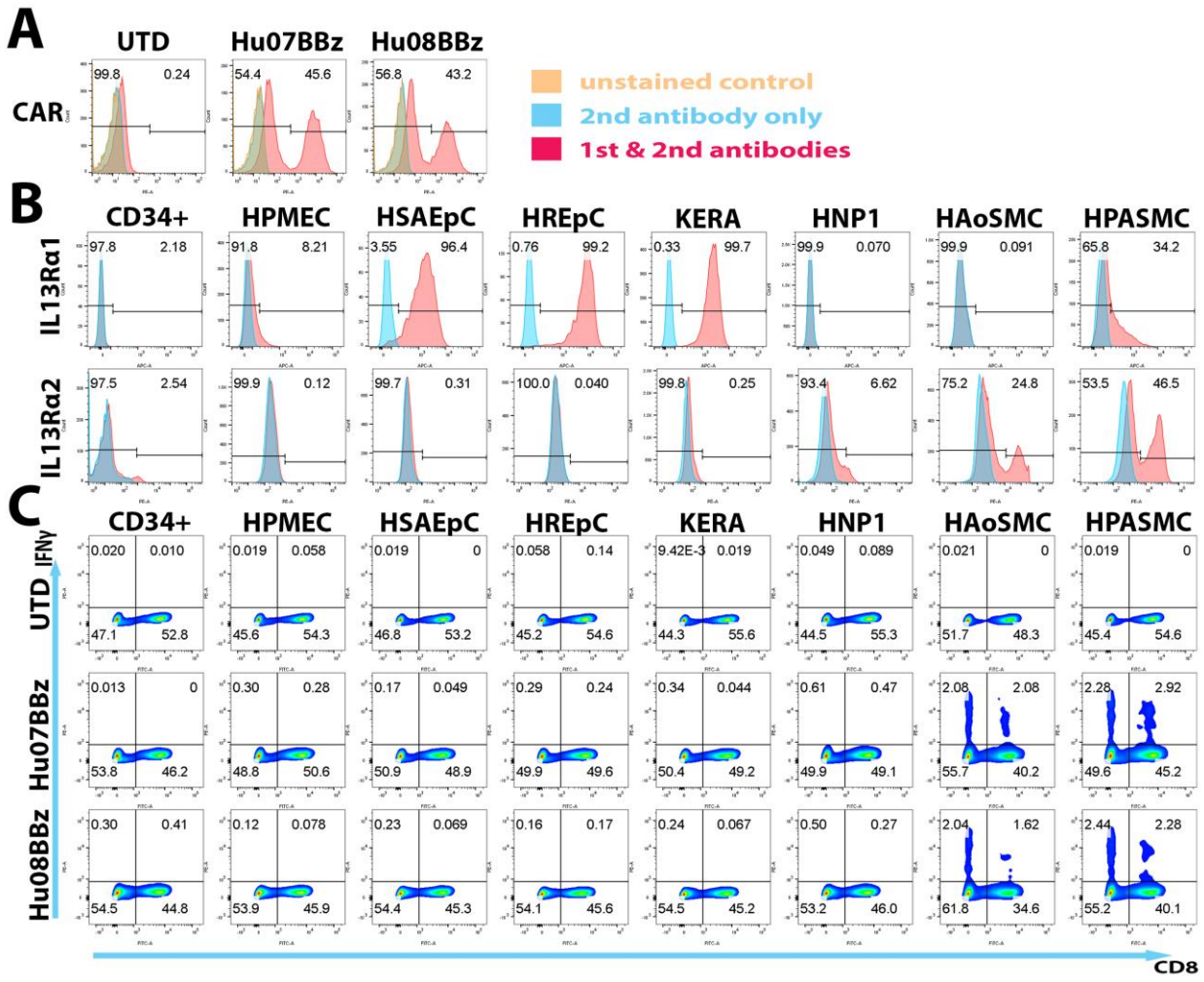

Fig. S3. Humanized IL13Rα2 targeting CAR T cells co-cultured with human normal cell types. (**A**) Flow-based CAR expression staining of the humanized IL13Rα2 CAR transduced T cells used in the co-culture experiments. (**B**) Flow cytometry of IL13Rα1 and IL13Rα2 expression analysis on the human normal cells (CD34 positive bone marrow cells, human pulmonary microvascular endothelial cells, human small airway epithelial cells, human renal epithelial cells, human keratinocytes, human neuronal progenitor cells, human aortic smooth muscle cells and human pulmonary artery smooth muscle cells) with the isotype antibodies control in blue. (**C**) Flow-based intracellular

cytokine (IFNγ) staining of the humanized IL13Rα2 CAR T cells co-cultured with human normal cells in (B) controlled with un-transduced T cells (UTD). Human CD3 and CD8 was stained to distinguish the CD4 positive and CD8 positive subgroups of T cells along the x axis.

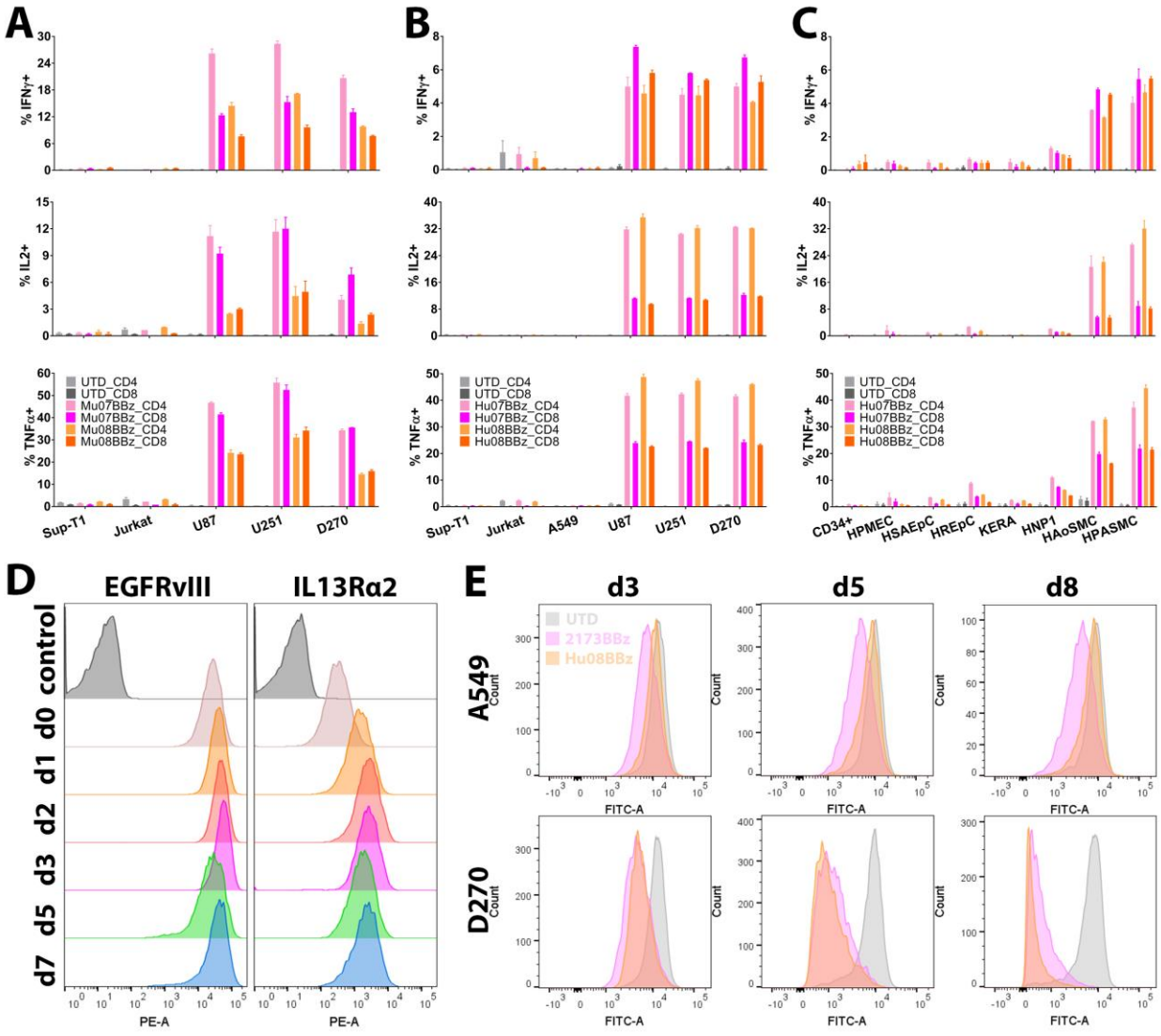

Fig. S4. Stimulation and expansion of IL13Rα2 targeting CAR T cells co-cultured *in vitro*.

(**A-C**) Flow-based intracellular cytokine (IFNγ, IL2 and TNFα) staining of murine

IL13Rα2 CAR T cells co-cultured with human tumor cell lines (A), humanized IL13Rα2 CAR T cells co-cultured with human tumor cell lines (B) and humanized IL13Rα2 CAR T cells co-cultured with human normal cells (C). The percentage of cytokine positive T cells was illustrated in the CD4 and CD8 positive subgroups. (**D**) Flow-based EGFRvIII and IL13Rα2 expression on the D270 tumor cell line of day 0, 1, 2, 3, 5 and 7 cultured *in vitro*, controlled with control antibodies. (**E**) Flow cytometry determined T cell proliferation assay with CFSE staining was performed on UTD T cells, 2173BBz and Hu08BBz CAR positive T cells on day 3, 5 and 8 co-culturing with D270 cell line controlled with A549 cell line. Data are presented as means ± SEM.

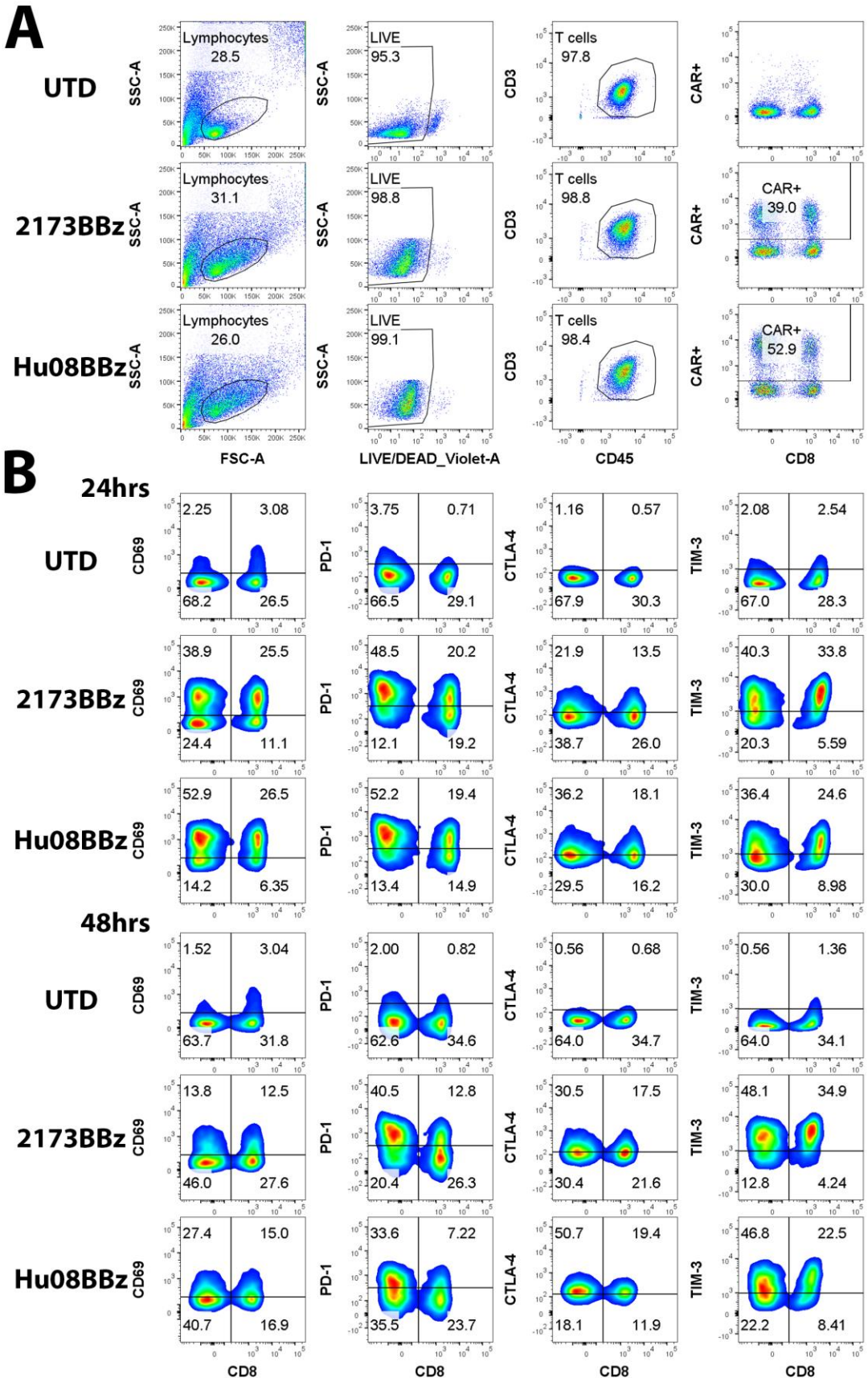
A
UTD
2173BBz
Hu08BBz
SSC-A
FSC-A
Lymphocytes
28.5
Lymphocytes
31.1
Lymphocytes
26.0
LIVE
95.3
LIVE
98.8
LIVE
99.1
LIVE/DEAD_Violet-A
CD3
CD45
T cells
97.8
T cells
98.8
T cells
98.4
CAR+
CD8
CAR+
39.0
CAR+
52.9
B
24hrs
48hrs
UTD
2173BBz
Hu08BBz
CD69
PD-1
CTLA-4
TIM-3
CD8
2.25
3.08
68.2
26.5
3.75
0.71
66.5
29.1
1.16
0.57
67.9
30.3
2.08
2.54
67.0
28.3
38.9
25.5
24.4
11.1
48.5
20.2
12.1
19.2
21.9
13.5
38.7
26.0
40.3
33.8
20.3
5.59
52.9
26.5
14.2
6.35
52.2
19.4
13.4
14.9
36.2
18.1
29.5
16.2
36.4
24.6
30.0
8.98
1.52
3.04
63.7
31.8
2.00
0.82
62.6
34.6
0.56
0.68
64.0
34.7
0.56
1.36
64.0
34.1
13.8
12.5
46.0
27.6
40.5
12.8
20.4
26.3
30.5
17.5
30.4
21.6
48.1
34.9
12.8
4.24
27.4
15.0
40.7
16.9
33.6
7.22
35.5
23.7
50.7
19.4
18.1
11.9
46.8
22.5
22.2
8.41

Fig. S5. Surface markers staining on CAR T cells co-cultured *in vitro*. (**A**) Representative gating scheme was illustrated with the samples of UTD T cells, 2173BBz and Hu08BBz CAR T cells co-cultured with D270 cell line for 48hrs. CD45+, CD3+ live lymphocytes were gated, expression of T cell surface markers was analyzed and compared among CAR+ T cells and UTD T cells. (**B**) The expression of CD69, PD-1, CTLA-4 and TIM-3 on the CD4+ and CD8+ T cells was determined by flow-cytometry, by staining with fluorochrome-conjugated corresponding antibodies after 24hrs or 48hrs co-culture. Representative expression results were illustrated in D270 cell line co-cultured UTD T cells and CAR+ T cells.

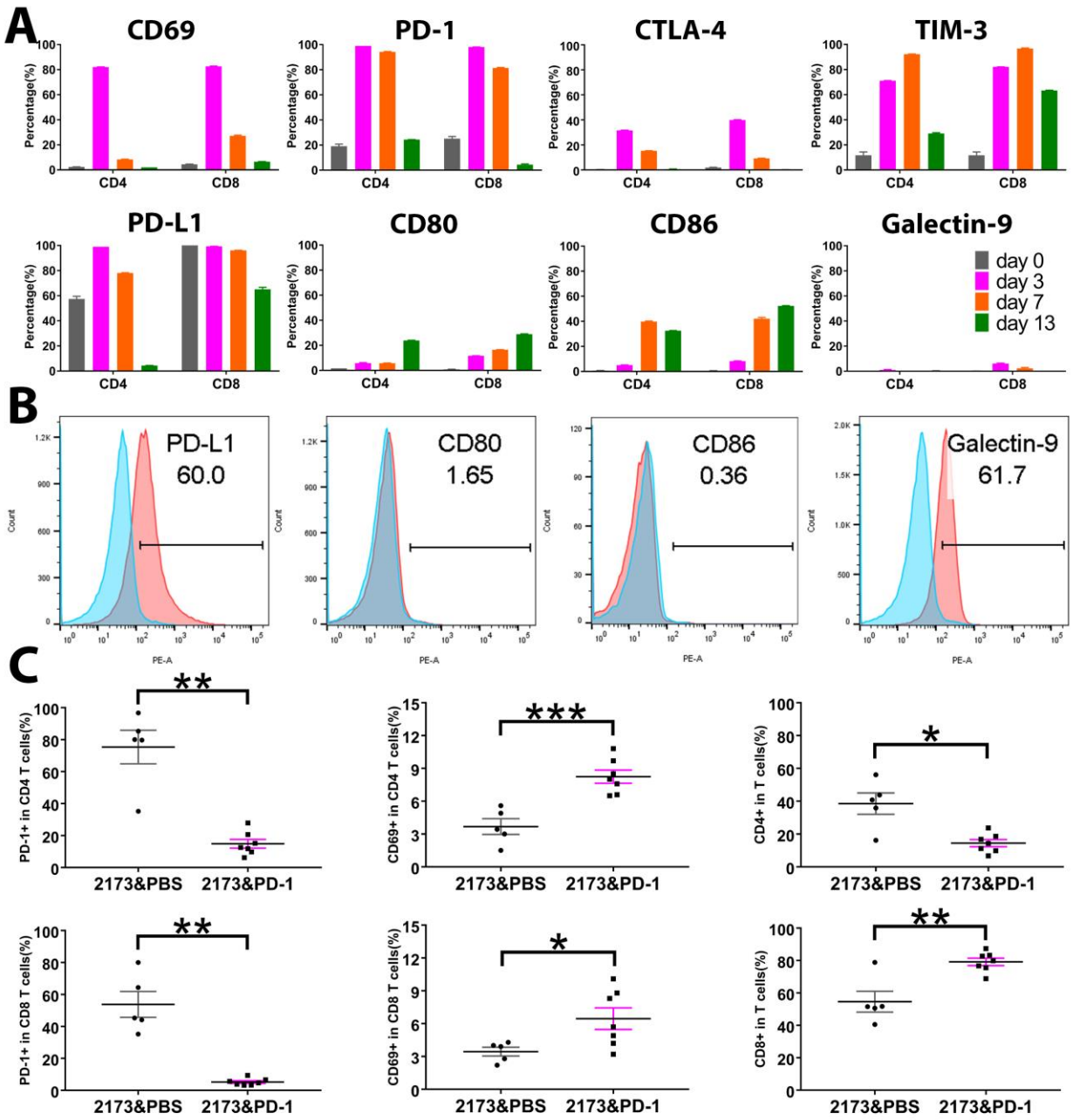

Fig. S6. Checkpoint receptor and ligand expressed and involved in the activity of CAR T cells *in vivo*. (**A**) Flow based detection of checkpoint receptors (PD-1, CTLA-4 and TIM-3) and their ligands (PD-L1, CD80, CD86 and galectin-9) in CD4 and CD8 positive T cell subgroups during T cell *in vitro* expansion with anti-CD3 and anti-CD28 beads on day 0, 3, 7 and 13. (**B**)) Flow-based detection of checkpoint receptor ligand (PD-L1,

CD80, CD86 and galectin-9) expression analysis on the D270 glioma cell line with the isotype antibodies control in blue. (**C**) Human PD-1, CD69, CD4 and CD8 staining on human $CD3^+$ T cells in the mouse spleen *ex vivo* after 2173BBz CAR T cells infusion combined with anti-PD-1 checkpoint blockade in a D270 subcutaneously implanted NSG mouse model. Data shown as the percentage of positive cells. Statistically significant differences were calculated by unpaired *t* test. **P*<0.05, ***P*<0.01, ****P*<0.001. Data are presented as means ± SEM.

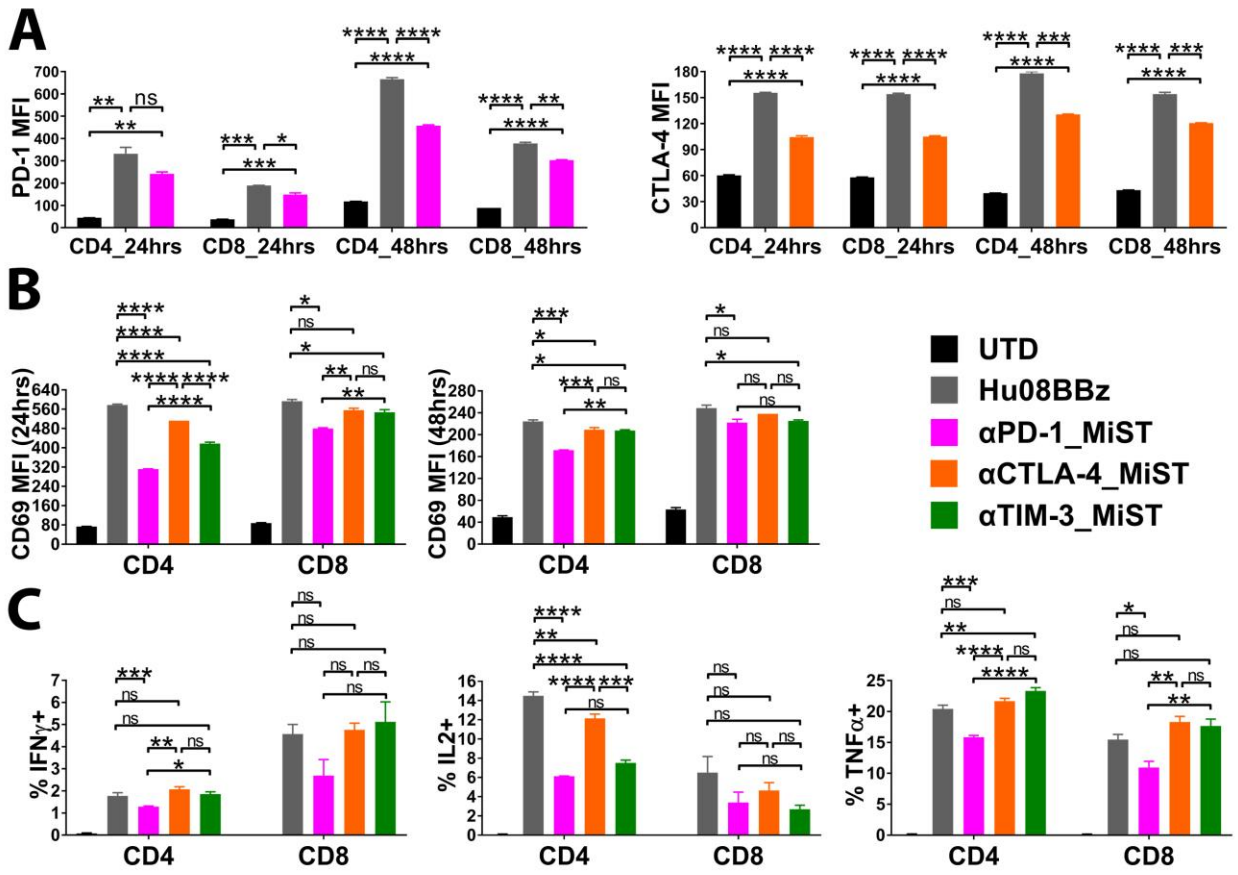

Fig. S7. MiST was co-cultured with target cells and analyzed *in vitro*. (**A**) Un-transduced T cells, IL13Rα2 targeting (Hu08BBz) CAR T cells and minibody secreting Hu08BBz

CAR T cells (anti-PD1 and anti-CTLA4 MiST) were co-cultured with D270 tumor cell line. Median fluorescence intensity (MFI) was quantified by BV711-conjugated anti-PD1 antibody and PE-conjugated anti-CTLA-4 antibody staining on CD4 and CD8 subgroups of CAR positive T cells after 24hrs or 48hrs co-culture. (**B**) The stimulation of IL13Rα2 (Hu08BBz) targeting CAR T cells and minibody secretion ones was evaluated after co-culture with D270 tumor cell line; median fluorescence intensity (MFI) was quantified by FITC-conjugated anti-CD69 antibody staining on CD4 and CD8 subgroups of CAR positive T cells after 24hrs or 48hrs co-culture. (**C**) The percentage of cytokine (IFNγ, IL2 and TNFα) staining positive T cells in CD4 and CD8 positive T cell subgroups was analyzed for IL13Rα2 targeting (Hu08BBz) CAR T cells and minibody secreted cells after co-culture with D270 target tumor cell lines. Statistically significant differences were calculated by one-way ANOVA with post hoc Tukey test. ns, not significant; **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001. Data are presented as means ± SEM.

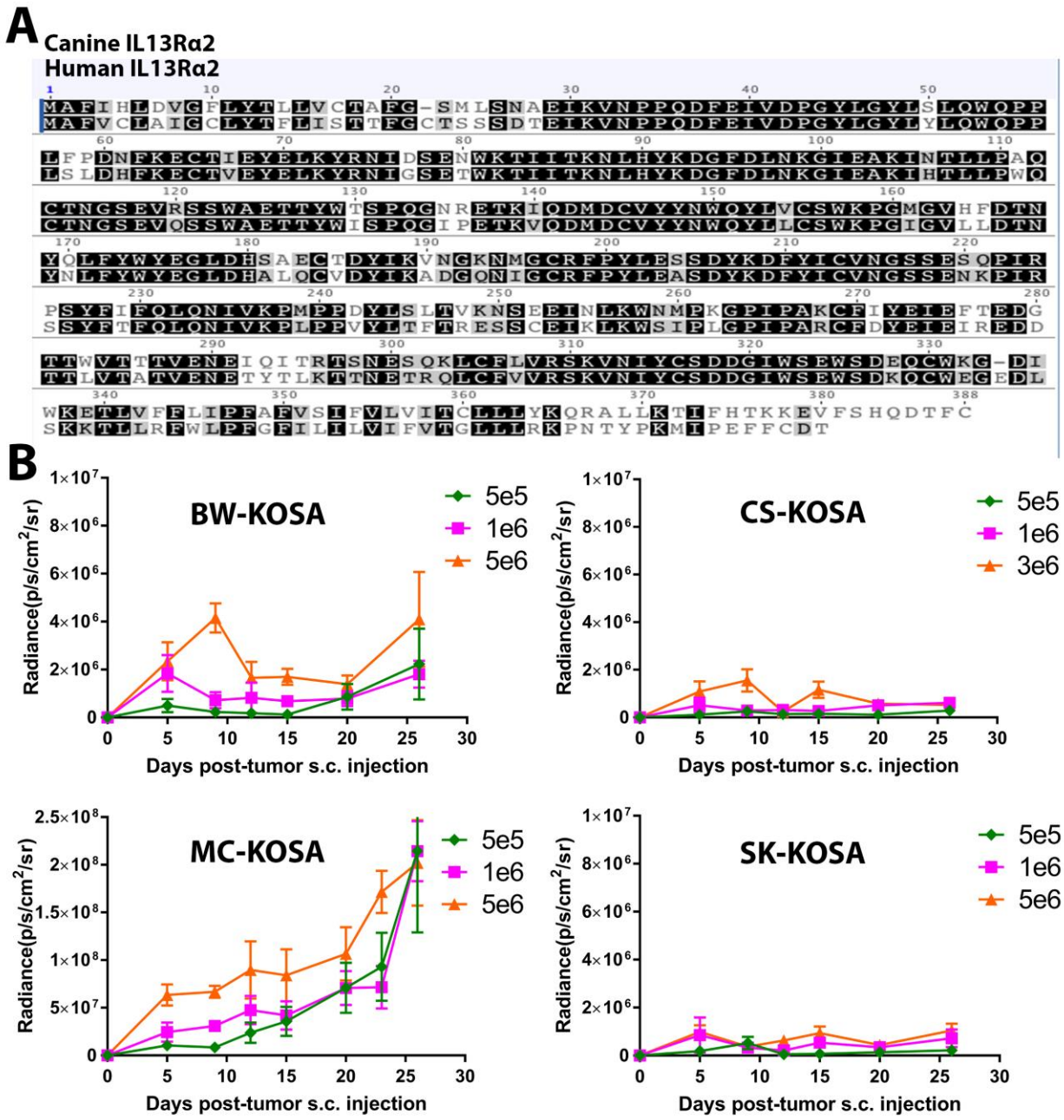

Fig. S8. The amino acid sequence of IL13Rα2 and canine osteosarcoma mouse models. (**A**) The amino acid sequences of human and canine IL13Rα2 were illustrated and compared with the software of Geneious. (**B**) Canine osteosarcoma tumor cell lines (BW-KOSA, CS-KOSA, MC-KOSA and SK-KOSA) were subcutaneously implanted into the right

flank of NSG mice with different doses. Bioluminescence imaging was repeatedly performed to evaluate the tumor growth in each group. Data are presented as means ± SEM.

Movies S1. D270 glioma cell line orthotopic implanted NSG mouse model. GFP transduced D270 glioma cells were intracranially implanted into the mouse brain. Tumor cells were visualized in the mouse brain with two-photon microscope after skull thinning. bar scale=90μm.

Movies S2. Human T cells in the mouse spleen. Human CAR T cells were labelled with CellTrace Violet (blue) and TRITC (red), then intravenously transplanted into an orthotopic glioma mouse model. Mouse spleen was removed and placed in media to be visualized in the two-photon microscope. bar scale=90μm.