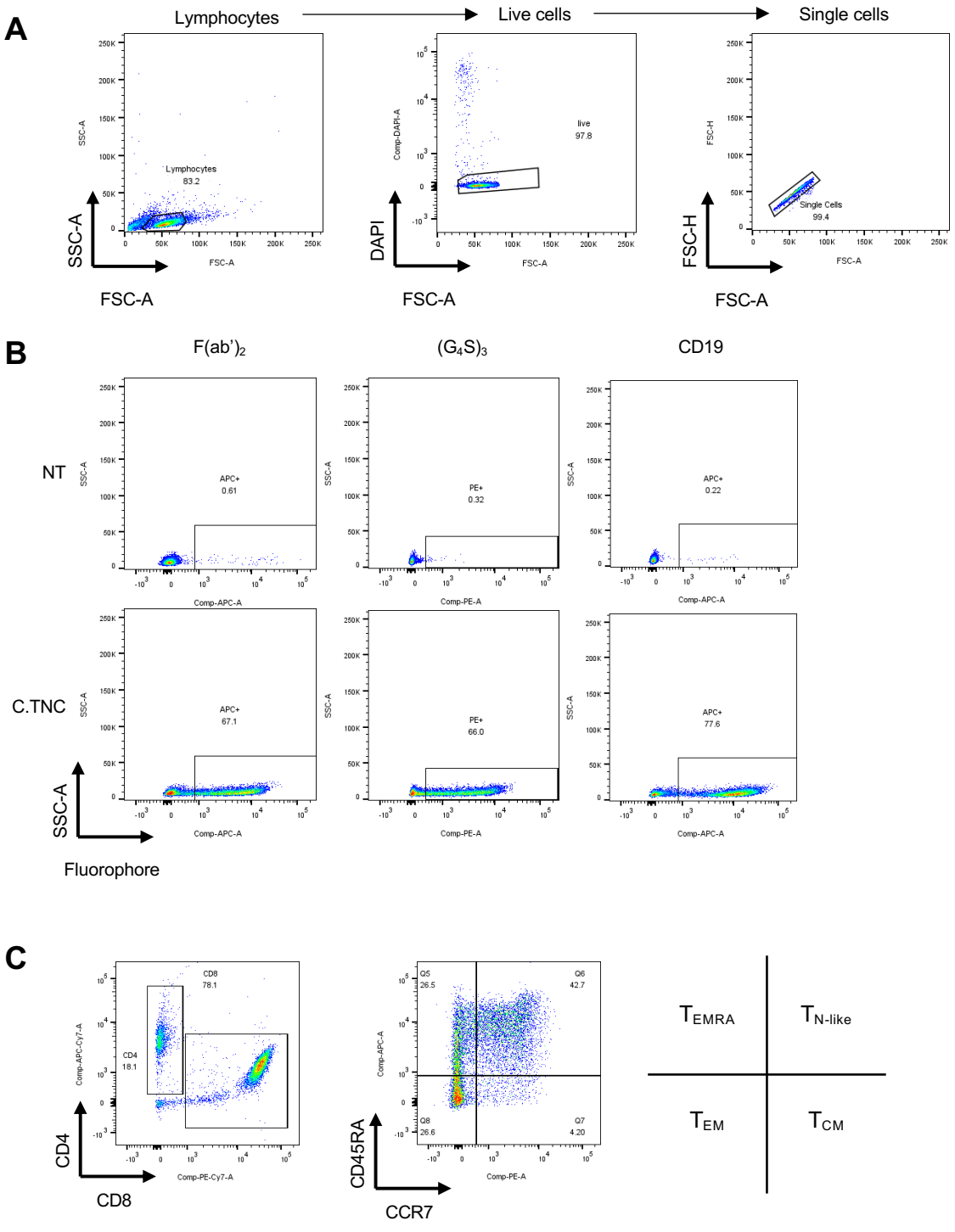
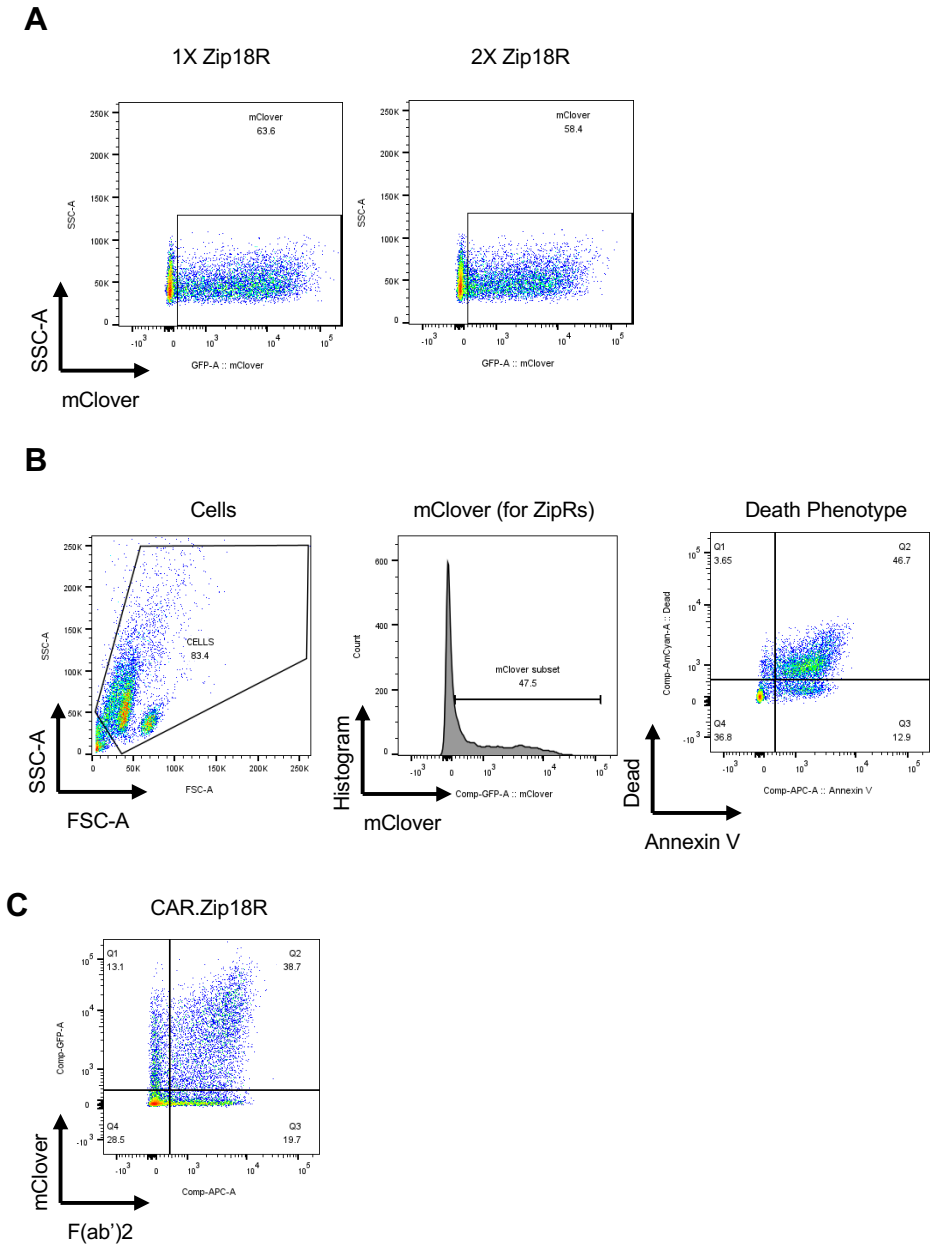
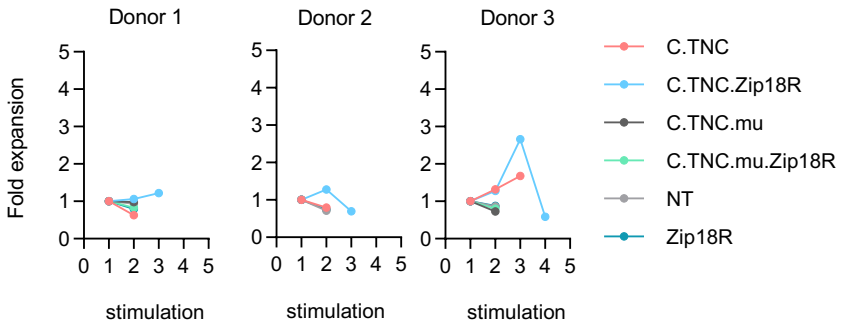


SFig 1. Representative transduction and phenotype flow cytometry gating strategy. (**A**) Gating for live, singlet T cells. (**B**) CAR-positive T cell populations based on F(ab')2, G4S, and CD19 staining. Top, NT cells; bottom, C.TNC-CAR T cells. (**C**) Representative phenotype gates.



SFig 2. Flow cytometric gating strategy to detect CAR.Zip18R T cells. (**A**) CAR and mClover transduction representative gating starting from live, singlet T cells. (**B**) Apoptosis assay gates. mClover gate only used for ZipR constructs. Dead+, Annexin V+: dead; Dead -, Annexin V+: apoptotic; Dead+, Annexin V-: necrotic; Dead-, Annexin V-: live. (**C**) CAR and Zip18R transduction representative gating from live, single cells.



SFig 3. Repeat stimulation assay with C.TNC-CAR T cell populations. T cells were stimulated with LM7.GFP.ffLuc at an E:T ratio of 2:1 (for scheme, see Figure 4B). Each graph represents one donor.

A

CAR: G11.SH.CD28TM.CD28.zeta.2A.tCD19
RED: Leader Sequence
Blue: G11 scFv (linker)
ORANGE: Hinge
BROWN: CD28 Transmembrane Domain
GREY: CD28.zeta
GREEN:2A
PINK: tCD19

MDWIWRILFLVGAATGAHSEVQLLESGGGLVQPGGSLRLSCAASGFTFSGSRMGWVRQAPGKGLEWVSAINEEGGQTYYADSVKGRFTISRDNSKNTLYLQMNSLR
AEDTAVYYCAKHPPHRPFDYWGQGTLVTVSSGGGGSGGGGSGGGGSSSELTQDPAVSVALGQTVRITCQGDSLRLYYASWYQQKPGQAPVLVIYGKNNRPSGIPDR
FSGSSSGNTASLTITGAQAEDEADYYCNSSHGPRRPVVFGGGTKLTVLGDLEPKSCDKTHTCPPCPDPKFWVLVVVGGVLACYSLLVTVAFIIFWVRSKRSRLLHS
DYMNMTPRRPGPTRKHYQPYAPPRDFAAYRSRVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSEIG
MKGERRRGKGHDGLYQGLSTATKDTYDALHMQALPPRASRAEGRGSLLTCGDVEENPGPMPPPRLLFFLLFLTPMEVRPEEPLVVKVEEGDNAVLQCLKGTSDGPT
QQLTWSRESPLKPFLKLSLGLPGLGIHMRPLAIWLFIFNVSQQMGGFYLCQPGPPSEKAWQPGWTVNVEGSGELFRWNVSDLGGLGCGLKNRSSEGPSSPSGKLMS
PKLYVWAKDRPEIWEGEPPCLPPRDSLNQSLSQDLTMAPGSTLWLSCGVPPDSVSRGPLSWTHVHPKGPKSLLSLELKDDRPARDMWVMETGLLLPRATAQDAGKY
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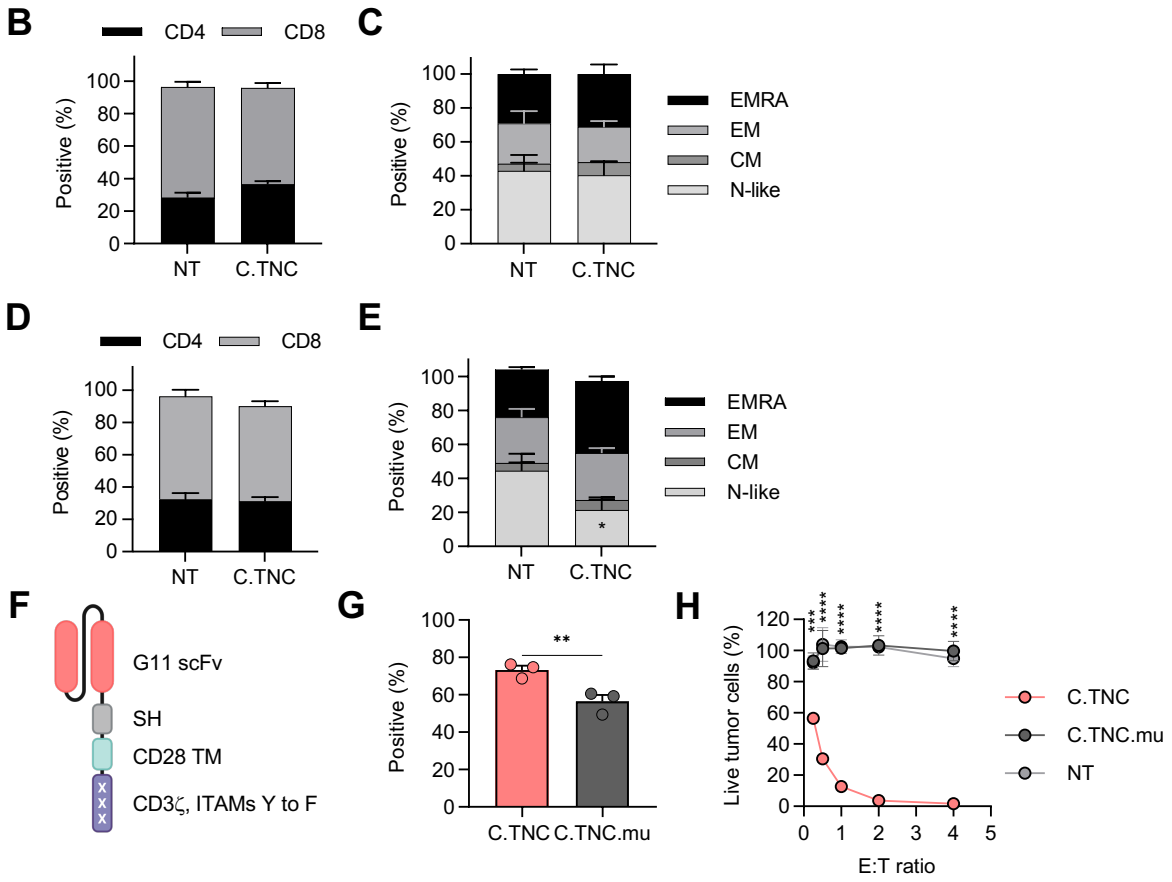
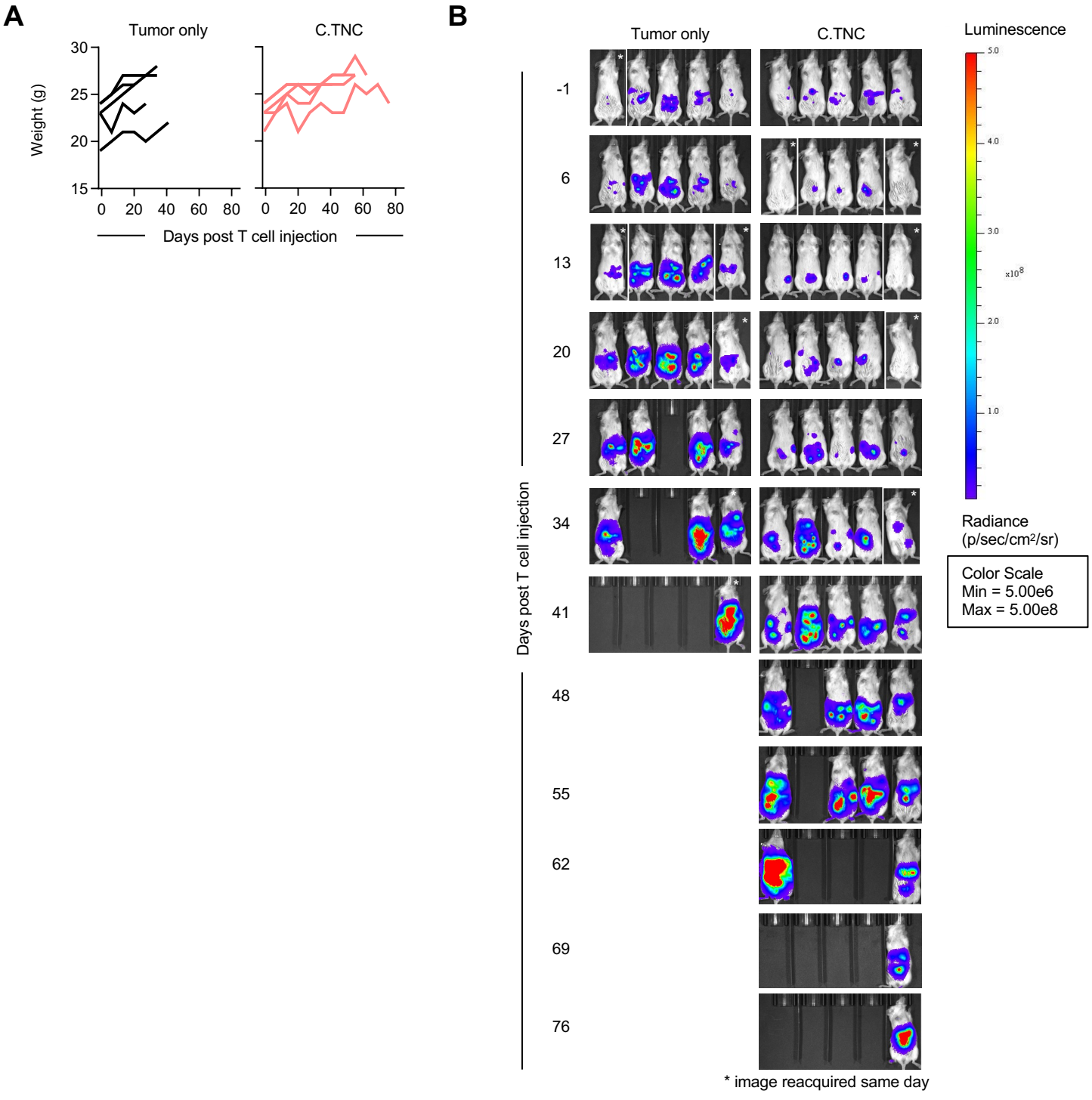
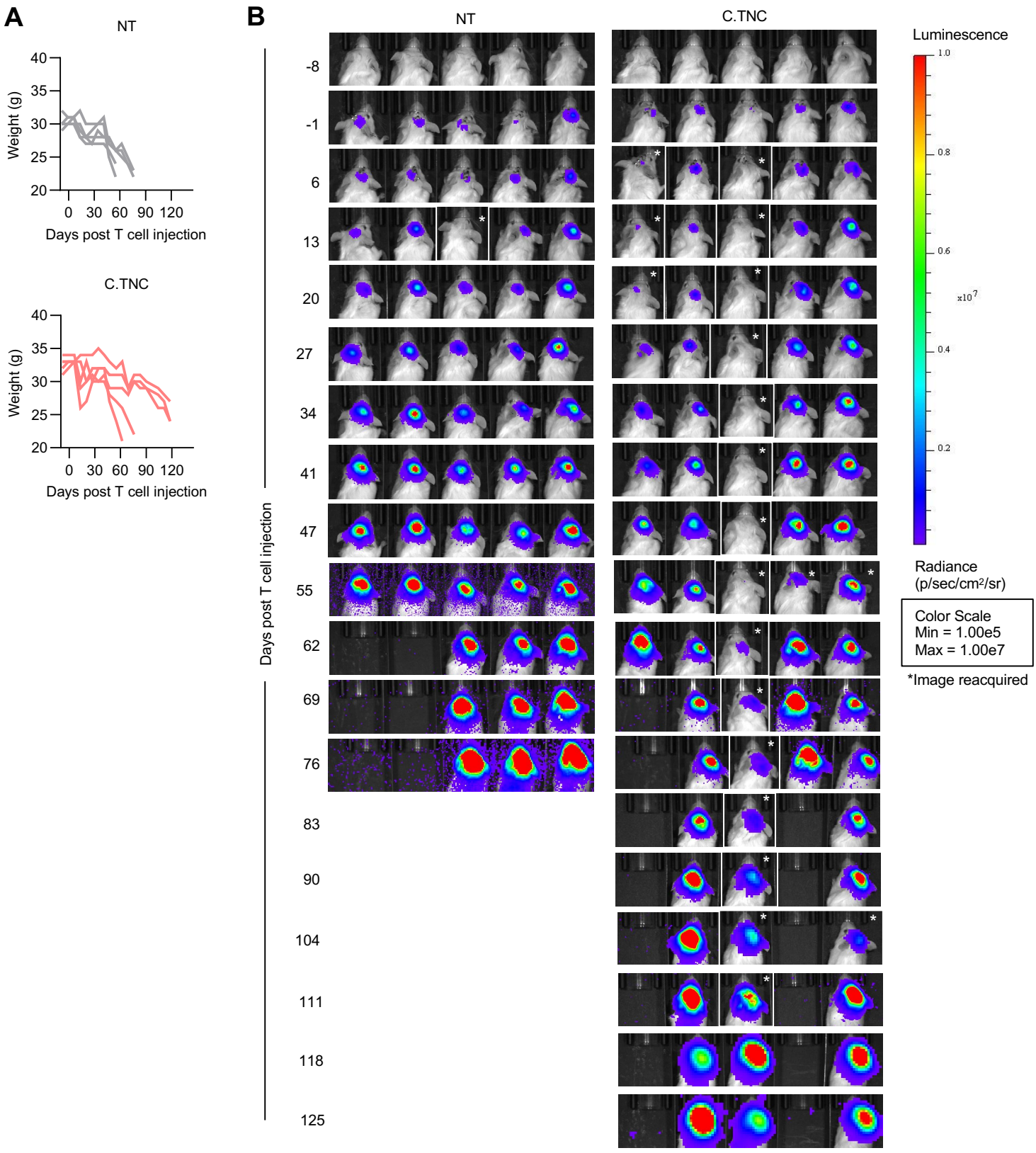


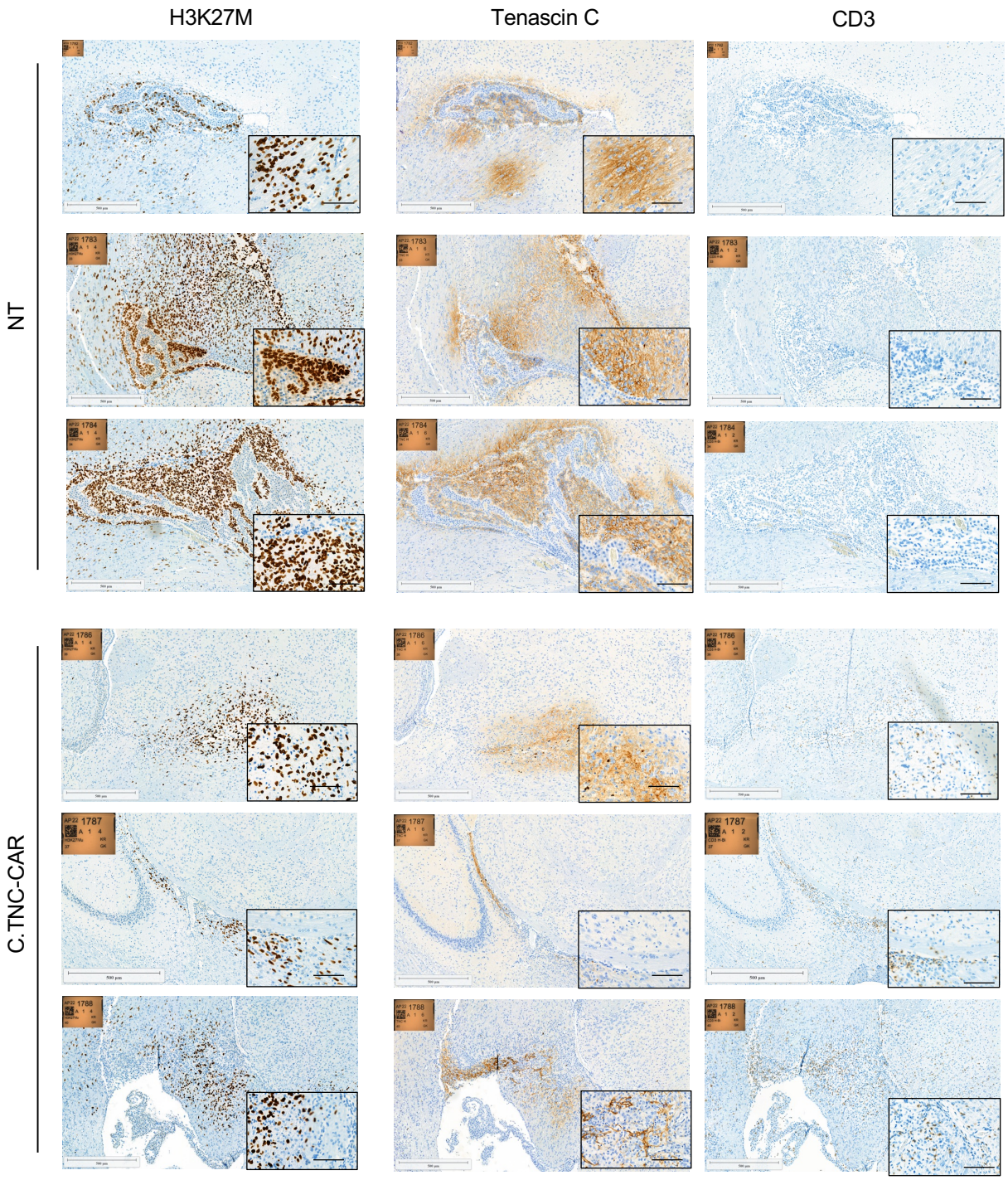
SFig 4. Characterization of C.TNC-CAR T cells *in vitro*. (**A**) Amino acid sequence of C.TNC-CAR. (**B**) CD4 and CD8 ratio and (**C**) phenotype (determined by CCR7 and CD45RA) measured by flow cytometry (mean + SEM, n=3). (**D**) CD4 and CD8 ratio and (**E**) phenotype after 48 hr coculture with LM7.GL cells measured by flow cytometry (mean + SEM, n=3), 2-way ANOVA, *:p<0.05. (**F**) C.TNC.mu-CAR scheme. Y to F substitutions in ITAMS highlighted. (**G**) T cell transduction of C.TNC.mu detected by F(ab')2 staining 7 days post-transduction (n=3, mean + SEM), Paired T-test, **:p<0.01. (**H**) 72-hour luciferase cytotoxicity assay with C.TNC-CAR T cells diluted with NT T cells to match TDX efficiencies (n=3, mean + SEM), 2-way ANOVA, ***:p<0.001, ****:p<0.0001.



SFig 5. Weight of mice and bioluminescence images for animal experiment shown in Figure 2H-J. (**A**) Weights for each cohort measured weekly (n=5 per cohort). (**B**) Bioluminescence images of mice.



SFig 6. Weight of mice and bioluminescence images for animal experiment shown in Figure 2K-M. (**A**) Weights for each cohort measured weekly (n=5 per cohort). (**B**) Bioluminescence images of mice.

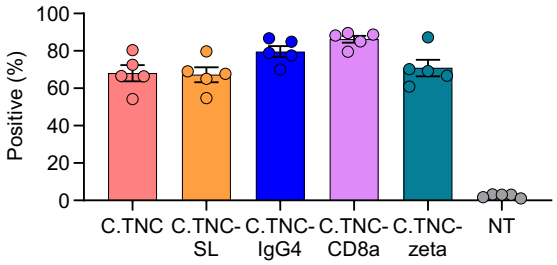


SFig 7. DIPG007 IHC post CAR T cell therapy. 1x10^6 DIPG.YFP.ffLuc cells were injected intracranially (i.c.), followed by 2x10^6 T cells injected i.c. 7 days later in 10–12-week-old male NSG mice. Tumors were harvested after 2 weeks post T cell treatment. IHC for H3K27M, Tenascin C, and CD3 was done. Images from 3 tumors (mice) is shown. Low magnification: 500 μm scale bar. High magnification: 100 μm scale bar.

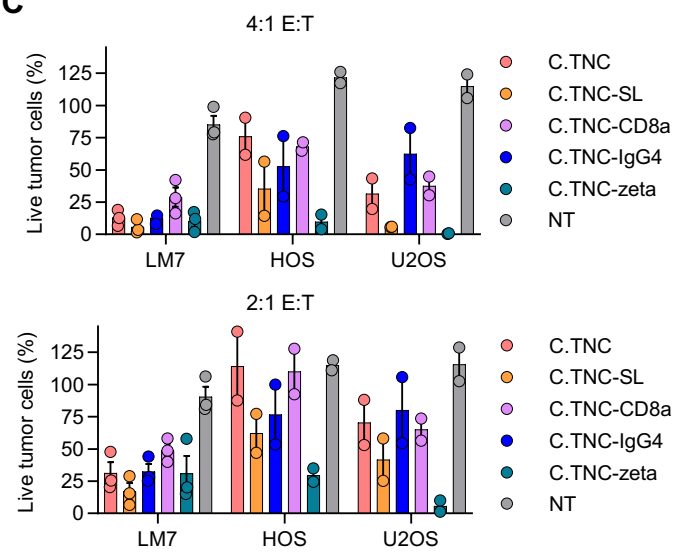
A

Construct	V_H	Linker	V_L	Hinge	TM	Costim.	ζ
C.TNC	G11	$(G_4S)_3$	G11	IgG1	CD28	CD28	CD3ζ
C.TNC-SL	G11	G_4S	G11	IgG1	CD28	CD28	CD3ζ
C.TNC-IgG4	G11	$(G_4S)_3$	G11	muIgG4	CD28	CD28	CD3ζ
C.TNC-CD8a	G11	$(G_4S)_3$	G11	CD8α	CD8α	CD28	CD3ζ
C.TNC-zeta	G11	$(G_4S)_3$	G11		CD3ζ		CD3ζ

B



C

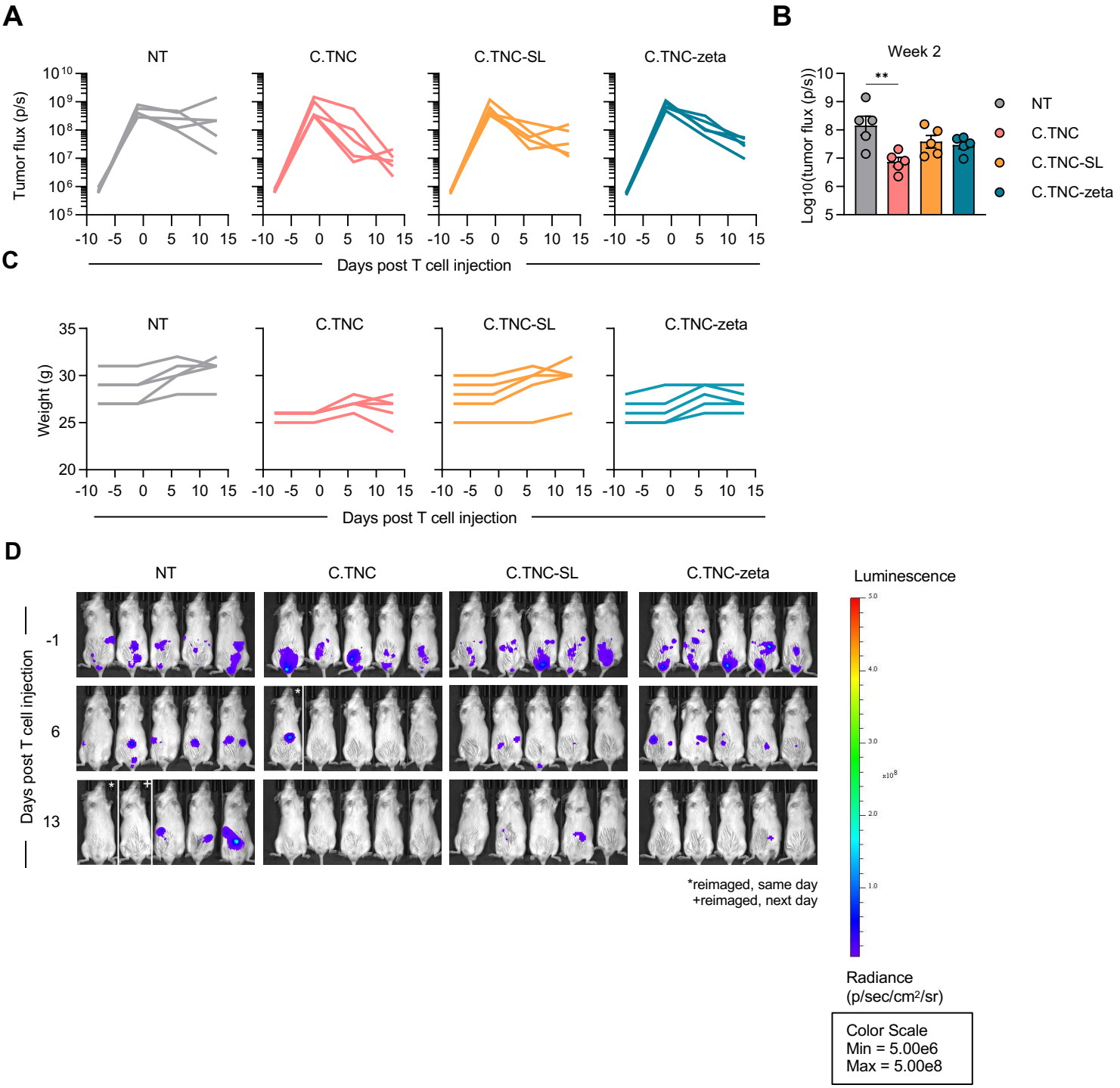


D

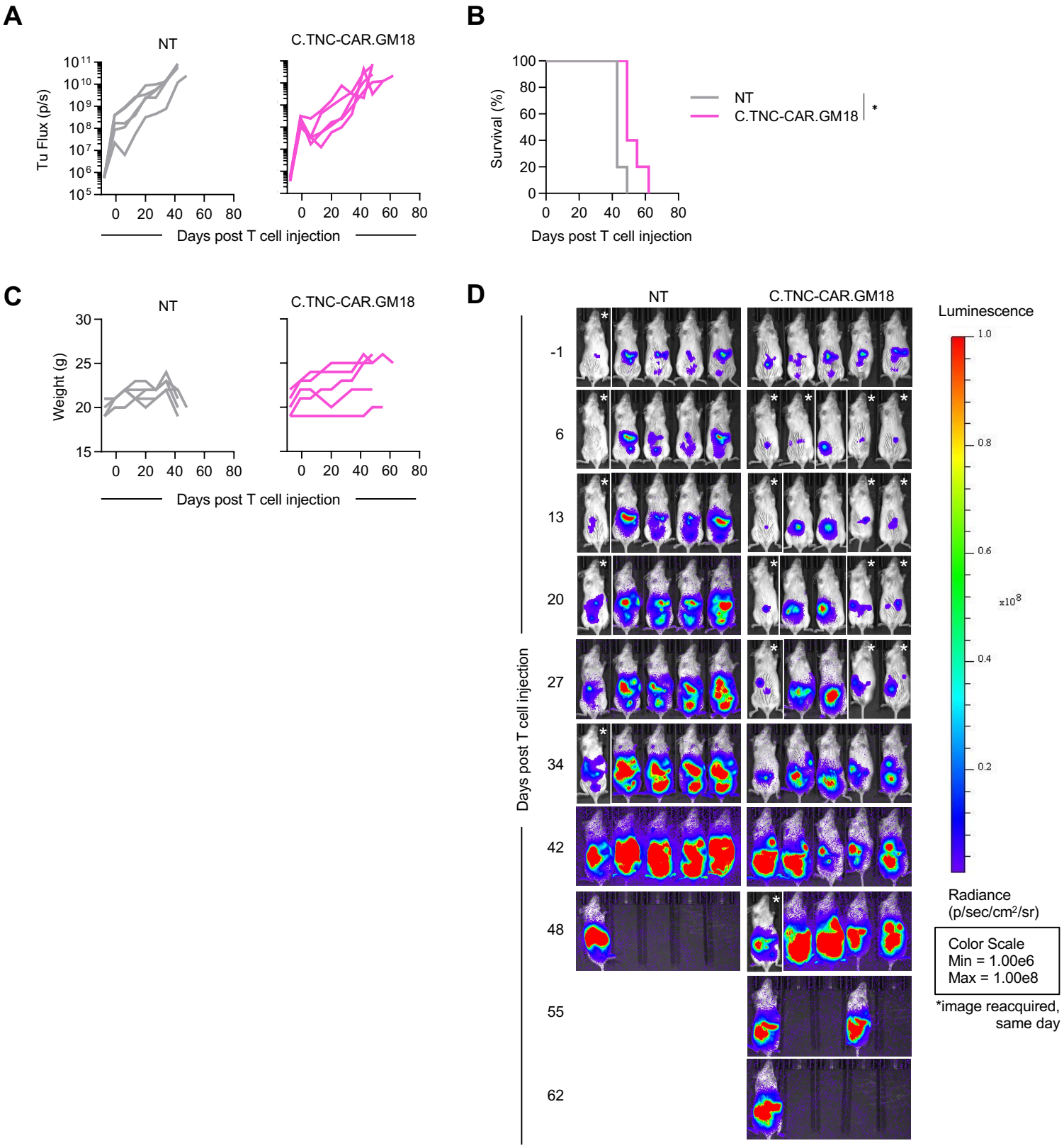
Condition	LM7		HOS		U2OS		
	2:1	4:1	2:1	4:1	2:1	4:1	SUM
C.TNC-SL	1	1	1	1	1	1	6
C.TNC-IgG4	0	0	1	1	-1	-1	0
C.TNC-CD8a	-1	-1	0	0	0	0	-2
C.TNC-zeta	0	0	1	1	1	1	4

Percent tumor cells remaining above C.TNC: -1
Percent tumor cells remaining similar to C.TNC: 0
Percent tumor cells remaining below C.TNC: 1

SFig 8. Influence of CAR design on cytolytic effector function of C.TNC-CAR T cells. (**A**) Table of new C.TNC-CAR constructs. V_H, variable heavy chain; V_L, variable light chain; muIgG4, mutant IgG4; TM, transmembrane; Costim., costimulatory domain. (**B**) Transduction efficiency measured by percent F(ab')2-positive via flow cytometry on Day 7 post-transduction (n=5, mean + SEM). (**C**) Cytotoxicity after 24 hours at 4:1 and 2:1 E:T ratios as determined by a luciferase-based assay (n=2-3, mean +SEM). (**D**) Table summary of cytotoxicity results from C. Value -1 assigned if the average is above C.TNC, 0 if similar, and 1 if below. Sum calculated from each condition tested, those with positive values were used for *in vivo* studies.



SFig 9. Alternative C.TNC-CAR design does not improve antitumor activity of C.TNC-CAR T *cells in vivo*. (**A**) Tumor flux values from LM7.GFP.ffLuc i.p. study in 5-6-week-old NSG male mice. 1x10^6 LM7.GFP.ffLuc cells were injected i.p. and treated 7 days later with 1x10^6-CAR-positive T cells (n=5 per cohort). (**B**) Day 13 post T cell injections log-transformed tumor flux values summarized from E (mean +SEM), One-way ANOVA,**:p<0.01. (**C**) Weights for each cohort measured weekly (n=5 per cohort). (**D**) Images of mice.



SFig 10. Inducible GM18 does not improve antitumor activity of C.TNC-CAR T cells *in vivo*. 1x10^6 LM7.GFP.ffLuc cells were injected intraperitoneally (i.p.), followed by 1x10^6 sorted T cells injected i.p. 7 days later in 5–6-week-old female NSG mice. (**A**) Tumor flux values (n=5 per cohort). (**B**) Kaplan-Meier survival curve, Mantel-Cox, *:p<0.05. (**C**) Weights measured weekly. (**D**) Mouse images.