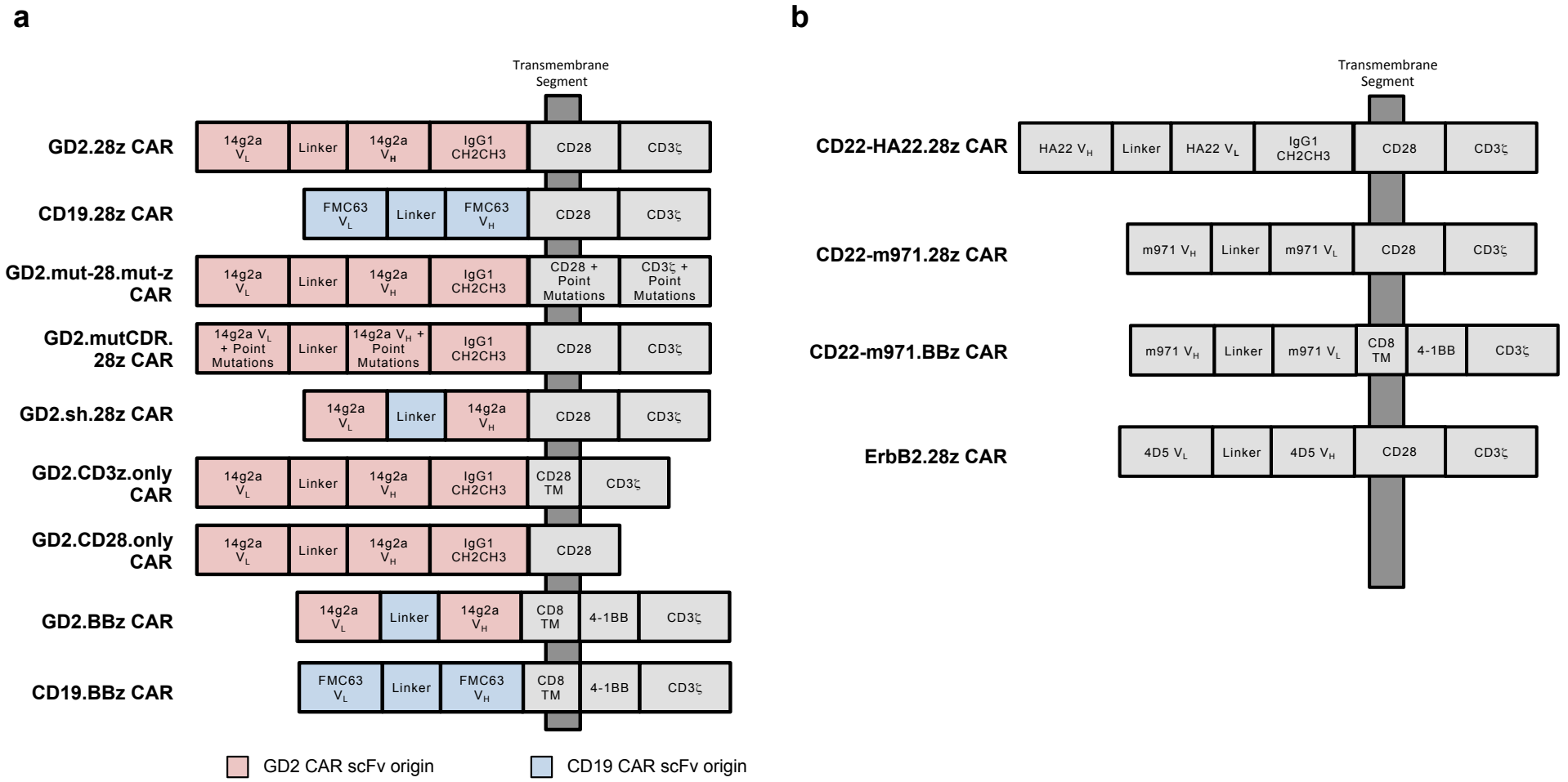
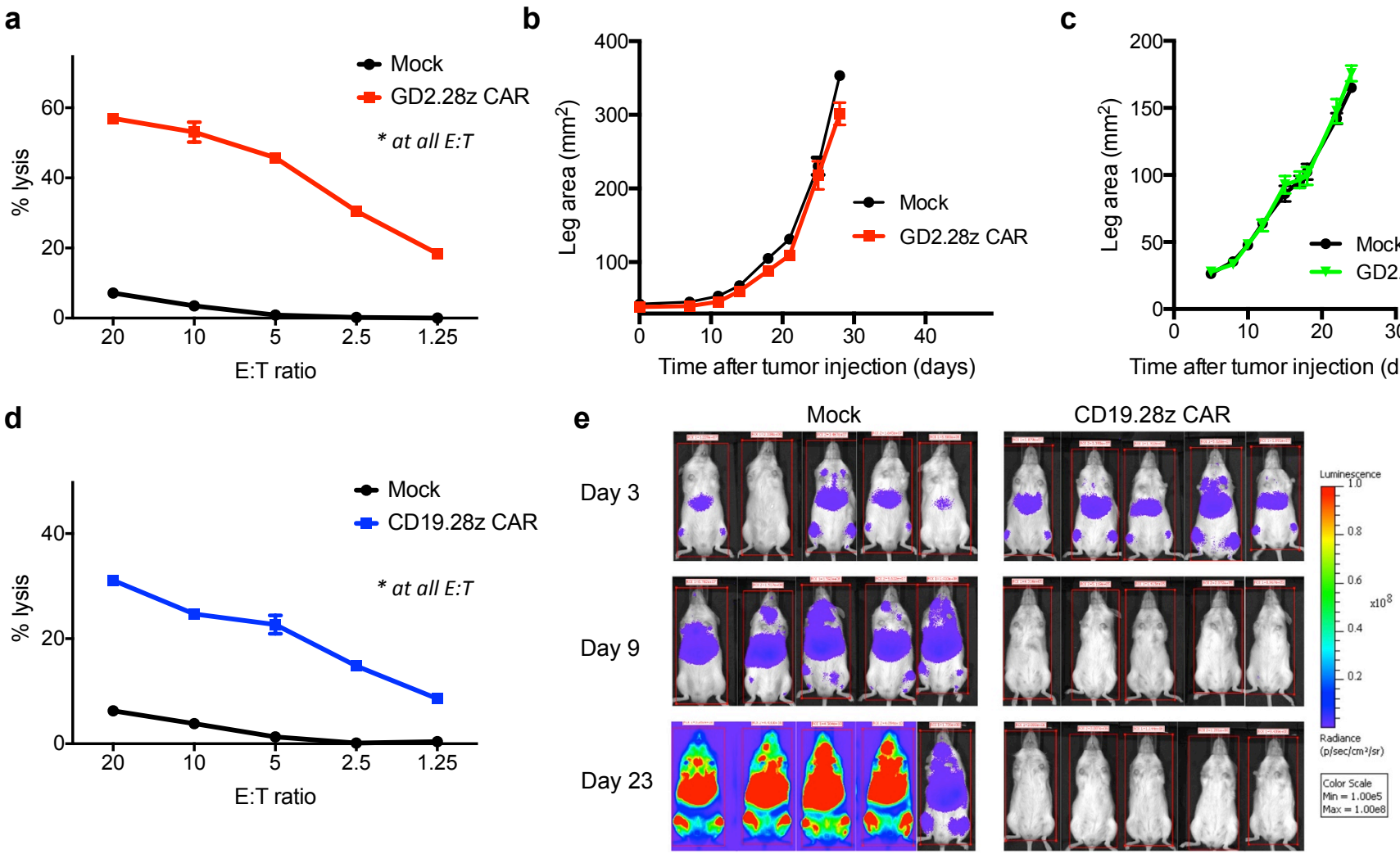
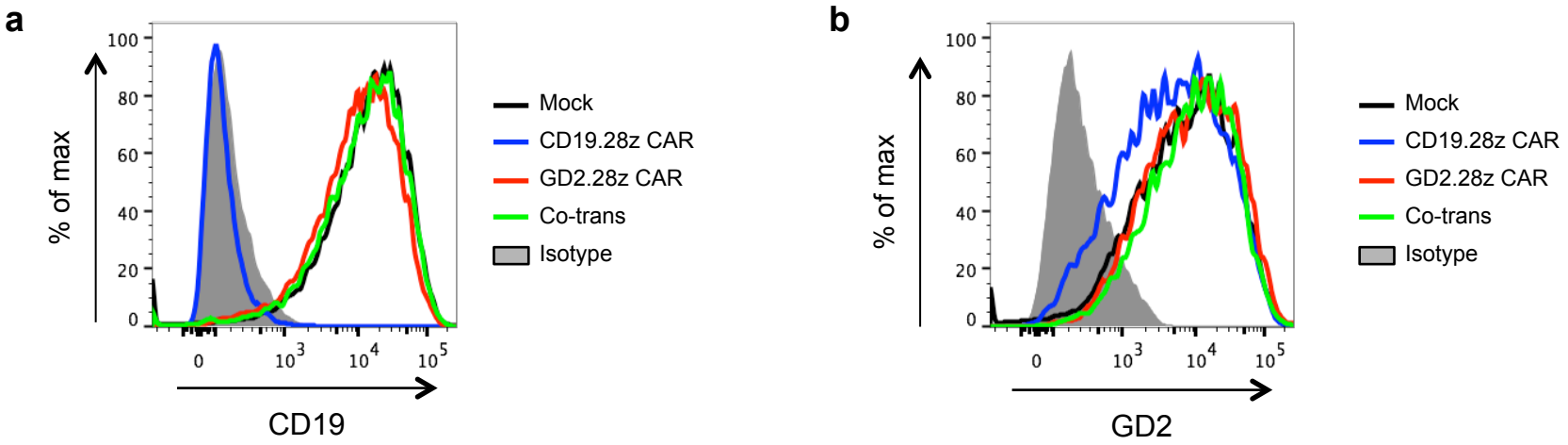


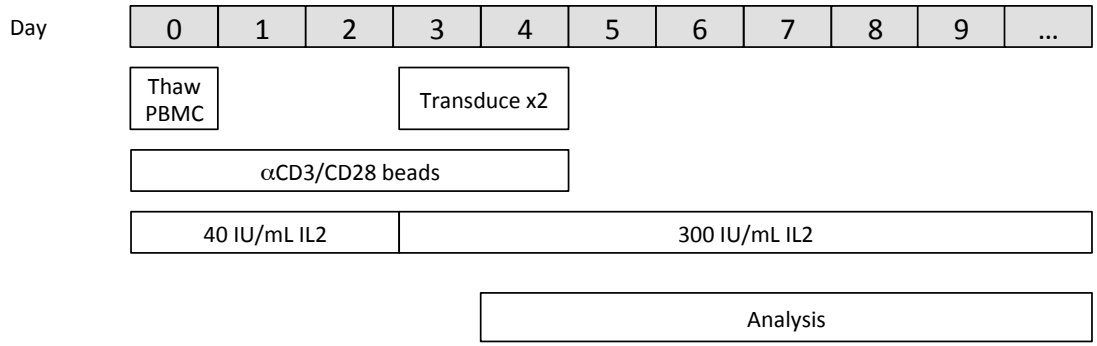
Supplementary Figure 1: Structure of CAR constructs. (**a**) The structures of the GD2 CARs, CD19 CARs, and GD2 CARs with mutations/ modifications are shown. Components of the original GD2.28z CAR (based on the 14g2a antibody) are designated in pink. Components of the original CD19.28z CAR (based on the FMC63 antibody) are designated in blue. Signaling domains are designated in grey. Domains incorporating point mutations are noted. (**b**) Structure of ErbB2 and CD22 CAR (HA22 and m971) constructs. The CD22-HA22 CAR incorporates the IgG_1 CH_2CH_3 spacer domain found within the GD2 CAR, while the CD22-m971 and ErbB2 CARs do not. CD28z versions were generated for the CD22-HA22 CAR and the ErbB2 CAR, while both CD28z and BBz versions were generated for the CD22-m971 scFv.



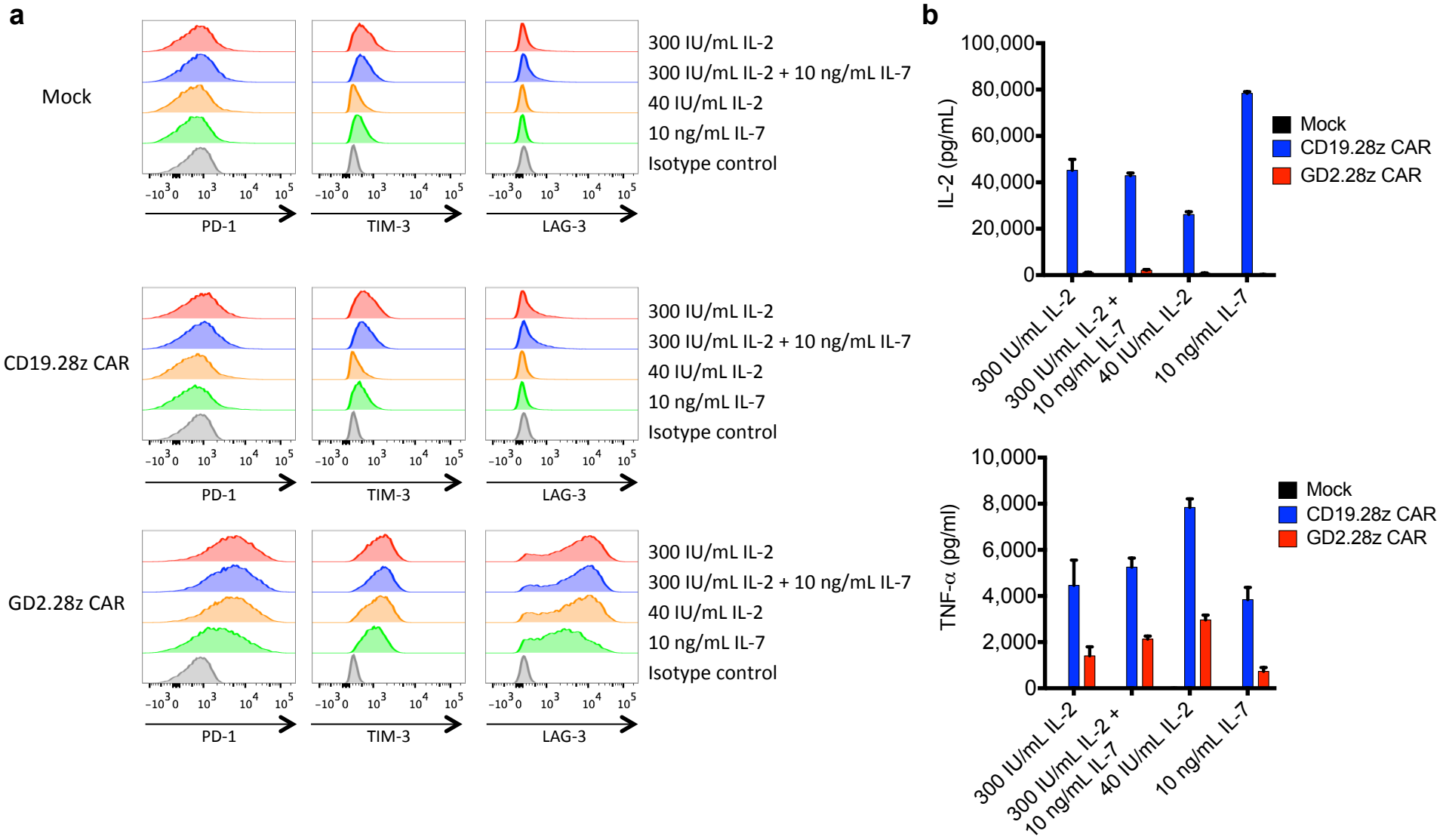
Supplementary Figure 2: GD2.28z and CD19.28z CAR T cells have comparable efficacy *in vitro* but different efficacies *in vivo*. (**a**) *In vitro* ^{51}chromium release assay of GD2.28z CAR T cells against the GD2+ 143B osteosarcoma cell line. Assay performed 9 days after initial activation. (**b**) Tumor growth curves of NSG mice inoculated with 5 x 10^6 143B periosteally on day 0, followed by adoptive transfer of 10^7 mock or transduced GD2.28z CAR T cells on day 3. n=8 mice/group. (**c**) Tumor growth curves of NSG mice inoculated with 10^6 143B periosteally on day 0, followed by adoptive transfer of 10^7 mock or transduced GD2.sh.28z CAR (no CH_2CH_3 spacer) T cells on day 3. n=5 mice/group. (**d**) *In vitro* ^{51}chromium release assay of CD19.28z CAR T cells against the CD19+ NALM6-GL leukemia cell line. Assay performed 9 days after initial activation. (**e**) Bioluminescence images from mice inoculated with 5 x 10^5 NALM6-GL on day 0, followed by adoptive transfer of 10^7 CD19.28z CAR T cells on day 3. * = $p<0.05$ by Student's T-test.



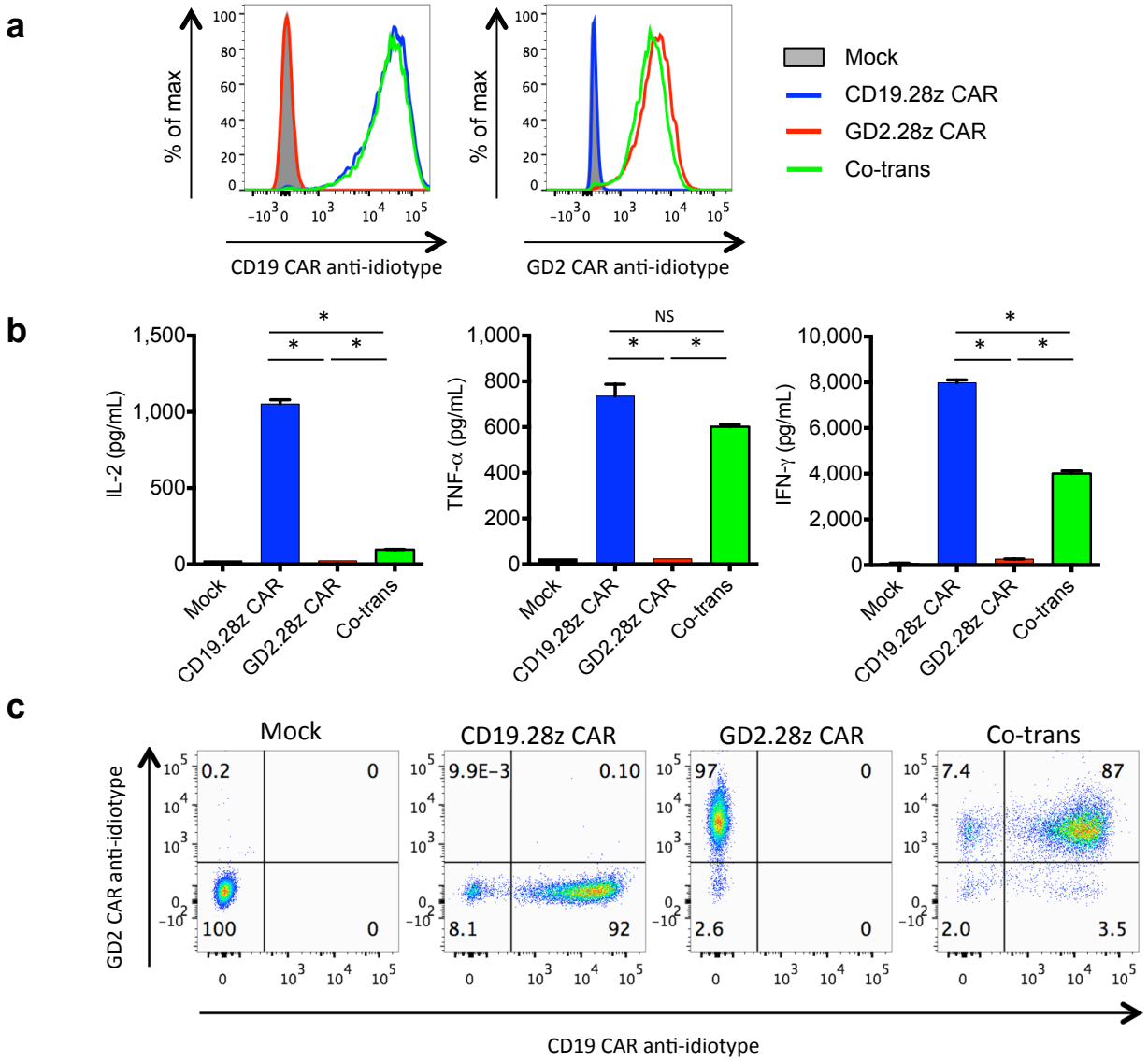
Supplementary Figure 3: Tumors that outgrow in CD19.28z CAR T cell treated mice lose CD19 expression. NSG mice were inoculated with 10^6 143B-CD19 on day 0, followed by adoptive transfer of 10^7 mock-transduced, CD19.28z CAR, GD2.28z CAR, or co-transduced (Co-trans; expressing both CD19.28z and GD2.28z CARs) T cells on day 14. Tumors were harvested on day 14 following T cell transfer. Homogenized tumor cells were analyzed by flow cytometry. Human tumor cells were distinguished from mouse stromal cells and adoptively transferred human T cells by HLA-ABC+CD45-. (**a**) CD19 expression and (**b**) GD2 expression on tumor outgrowths.



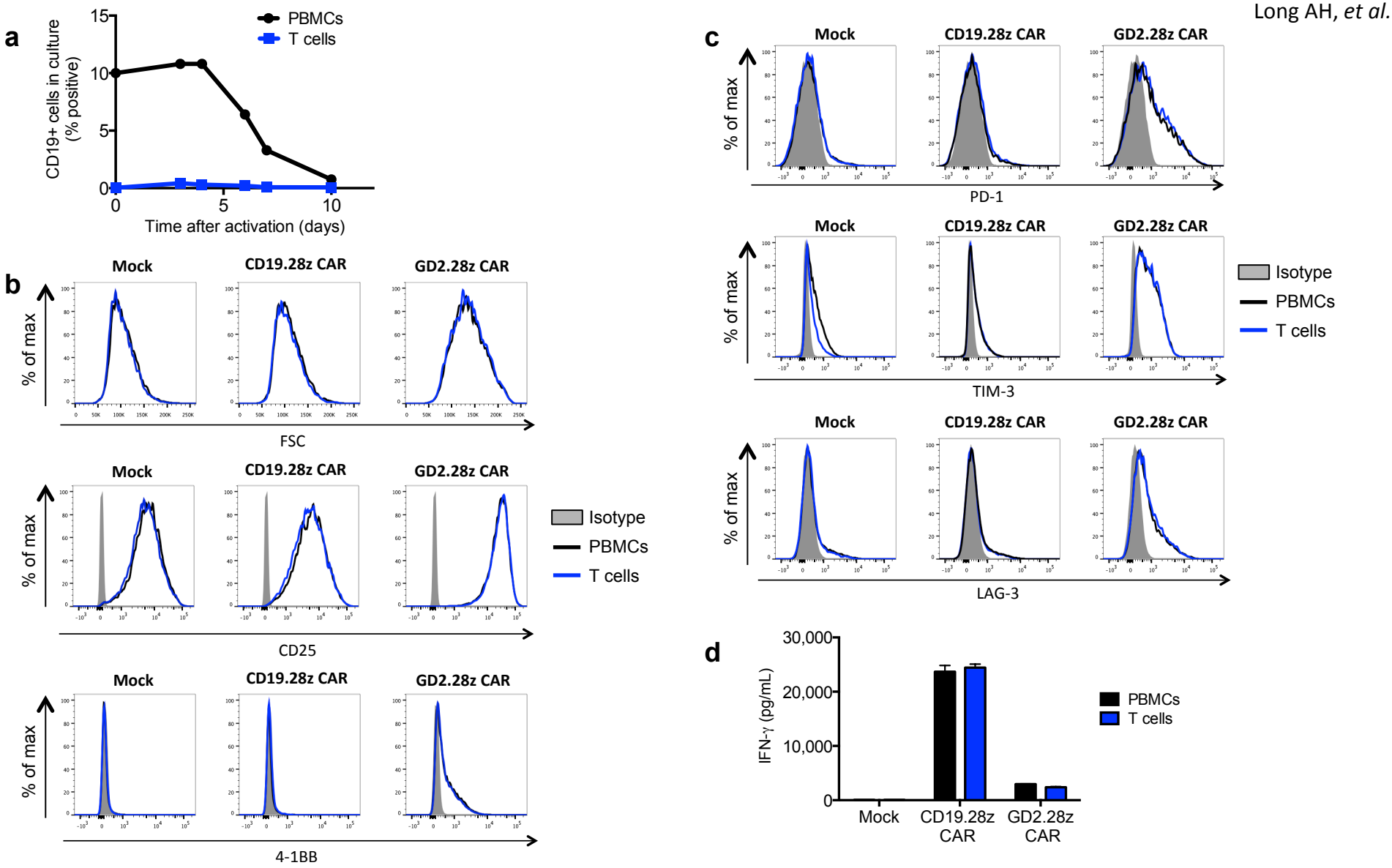
Supplementary Figure 4: CAR T cell activation protocol. PBMCs from healthy donors were activated with anti-CD3/CD28 beads (3:1 bead to cell ratio) and cultured in 40 IU/mL IL-2 for three days. On days 3 and 4 post activation, T cells were transduced with CAR retroviral supernatants and cultured in 300 IU/mL IL-2. On day 5, anti-CD3/CD28 beads were removed. T cells were replated at 3 x 10^5 / mL with fresh IL-2 every two days.



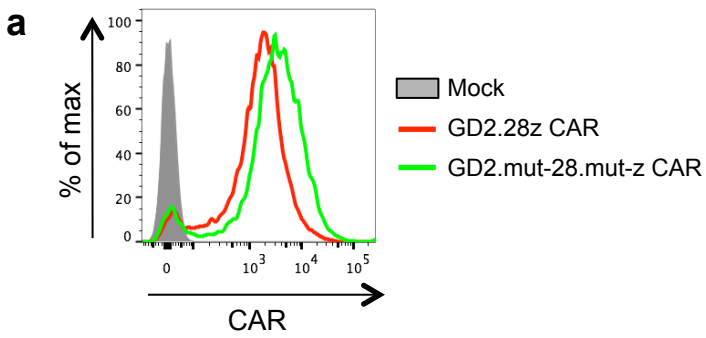
Supplementary Figure 5: Impact of IL-2 and IL-7 on exhausted phenotype of GD2.28z CAR T cells. Mock, CD19.28z or GD2.28z CAR T cells were cultured in different concentrations of IL-2 and IL-7. (**a**) Exhaustion marker expression was evaluated at day 9 following initial activation. Cells were cultured in 40 IU/mL IL-2 during activation and switched to 300 IU/mL IL-2 at transduction (red; original protocol), 40 IU/mL IL-2 during activation and switched to 300 IU/mL IL-2 + 10 ng/mL IL-7 (blue), 40 IU/mL IL-2 throughout *in vitro* culture (orange) or 10 ng/mL IL-7 throughout *in vitro* culture (green). (**b**) Cytokine production following co-incubation with 143B-CD19 for 24 hours beginning day 9 of *ex vivo* culture.



Supplementary Figure 6: Expression of the GD2.28z CAR has a dominant inhibitory effect on CAR T cell function. (**a**) CAR expression of post-flow sorted CAR T cells, demonstrating equivalent MFI of CAR receptor expression on co-transduced (Co-trans) vs. single transduced (CD19.28z CAR and GD2.28z CAR) T cells. (**b**) Cytokine production of flow-sorted single-transduced and co-transduced CAR T cells, co-incubated with NALM6-GL (CD19+GD2-) for 24 hours, beginning on day 9 of *ex vivo* culture. n=3 replicates/group. T cells with media: <5 pg/mL IL-2, TNF-α and IFN-γ. (**c**) Purity of CAR expression in co-transduced and single transduced CAR T cells used in *in vivo* experiments to treat 143B-CD19 bearing NSG mice. * = $p<0.05$ by Student's T-test.

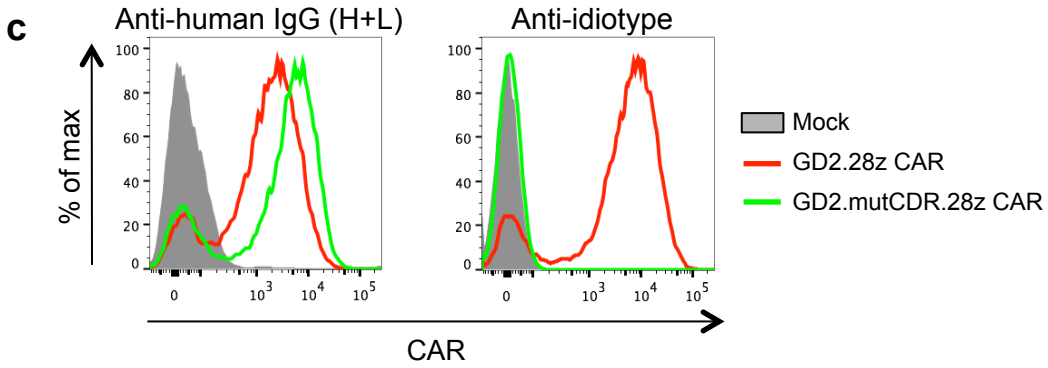


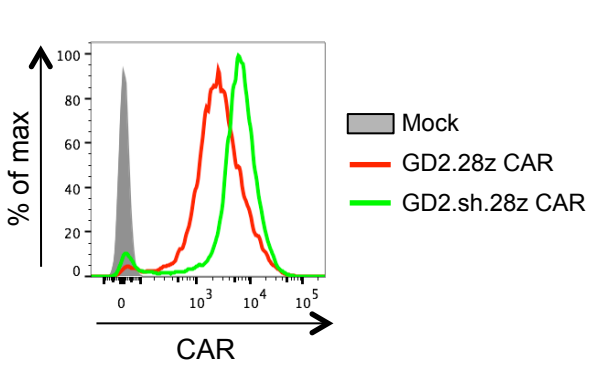
Supplementary Figure 7: Removing CD19+ cells from culture system does not affect exhausted phenotype of CAR T cells. CAR T cells were generated from monocyte depleted PBMCs (original protocol) or with T cells isolated from PBMCs via negative magnetic selection. (**a**) Flow cytometric quantification of CD19+ cells in the culture system, demonstrating that T cell selection on day 0 led to <0.5% of CD19+ cells in the culture system throughout *ex vivo* expansion. (**b**) Activation and (**c**) exhaustion marker expression of CAR T cells generated from PBMCs or isolated T cells. (**d**) IFN-γ production of CAR T cells generated from PBMCs or isolated T cells, co-incubated with 143B-CD19 (CD19+GD2+) for 24 hours beginning on day 9 of *ex vivo* culture.

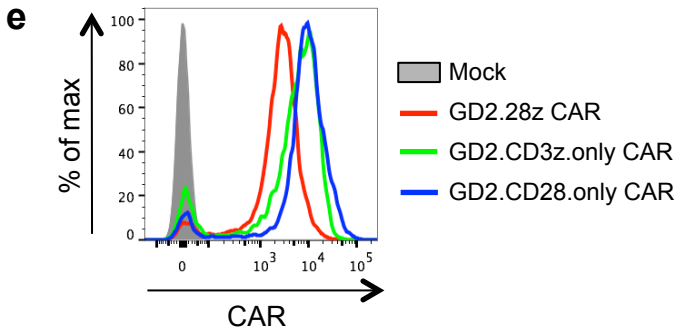


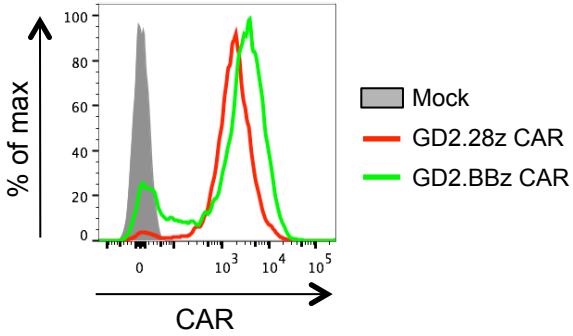
b

	Light Chain	Heavy Chain
CDR1	SLVGAHRNGNTY	GSSFTGYN
CDR2	KVS	IDPYYGGT
CDR3	SQSTHVPPLT	VSGMEY

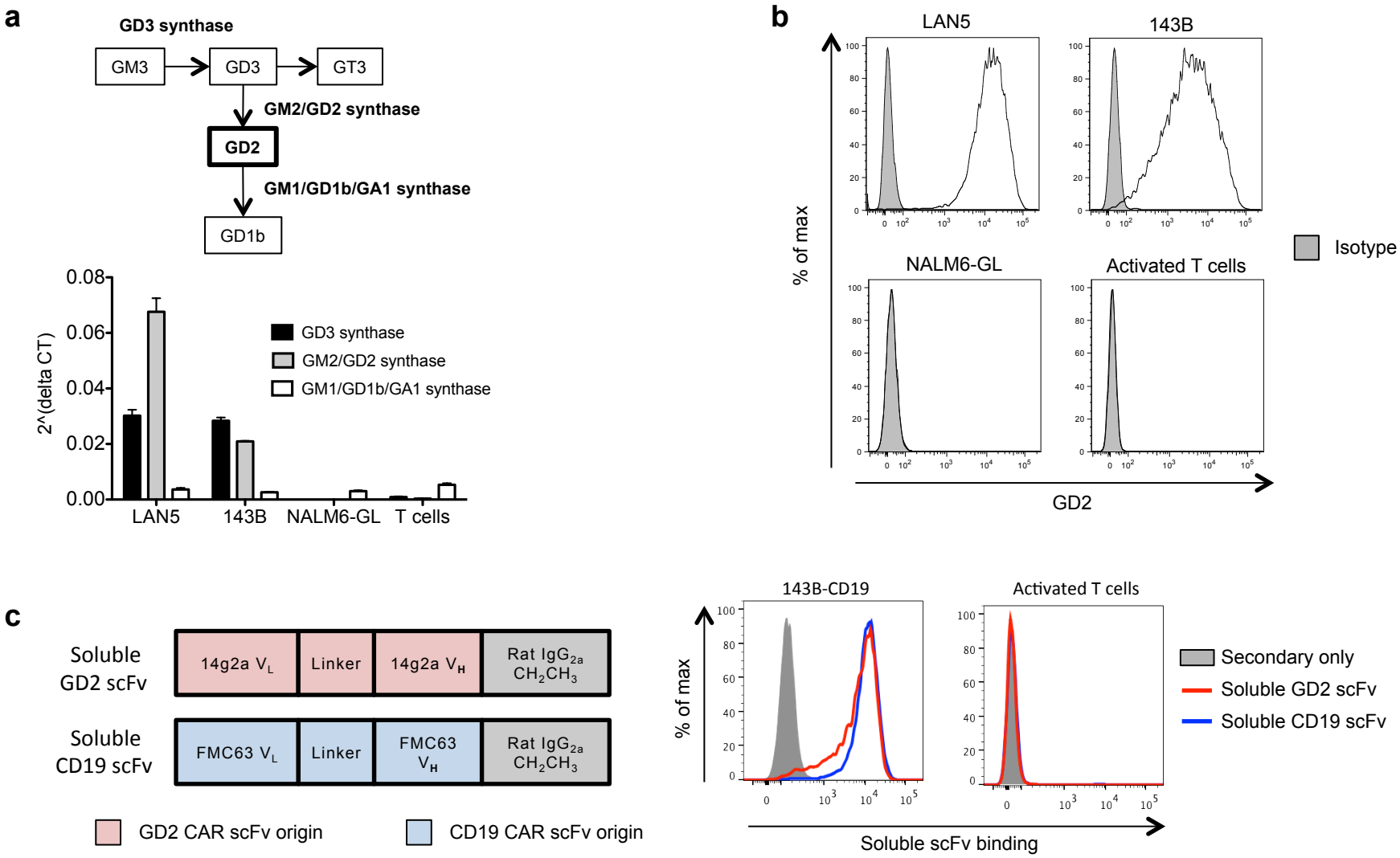




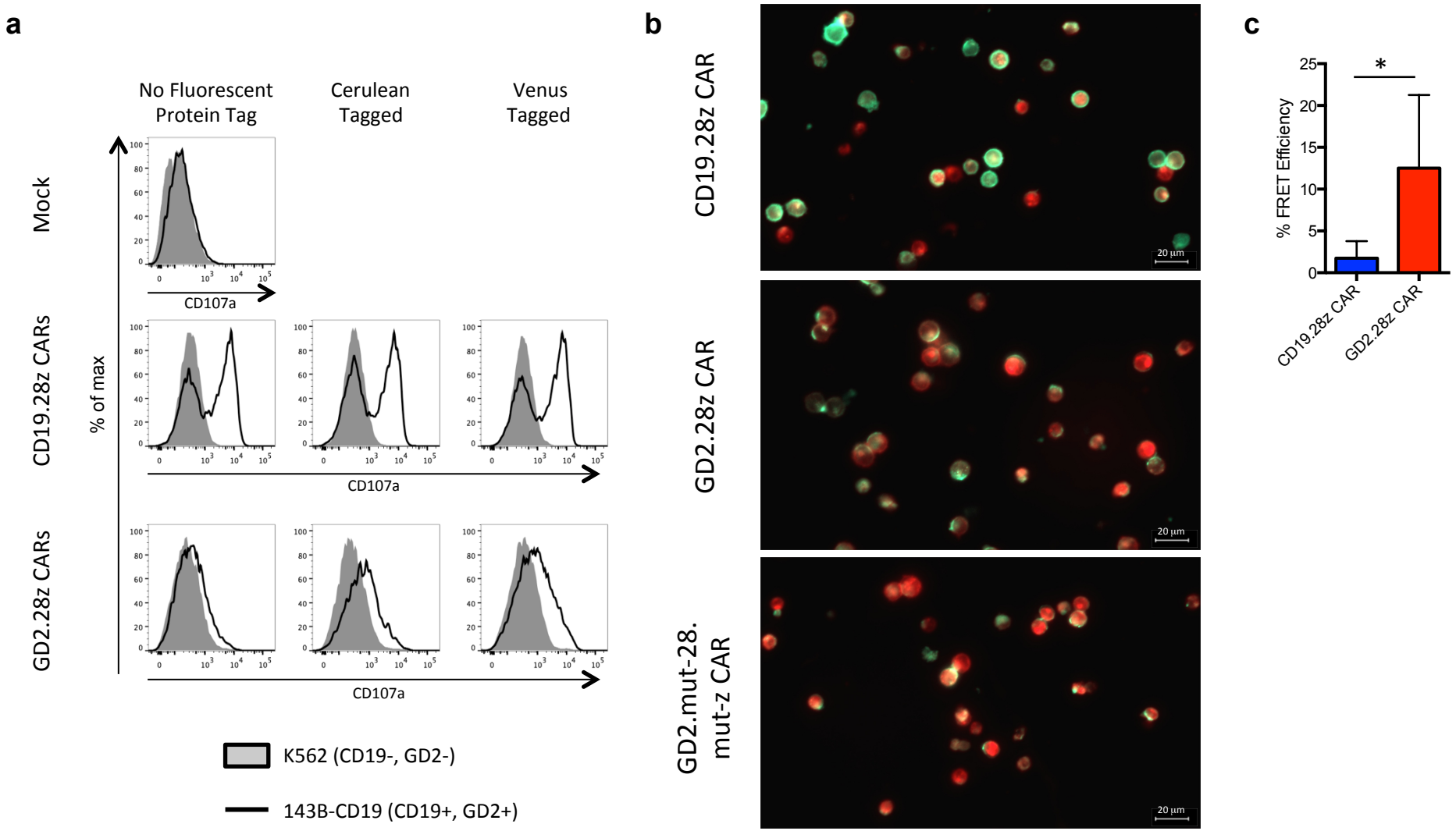




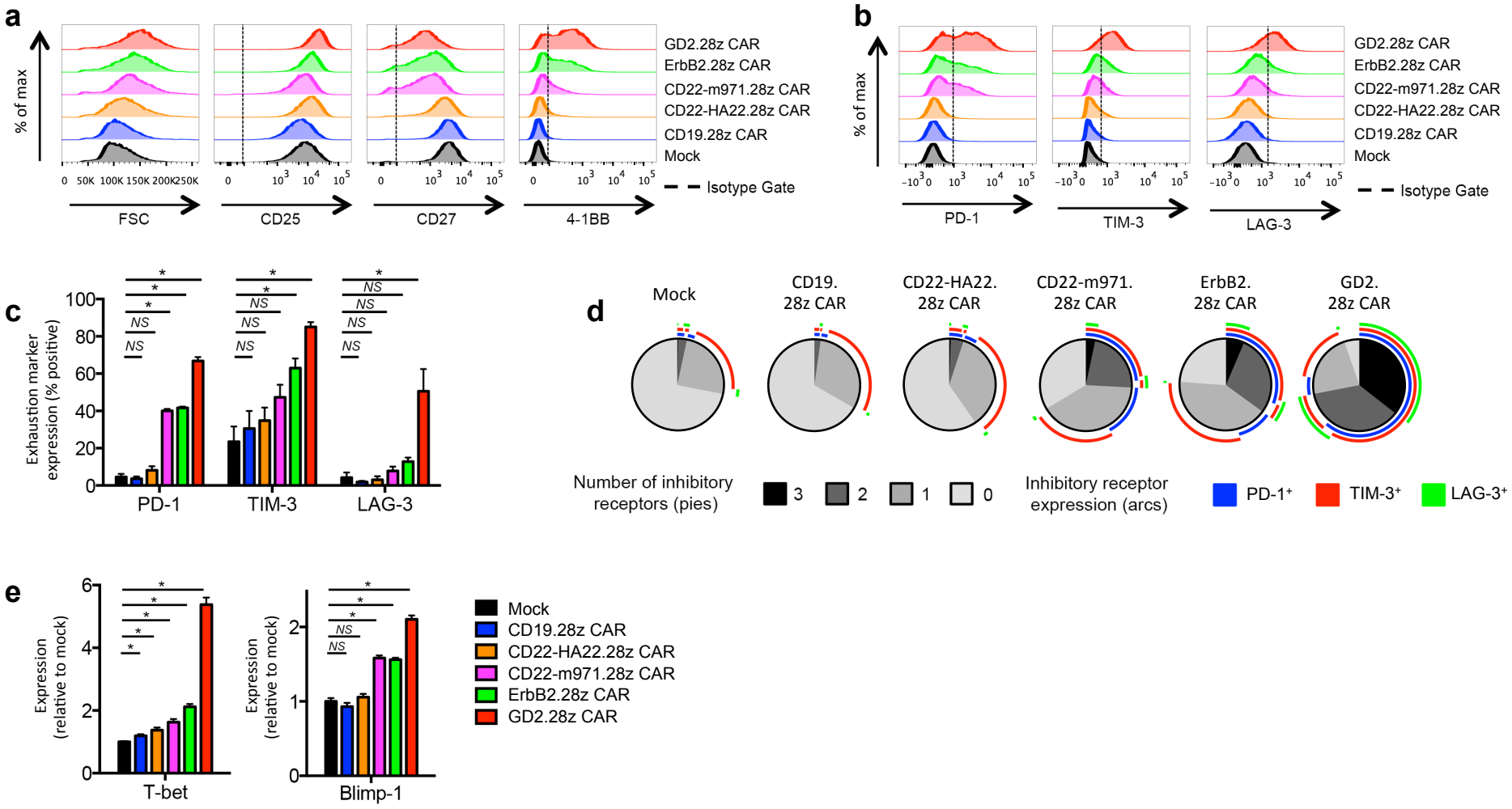
Supplementary Figure 8: GD2 CAR mutants. (**a**) Surface expression of GD2.mut-28.mut-z CAR (GD2.28z CAR with point mutations introduced in the signaling domains). Assessed by anti-idiotype staining. (**b**) Point mutations introduced into the GD2.28z CAR antigen binding domain to produce the GD2.mutCDR.28z CAR. The complementary determining regions (CDR) of the GD2.28z CAR scFv were identified using the iMGT V-QUEST tool[57]. Five residues with charged or large side chains were identified and mutated to alanines (red), and two amino acids (GA) were introduced into the CDR1 region of the light chain (underlined). (**c**) Surface expression the GD2.mutCDR.28z CAR. Assessed by anti-human H+L antibody, left. Ability to bind anti-idiotype antibody also assessed, right. (**d**) Surface expression of the GD2.sh.28z CAR (GD2.28z CAR with removed IgG_1 CH_2CH_3 spacer and changed scFv linker). Assessed by anti-idiotype staining. (**e-f**) Surface expression of GD2 CARs with signaling domain manipulations to include only CD3-ζ (GD2.CD3z.only CAR), only CD28 (GD2.CD28.only CAR), or the 4-1BB costimulatory domain (GD2.BBz CAR). Assessed by anti-idiotype staining.



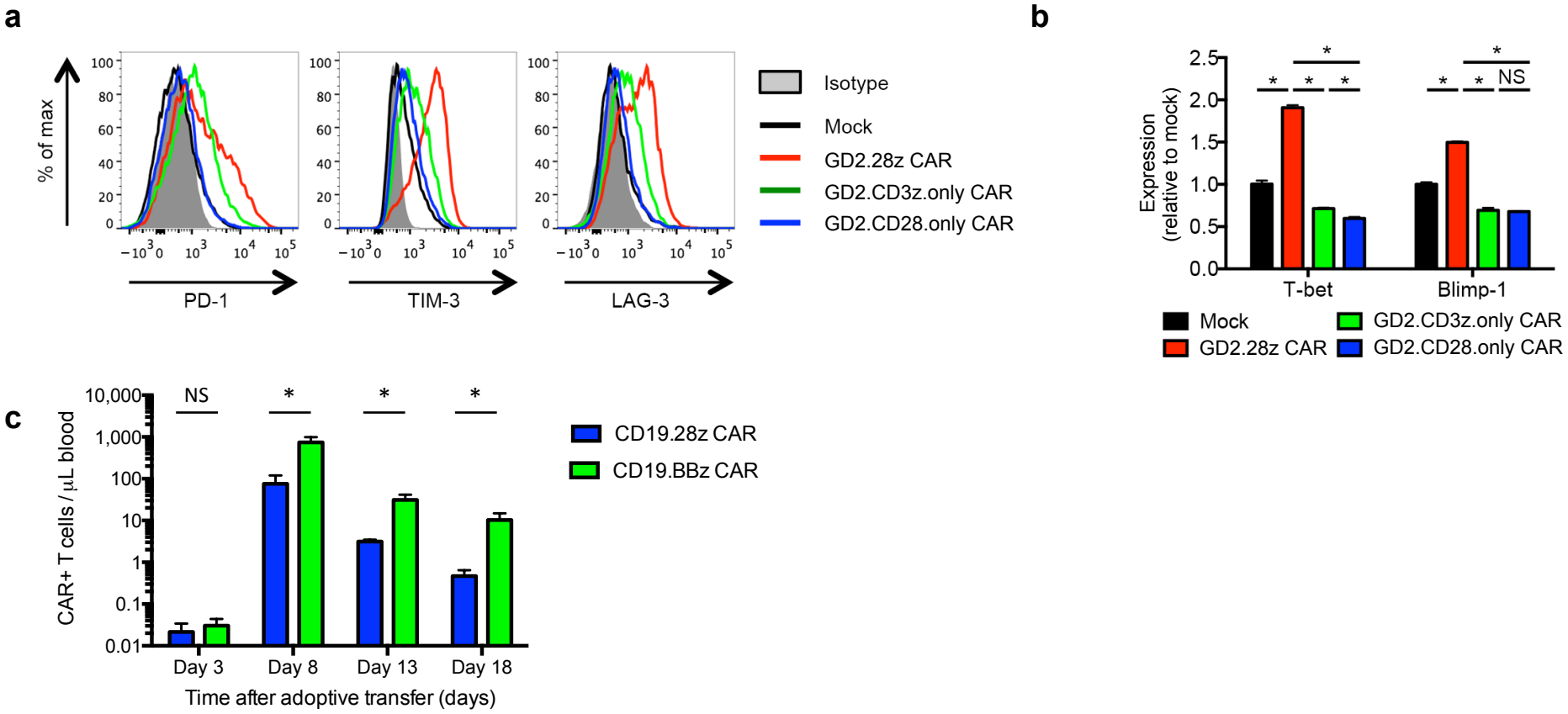
Supplementary Figure 9: Activated T cells do not express GD2. (**a**) qRT-PCR expression of enzymes involved in GD2 synthesis (GD3 synthase and GM2/GD2 synthase) and degradation (GM1/GD1b/GA1 synthase). (**b**) Representative flow expression of GD2 on tumors and activated T cells (7 days post activation). (**c**) Soluble versions of the GD2 and CD19 scFvs were synthesized to evaluate potential binding to cross-reactive antigens on activated T cells. The scFv regions from the GD2 and CD19 CARs were linked to the CH_2CH_3 domain of rat IgG_{2a} to facilitate detection. 143B-CD19 tumor cells or activated T cells were incubated in the presence of soluble scFvs and then stained with an anti-rat IgG $F(ab')_2$ secondary.



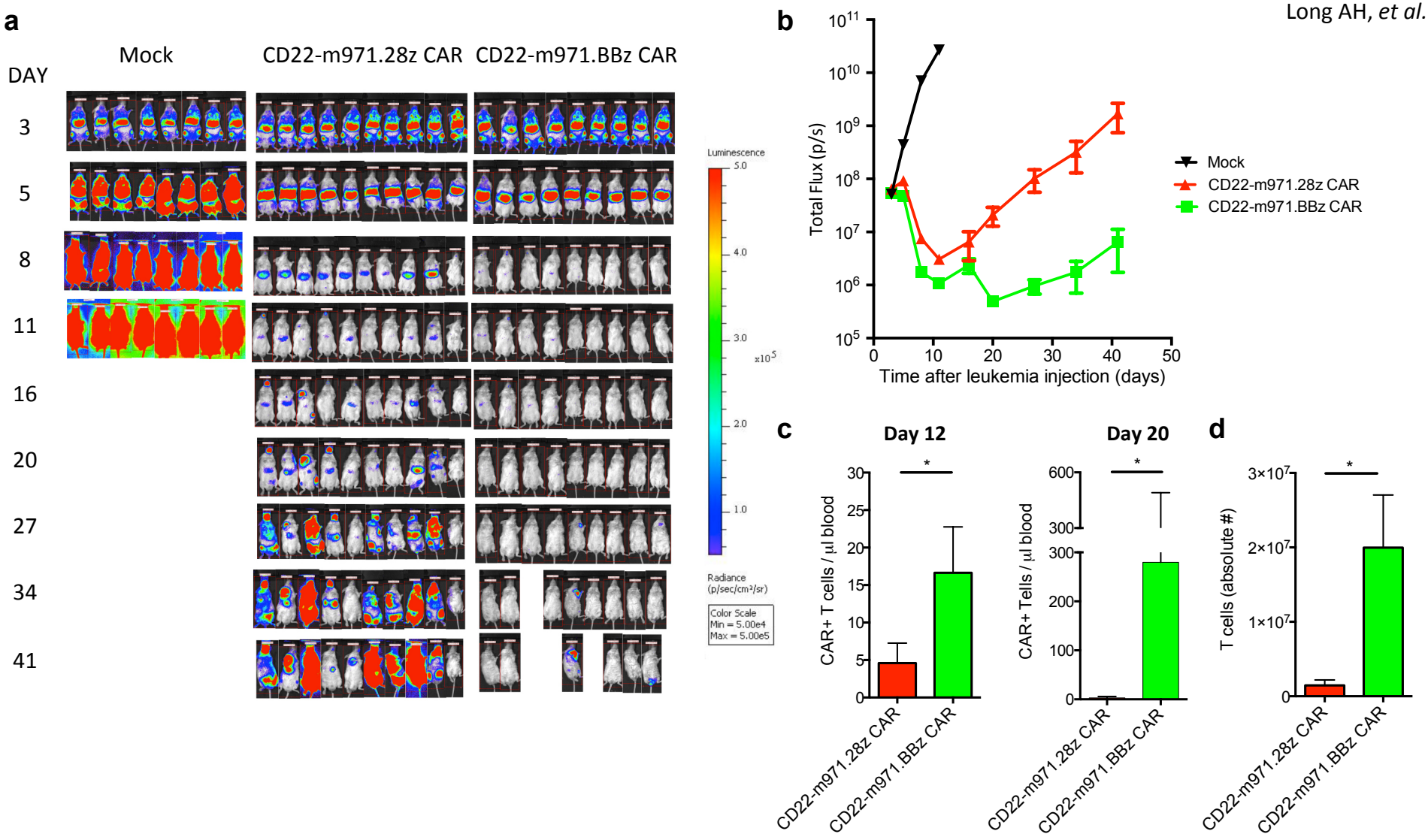
Supplementary Figure 10: Characterization of CAR-fluorescent protein fusions. (**a**) CAR-fluorescent protein fusions retain their functionality. T cells expressing either the untagged of fluorescent protein tagged CARs were co-incubated with 143B-CD19 tumor and degranulation evaluated by CD107a expression. Degranulation was assessed on day 9 of *ex vivo* expansion. (**b**) Fluorescent microscopy images of T cells expressing CAR-Cerulean protein fusions. T cells expressing Cerulean tagged CARs (cyan) at day 9 following initial activation. Cell membranes were stained with DiD lipophilic dye (red). (**c**) FRET signal measured from T cells co-transduced and flow sorted to co-express CAR-Cerulean and CAR-Venus fusion proteins. * = $p<0.05$ by Student's T-test.



Supplementary Figure 11: Tonic activation and early exhaustion across a spectrum of CARs. (**a**) Activation marker (day 7) and (**b**) exhaustion marker (day 9) expression on T cells expressing an ErbB2.28z CAR (4D5 scFv) or a CD22.28z CAR (m971 scFv derived or HA22 scFv derived) compared to untransduced-mock, CD19.28z CAR or GD2.28z CAR T cells. Dotted line represents +/- gate defined by isotype controls. Representative of 3 donors. (**c**) Quantification of exhaustion marker expression in (b) pooled from 3 unique donors, 9 days following initial activation. (**d**) SPICE[55] analysis of exhaustion markers expression in (c). (**e**) ΔΔCT q-RT-PCR expression levels of transcription factors associated with T cell exhaustion, calculated relative to mock-transduced T cell expression levels, 9 days following initial activation. n=3 technical replicates; representative of 3 donors. * = $p<0.05$ by Student's T-test.

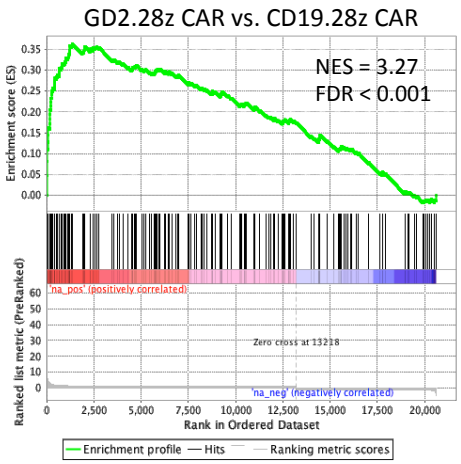


Supplementary Figure 12: Incorporation of CD28 endodomain augments exhaustion whereas 4-1BB endodomain ameliorates exhaustion of CAR T cells. (**a**) Exhaustion marker and (**b**) exhaustion-associated transcription factor expression in GD2 CAR T cells with isolated signaling components (either CD28 signaling only or CD3-ζ signaling only). ΔΔCT q-RT-PCR expression levels of transcription factors associated with T cell exhaustion were calculated relative to mock-transduced T cell expression levels, 9-11 days following initial activation. n=3 technical replicates; representative of 3 donors. (**c**) Quantification of T cells within the blood of NSG mice inoculated with 10^7 NALM6-GL on day 0, followed by adoptive transfer of 5 x 10^6 CAR T cells on day 7. Blood T cells were quantified on day 3-18 following adoptive transfer.



Supplementary Figure 13: 4-1BB costimulatory domain enhances *in vivo* efficacy and persistence of CD22-m971 CAR T cells. (**a**) Bioluminescence images from NSG mice inoculated with 10^6 NALM6-GL on day 0, followed by adoptive transfer of 1.5 x 10^7 mock, CD22-m971.28z or CD22-m971.BBz CAR T cells on day 3. (**b**) Quantification of bioluminescent signal intensity over time from (a) representing tumor burden. (**c**) Quantification of T cells within the blood of mice from (a) on day 12 and 20 following adoptive transfer. n=10 mice/group. (**d**) Quantification of T cells within the spleens of mice from (a) on day 24 following adoptive transfer. n=10 mice/group. * = p<0.05 by Student's T-test.

a



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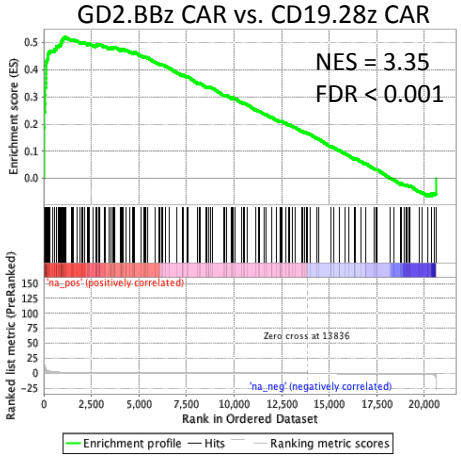

c

Name	NES	p value	FDR	Description
ELVIDGE_HYPOXIA_UP	6.49	0.00	0.00	Upregulated by hypoxia in breast cancer cell line
ELVIDGE_HYPOXIA_BY_DMOG_UP	6.14	0.00	0.00	Upregulated by exposure to hypoxia mimetic DMOG in breast cancer line
ELVIDGE_HIF1A_AND_HIF2A_TARGETS_DN	6.03	0.00	0.00	Downregulated following siRNA knockdown of HIF1a and HIF2a in breast cancer cell line
ELVIDGE_HIF1A_TARGETS_DN	5.86	0.00	0.00	Down regulated following siRNA knockdown of HIF1a in breast cancer cell line
MENSE_HYPOXIA_UP	5.73	0.00	0.00	Upregulated by hypoxia in HeLa cells and astrocytes
MANALO_HYPOXIA_UP	4.77	0.00	0.00	Upregulated by hypoxia and constitutively active HIF1a in vascular endothelium
GROSS_HYPOXIA_VIA_ELK3_DN	4.58	0.00	0.00	Downregulated upon knockdown of ELK3 in skin endothelium cells at hypoxia
QI_HYPOXIA	4.58	0.00	0.00	Upregulated by hypoxia in prostate cancer line
WINTER_HYPOXIA_METAGENE	4.48	0.00	0.00	Regulated by hypoxia, as reported in literature metagene analysis
FARDIN_HYPOXIA_11	4.35	0.00	0.00	Regulated by hypoxia in neuroblastoma cell lines

Supplementary Figure 14: GD2 CAR T cells have altered transcriptional profile consistent with exhaustion. Gene set enrichment analysis (GSEA) was performed using the unfiltered rank list comparing (**a**) GD2.28z CAR vs CD19.28z CAR T cells, or (**b**) GD2.BBz CAR vs. CD19.28z CAR T cells. Analyses were performed against previously reported sets of genes upregulated in exhausted vs. effector T cells (down regulated in effector vs. exhausted T cells; GSE9650_EFFECTOR_VS_EXHAUSTED_CD8_TCELL_DN) in the murine chronic LCMV model of exhaustion[42]. (**c**) Top 10 enriched gene sets related to hypoxia response identified following GSEA of unfiltered GD2.BBz CAR vs. GD2.28z CAR T cell rank list against the MSigDB C2 curated gene sets.