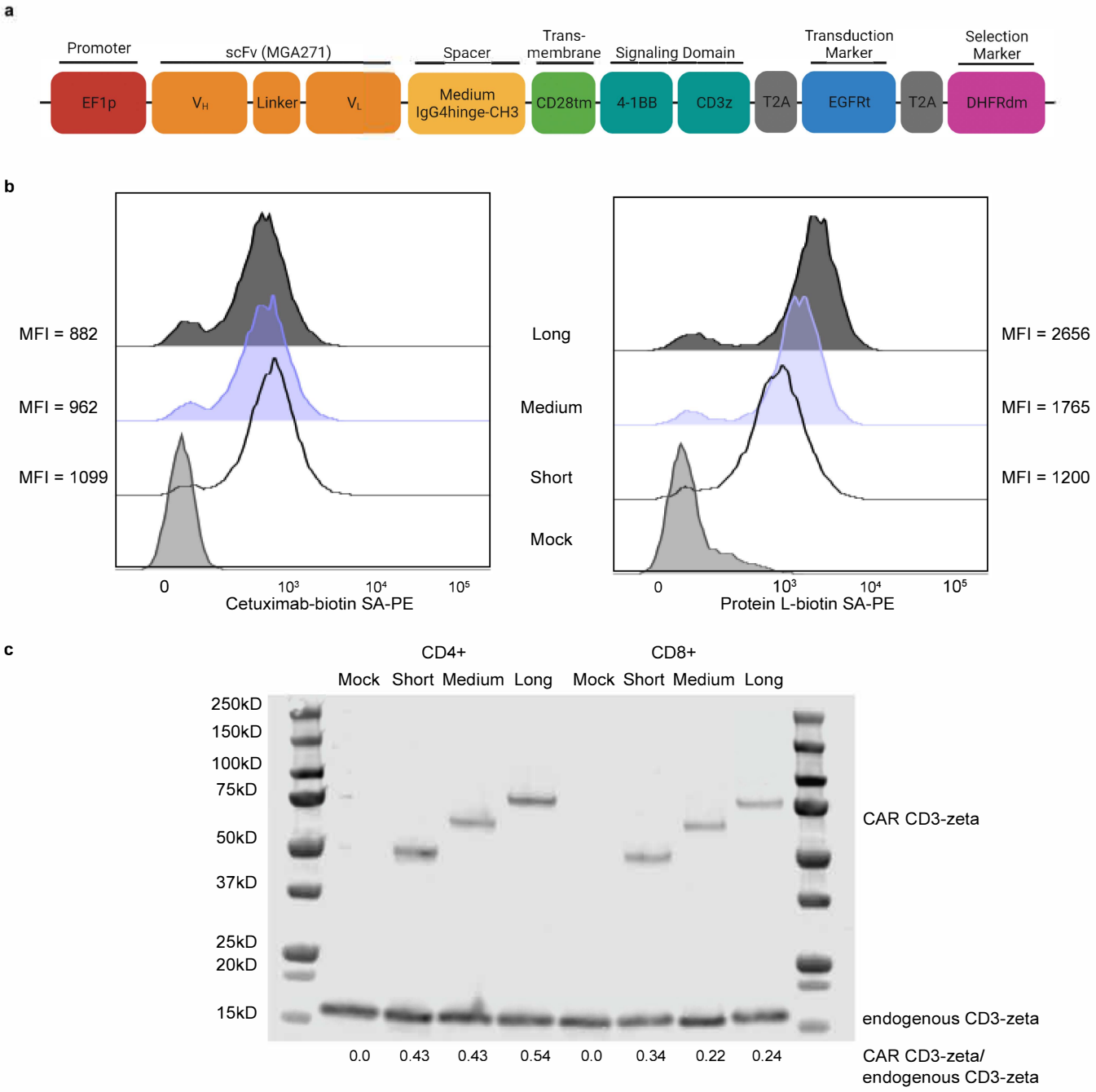
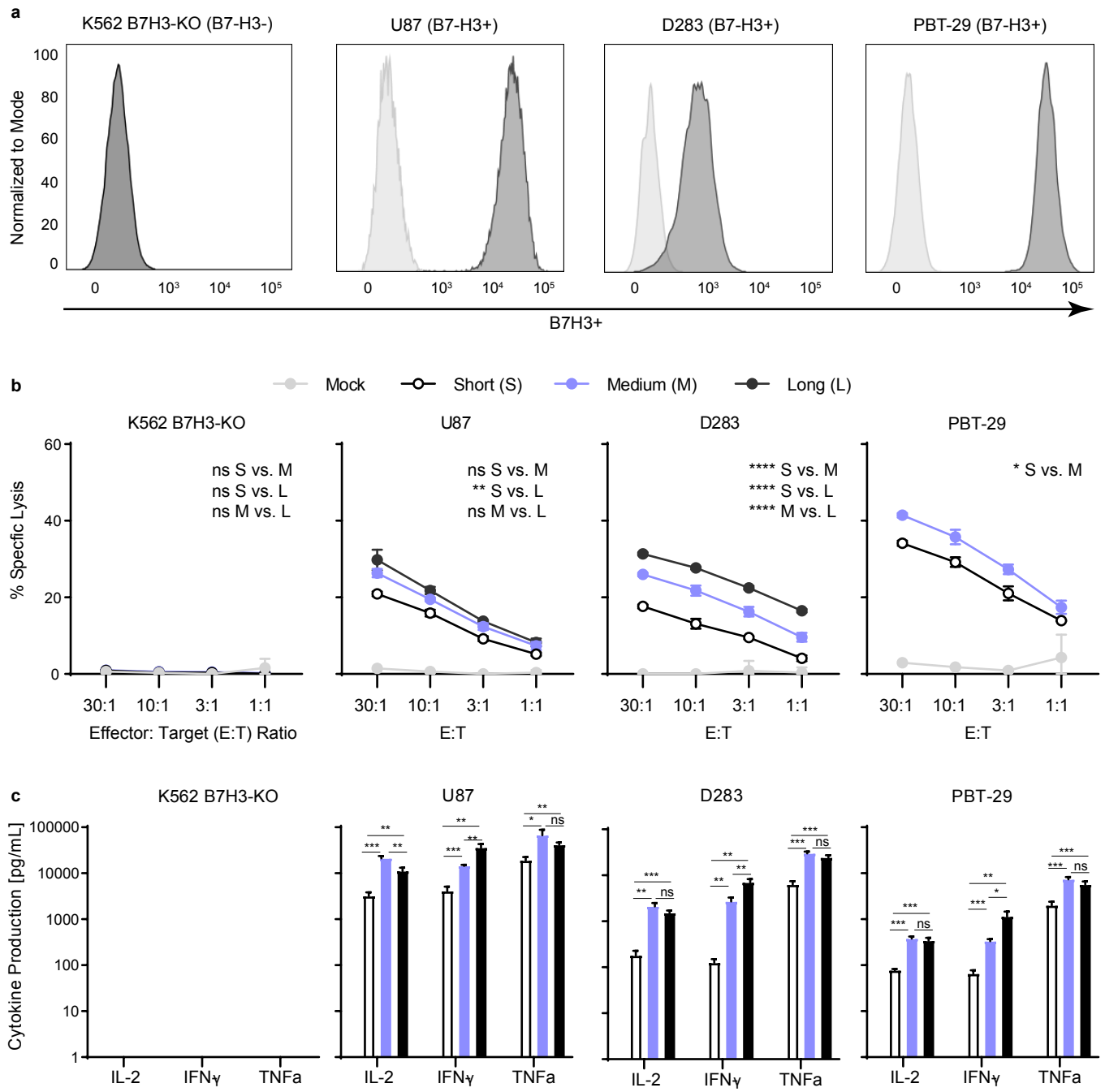
Supplementary Figure 1



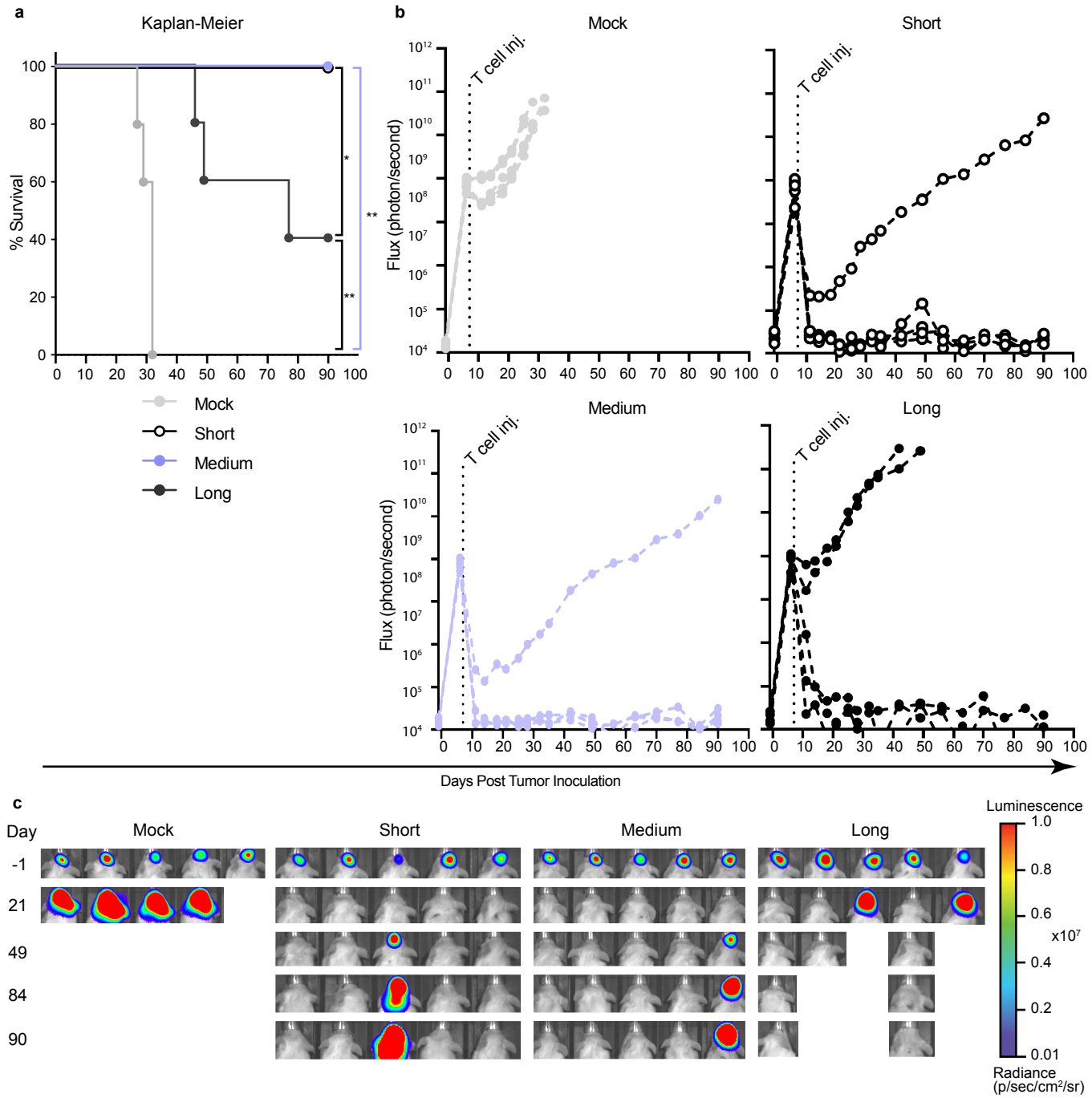
Supplementary Figure 1. B7-H3CAR spacer variants are efficiently expressed in T cells. (a) Schematic of 2nd generation B7-H3CAR. (b) Human CD8+ T cell surface expression of Short (S), Medium (M), and Long (L)-spacer variants of CAR (EGFRt+) cells detected by cetuximab and Protein-L. (c) CAR expression of untransduced Mock and S-, M-, and L-spacer B7-H3CARs detected by CD3-zeta-specific western blot. Band intensity normalized to endogenous CD3-zeta is indicated below each lane. A representative, complete western blot is shown.

Supplementary Figure 2



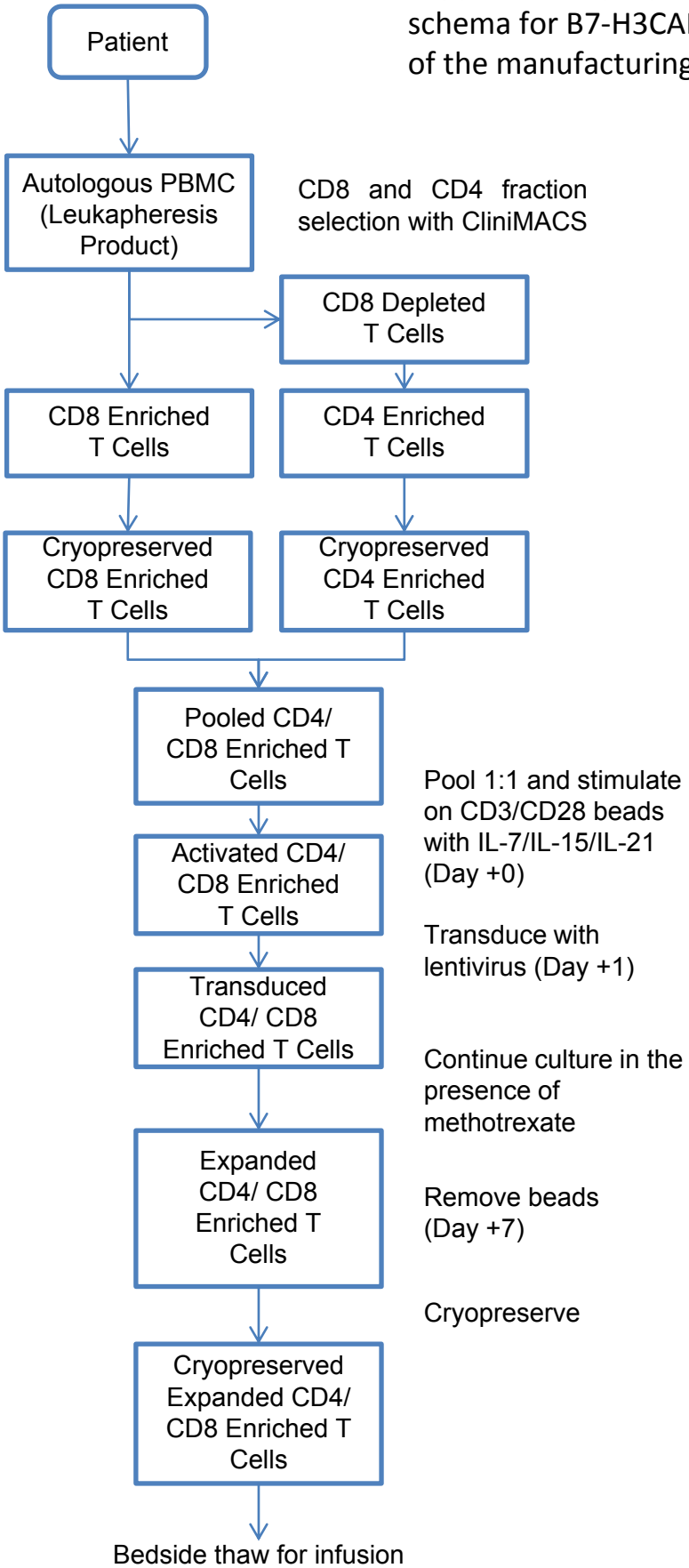
Supplementary Figure 2. B7-H3CARs are efficacious in vitro against B7-H3+ cancer models. (a) Flow analysis of B7-H3+ (U87, D283, PBT-29) and B7-H3- (K562 B7-H3-KO) cell cultures. (b) In vitro cytotoxicity of B7-H3CAR CD8+ T cells against cancer cell cultures. (c) Cytokine release assay following 24-hour co-cultures of B7-H3CAR CD8+ T cells with cancer cell cultures (2:1 ratio). ns = not significant. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. N = 3 technical replicates per condition and data presented as mean values ± SD.

Supplementary Figure 3

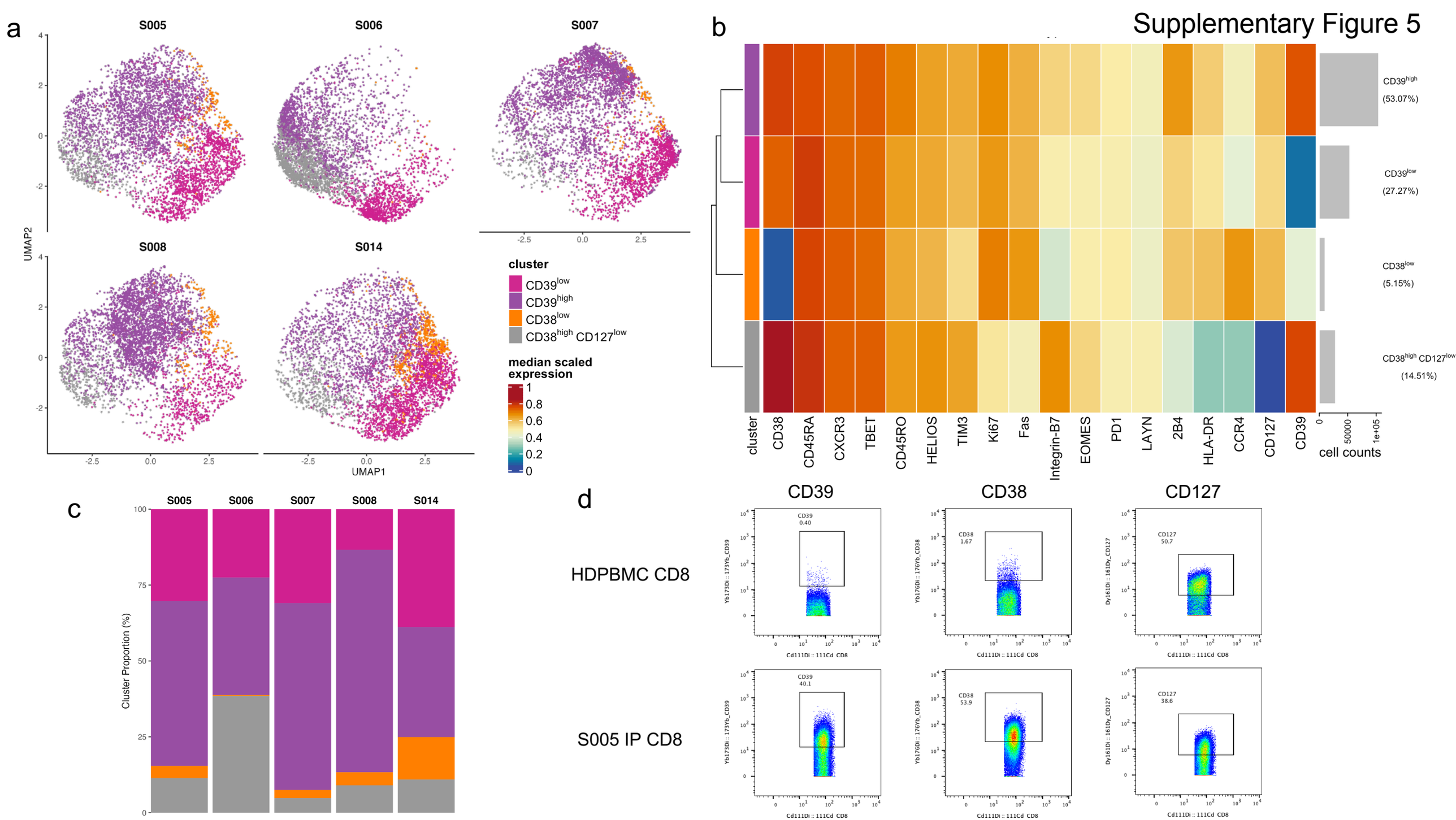


Supplementary Figure 3. B7-H3CARs are efficacious against in vivo B7-H3+ cancer models. Groups of mice were orthotopically inoculated with $2x10^5$ eGFP:ffluc U87 cells (Day 0) and $2x10^6$ B7-H3CAR CD8+ T cells (Day 7, dotted vertical line). (a) Kaplan-Meier analysis of survival. (Medium (M)- and Short (S)- vs. Long (L)-spacer, *p<0.05; S-, M-, and L-spacer vs. Mock, **p<0.005). (b,c) Serial bioluminescence and (c) imaging of tumor-bearing mice treated with Mock, S-, M-, or L-spacer B7-H3CAR CD8+ T cells. Bioluminescence imaging measured in region of interest (head). In vivo data are representative of two independent experiments. Data presented as mean values ± SD. N = 5 animals per group.

Supplementary Figure 4. Manufacturing schema for B7-H3CAR T cells. Flow diagram of the manufacturing process.

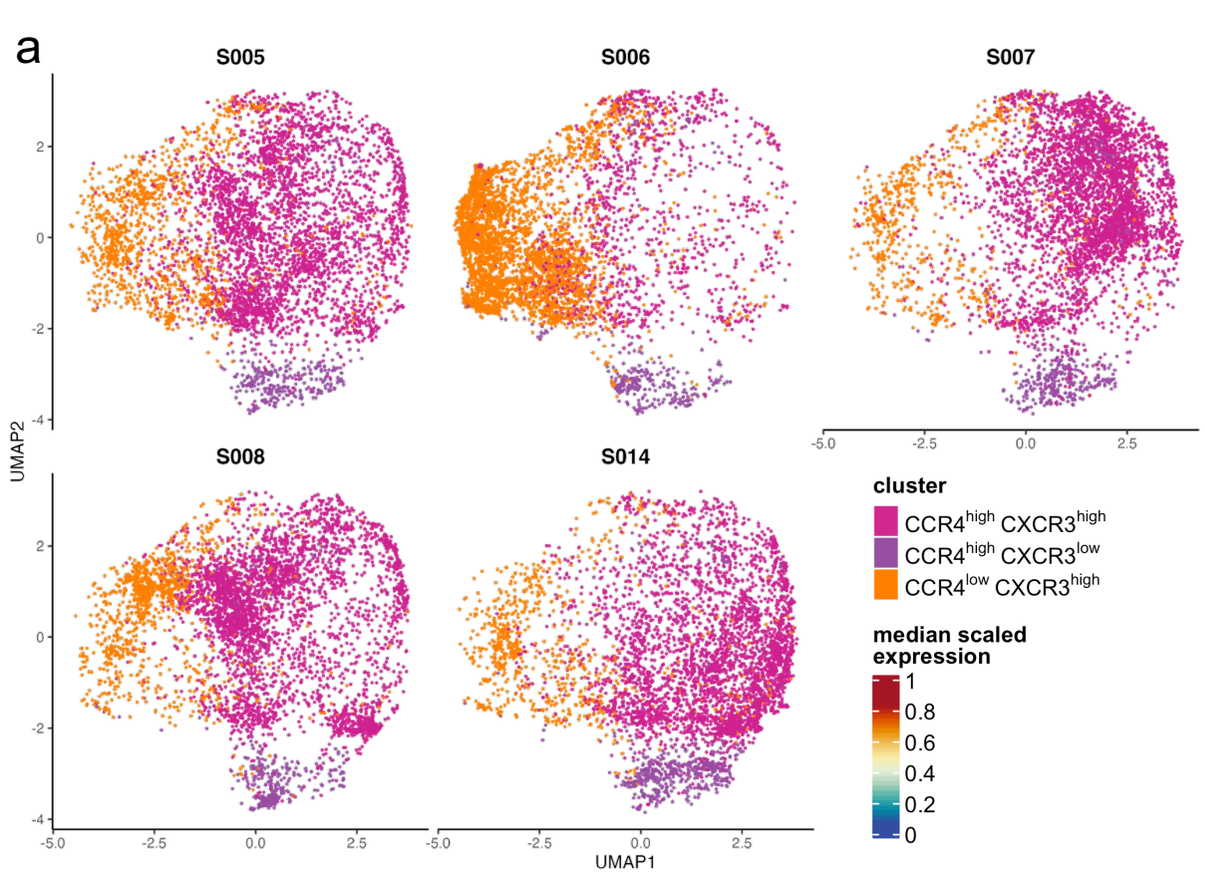


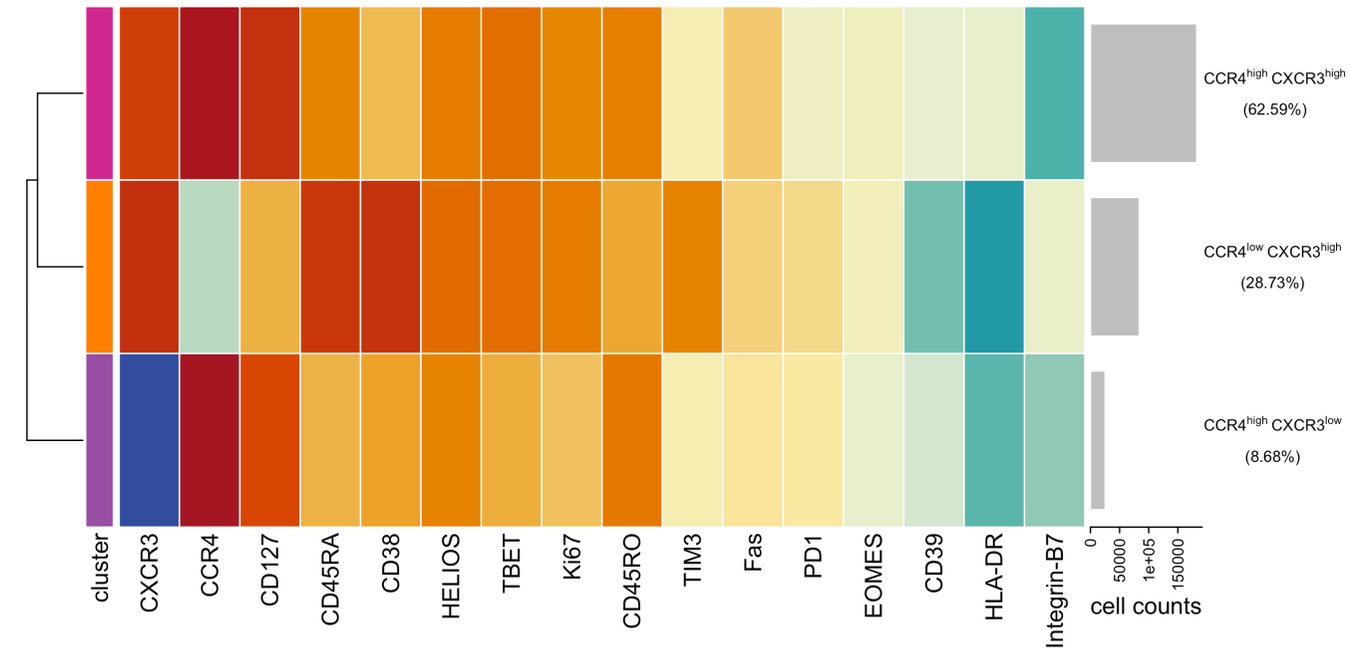
Supplementary Figure 5

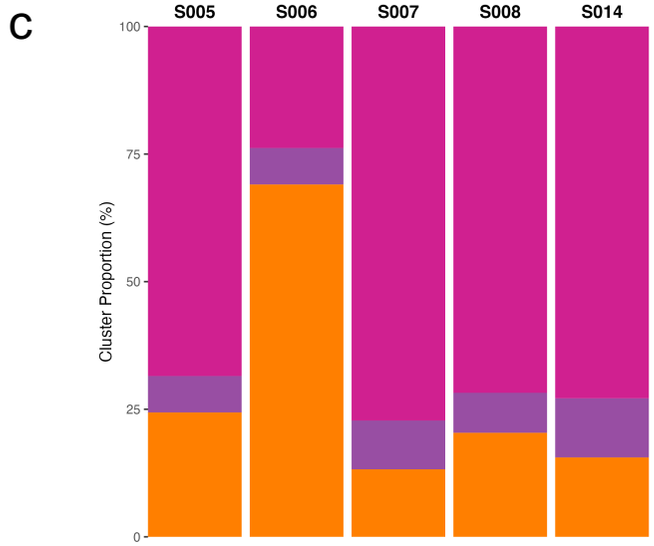


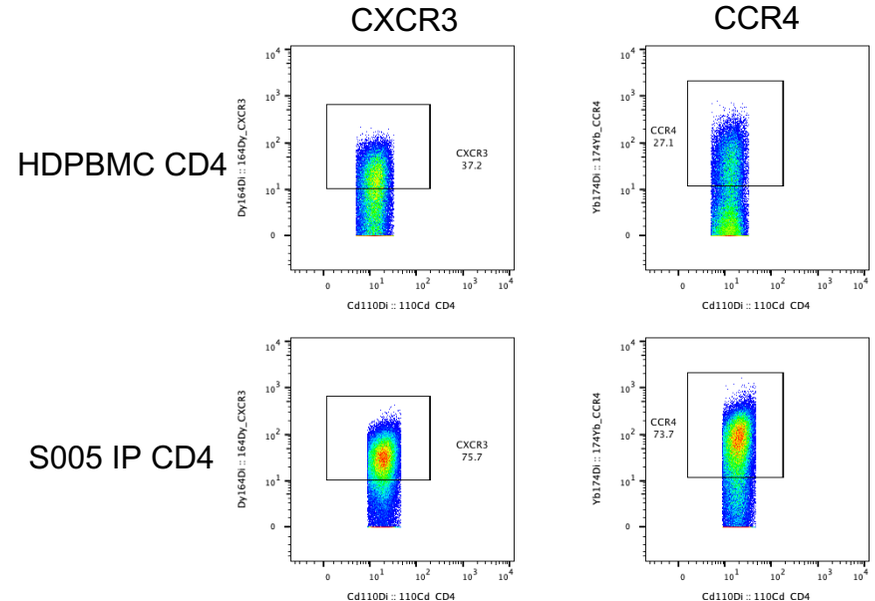
Supplementary Figure 5. Phenotypic analysis of unstimulated CD8+ infusion products by CyTOF. (a) Uniform Manifold Approximation and Projection (UMAP) representations of the cells. 5,000 cells from each product were used in the UMAP analysis. Cells in the UMAPs were colored by their cluster color designation. (b) Heatmap of cell clusters that constitute unstimulated infusion products of all five DIPG patients. Cell intensity expressions were first normalized from 0 to 1 for each marker. The heatmap was then colored using the median of the scaled intensity expressions (between 0 and 1) of each marker. Each row of the heatmap was annotated by the cluster color designation, descriptive cluster name, histogram of cell count, and percentage of cell counts. (c) Stacked bar graph of the cluster proportion within all five unstimulated products. (d) Representative 2-D flow plots of key markers of interest. The 2-D flow plots showcase results from a healthy donor PBMC sample and S005.

Supplementary Figure 6



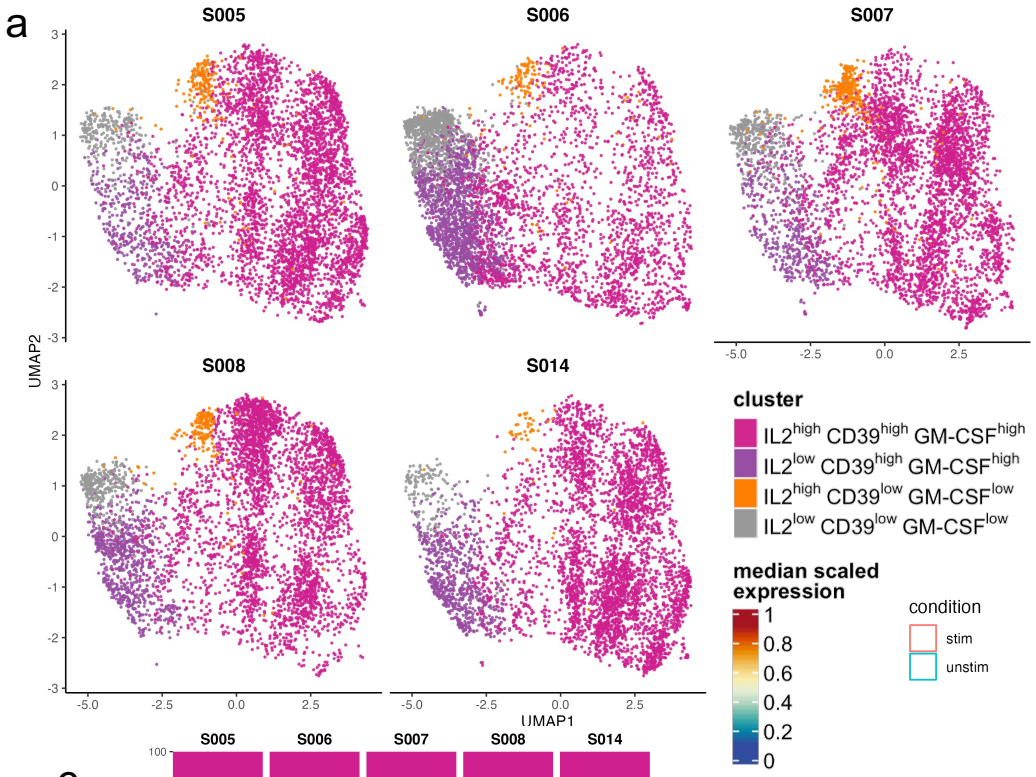


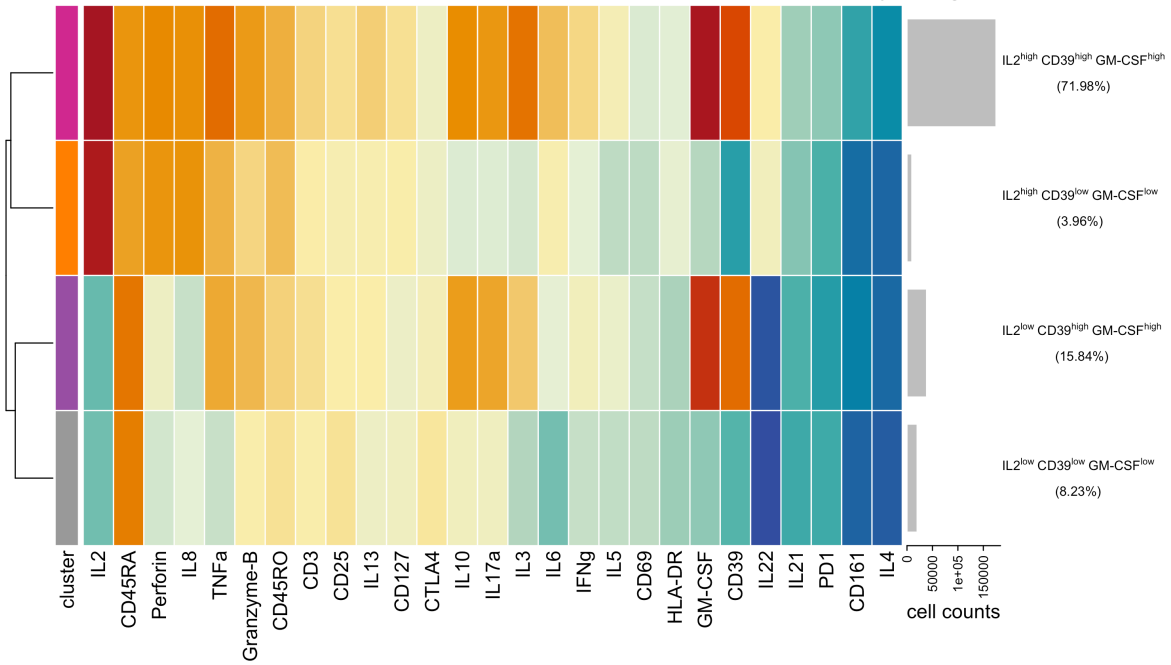


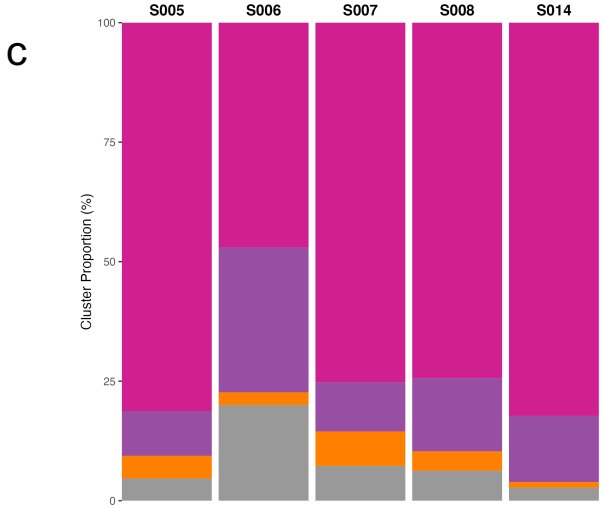


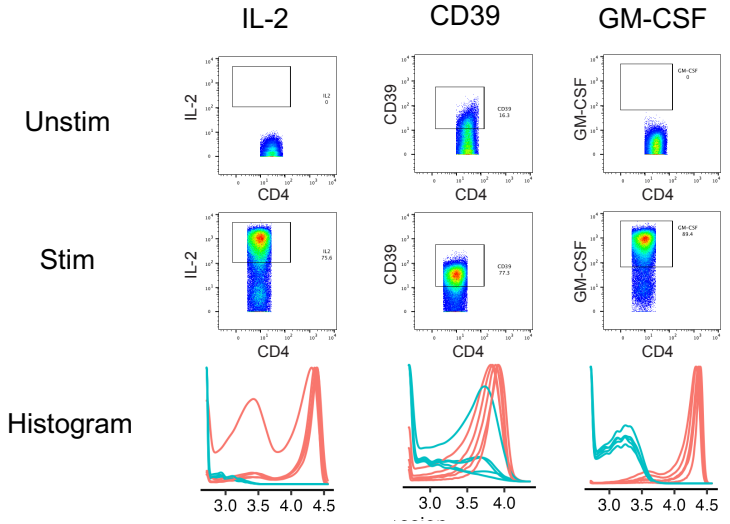
Supplementary Figure 6. Phenotypic analysis of unstimulated CD4+ infusion products by CyTOF. (a) Uniform Manifold Approximation and Projection (UMAP) representations of the cells. 5,000 cells from each product were used in the UMAP analysis. Cells in the UMAPs were colored by their cluster color designation. (b) Heatmap of cell clusters that constitute unstimulated infusion products of all five DIPG patients. Cell intensity expressions were first normalized from 0 to 1 for each marker. The heatmap was then colored using the median of the scaled intensity expressions (between 0 and 1) of each marker. Each row of the heatmap was annotated by the cluster color designation, descriptive cluster name, histogram of cell count, and percentage of cell counts. (c) Stacked bar graph of the cluster proportion within all five unstimulated products. (d) Representative 2-D flow plots of key markers of interest. The 2-D flow plots showcase results from a healthy donor PBMC sample and S005.

Supplementary Figure 7



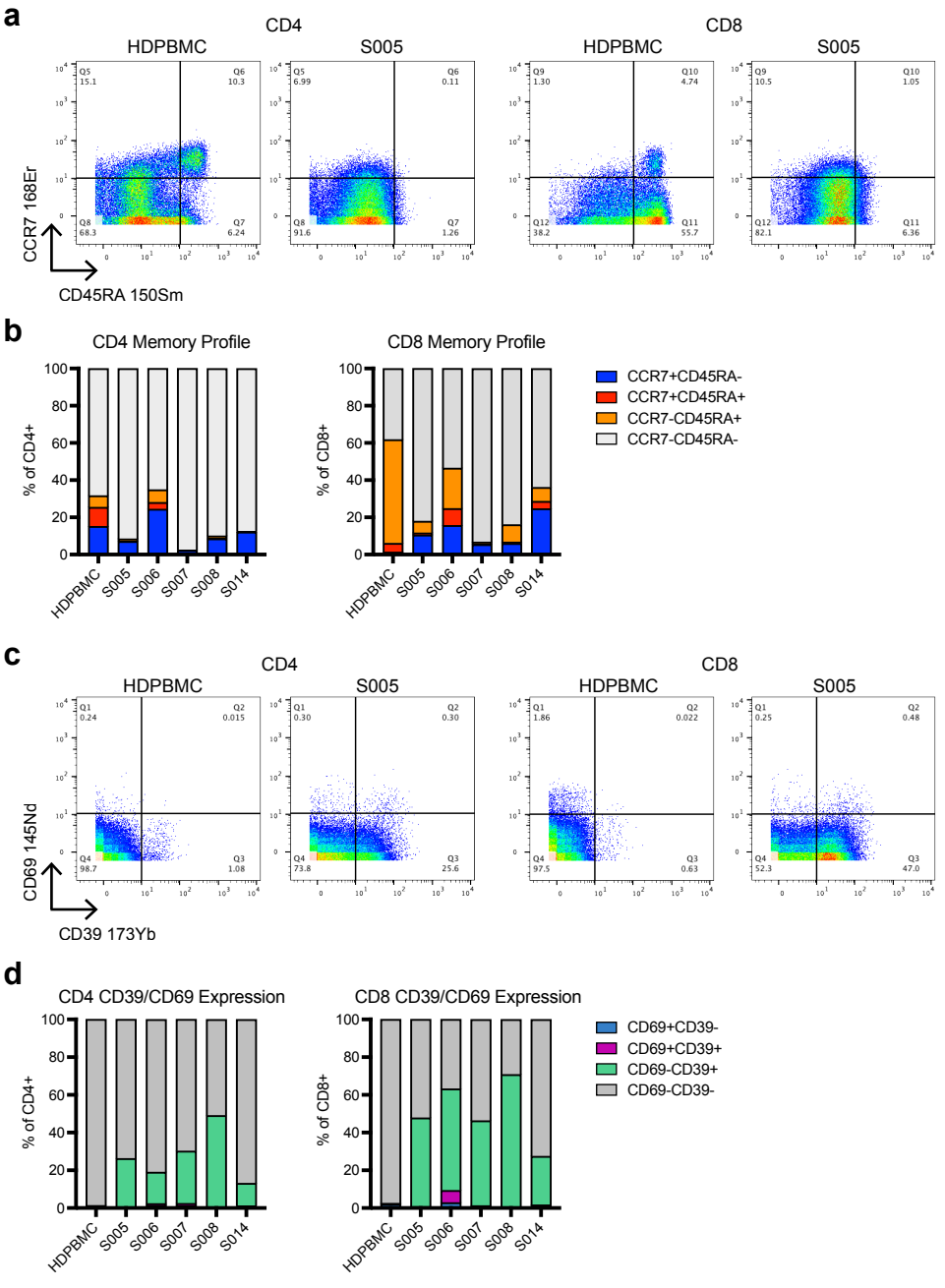






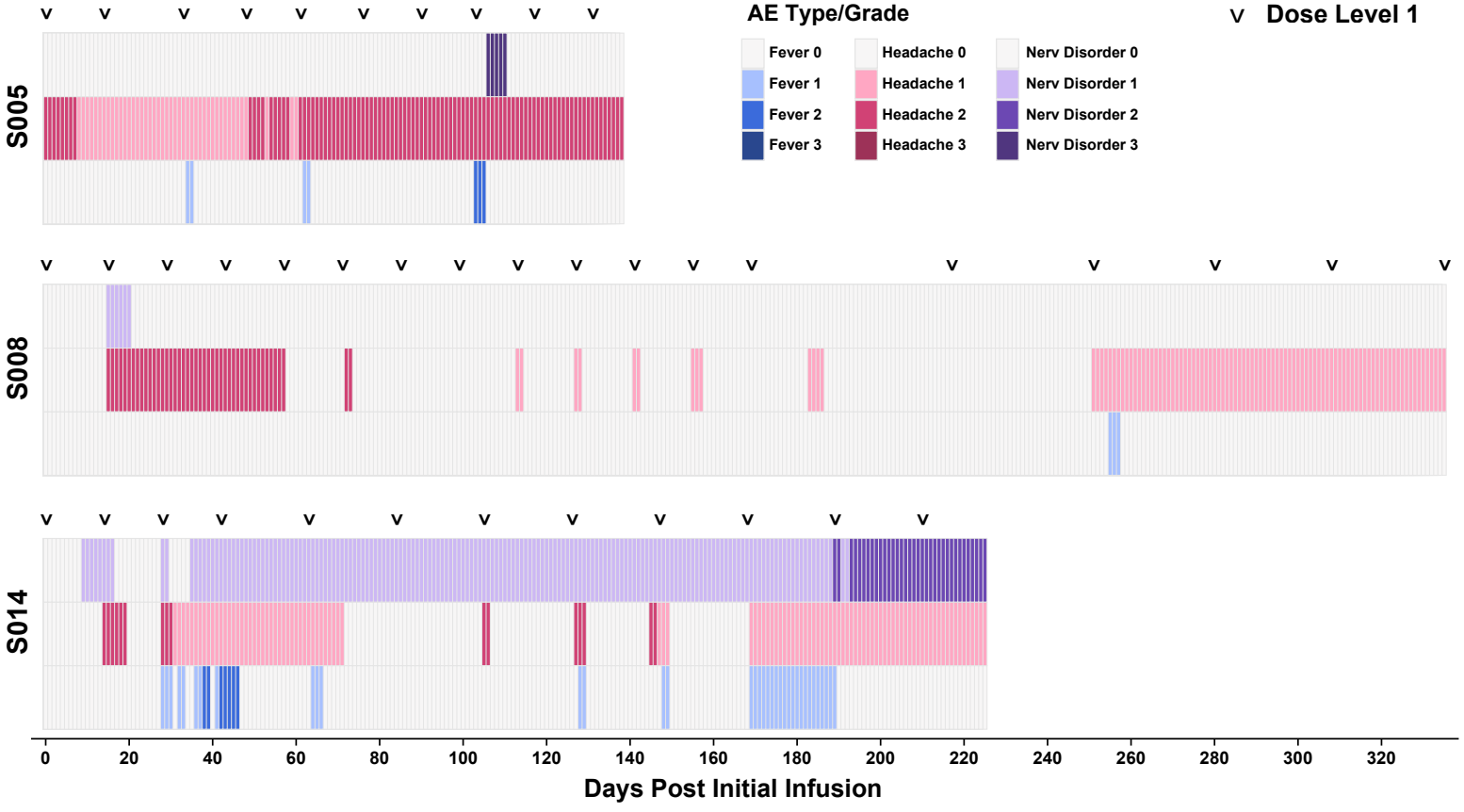
Supplementary Figure 7. Functional analysis of stimulated CD4+ infusion products by CyTOF. (a) Uniform Manifold Approximation and Projection (UMAP) representations of the cells. 5,000 cells from each product were used in the UMAP analysis. Cells in the UMAPs were colored by their cluster color designation. (b) Heatmap of cell clusters that constitute stimulated (by PMA/ionomycin) infusion products of all five DIPG patients. Cell intensity expressions were first normalized from 0 to 1 for each marker. The heatmap was then colored using the median of the scaled intensity expressions (between 0 and 1) of each marker. Each row of the heatmap was annotated by the cluster color designation, descriptive cluster name, histogram of cell count, and percentage of cell counts. (c) Stacked bar graph of the cluster proportion within all five stimulated products. (d) Representative cytometry scatter density plots and histograms to show expression of key markers of interest. The top two rows show representative results from S005. Scatter-density of cells are plotted by cytokine and CD39 expression (y-axis) versus CD4 expression (x-axis) for unstimulated (top row) versus PMA and ionomycin stimulated (second row) cells. The third row shows histograms from all five stimulated (red lines) and unstimulated (cyan lines) samples.

Supplementary Figure 8



Supplementary Figure 8. Memory profile and CD39/CD69 expression analysis of B7H3CAR T cell products. (a) Memory status of CD4+ and CD8+ CAR T cell infusion products. Representative CCR7 and CD45RA biaxial gating of CD4+ and CD8+ T cells from a healthy donor PBMC sample (HDPBMC) and S005's CAR T cell infusion product. (b) Stacked bar graphs display abundance of different memory phenotypes within the CD4+ and CD8+ CAR T cell infusion products based on CCR7 and CD45RA gating. (c) Representative CD69 and CD39 biaxial gating of CD4+ and CD8+ T cells from a healthy donor PBMC sample (HDPBMC) and S005's CAR T cell infusion product. (d) Stacked bar graphs display abundance of different populations within the CD4+ and CD8+ CAR T cell infusion products based on CD69 and CD39 gating.

Supplementary Figure 9



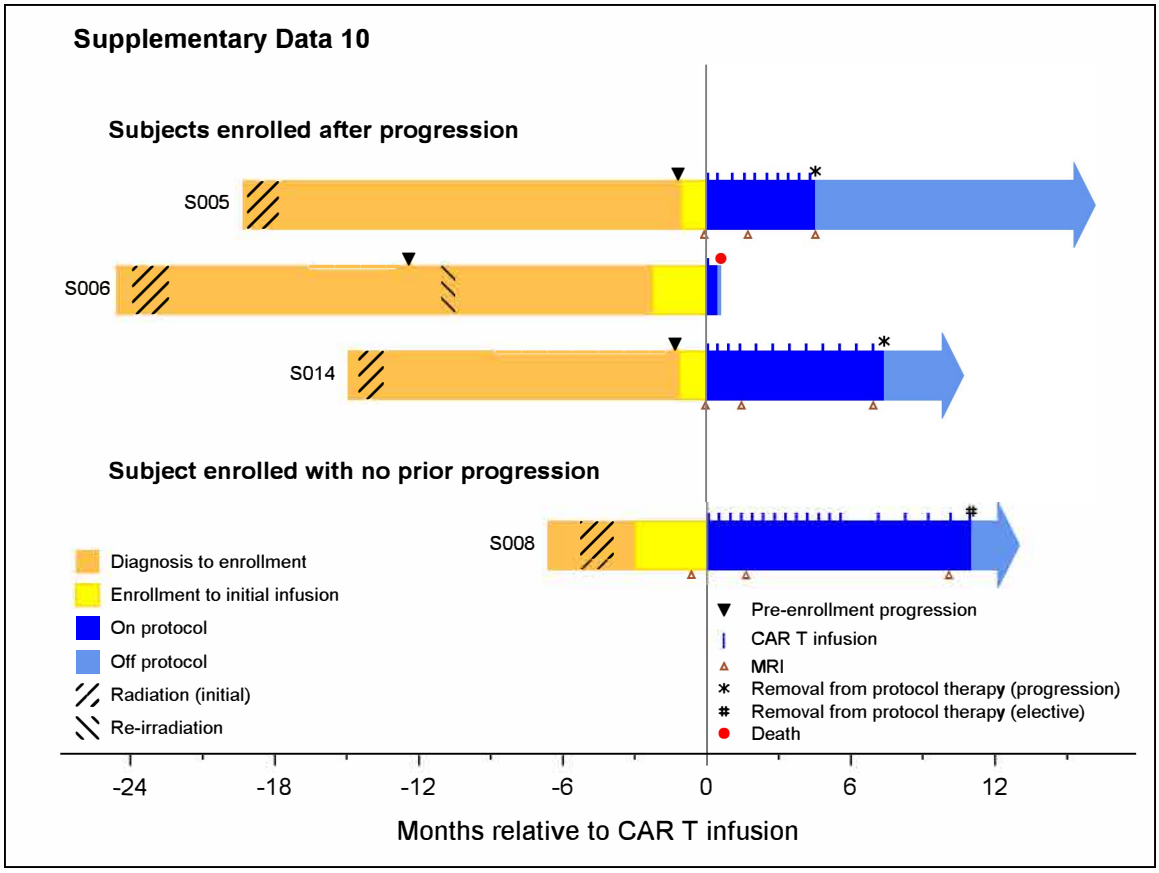
Supplementary Figure 9. Adverse event development over the course of treatments. Three adverse event (AE) types are plotted here: fever, headaches, and nervous system disorders (excluding headache). CAR T cell infusions indicated with arrows.

Subject	Body System Organ Class	Toxicity Type	Baseline Grade (if present at pre-Cr1W1 dose administration)	Maximum Grade Post CAR T
01659-S005	BLOOD AND LYMPHATIC SYSTEM DISORDERS	Anemia		1
		Eosinophilia		1
	CARDIAC DISORDERS	Sinus tachycardia		1
	GASTROINTESTINAL DISORDERS	Diarrhea		1
		Dyspepsia		1
		Dysphagia	1	2
		Fecal incontinence		1
		Nausea	1	2
		Vomiting		1
	GENERAL DISORDERS AND ADMINISTRATION SITE CONDITIONS	Fever		2
		Gait disturbance	1	2
	INJURY, POISONING AND PROCEDURAL COMPLICATIONS	Fall		2
	INVESTIGATIONS	Lymphocyte count decreased		2
		White blood cell decreased		1
	METABOLISM AND NUTRITION DISORDERS	Anorexia		1
		Hyponatremia		1
	MUSCULOSKELETAL AND CONNECTIVE TISSUE DISORDERS	Muscle weakness lower limb	1	2
		Muscle weakness trunk		2
		Muscle weakness upper limb	1	2
	NERVOUS SYSTEM DISORDERS	Dysarthria		3
		Headache		2
	PSYCHIATRIC DISORDERS	Anxiety	1	2
	RESPIRATORY, THORACIC AND MEDIASTINAL DISORDERS	Dyspnea		1
	VASCULAR DISORDERS	Hypertension		1

Supplementary Table 1. Treatment-emergent adverse events. CTCAE grading of all observed toxicities that constituted an increase from baseline. If the toxicity was not present pre-Course 1 Week 1 administration, then no baseline grade is shown.

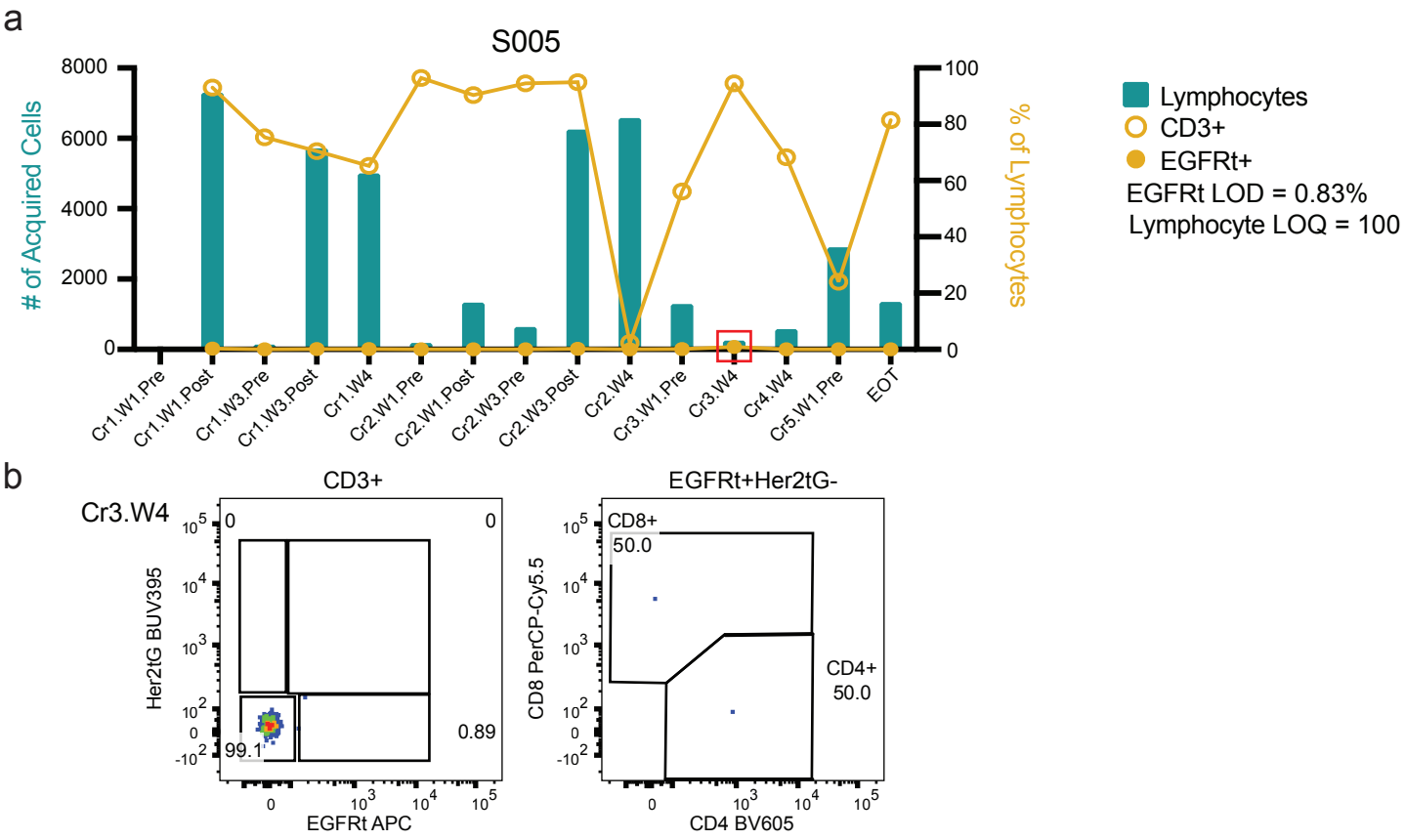
Subject	Body System Organ Class	Toxicity Type	Baseline Grade (if present at pre-Cr1W1 dose administration)	Maximum Grade Post CAR T
01659-S006	BLOOD AND LYMPHATIC SYSTEM DISORDERS	Anemia		1
	EAR AND LABYRINTH DISORDERS	Ear pain		1
	GASTROINTESTINAL DISORDERS	Constipation		1
		Nausea		2
		Vomiting		2
	GENERAL DISORDERS AND ADMINISTRATION SITE CONDITIONS	Fatigue		1
		Pain		2
	INVESTIGATIONS	Lymphocyte count decreased		1
	METABOLISM AND NUTRITION DISORDERS	Dehydration		2
		Hyponatremia	1	1
	NERVOUS SYSTEM DISORDERS	Dysarthria		3
	RESPIRATORY, THORACIC AND MEDIASTINAL DISORDERS	Dyspnea		2
		Hypoxia		3
		Productive cough		2
	VASCULAR DISORDERS	Hypertension		1
01659-S008	BLOOD AND LYMPHATIC SYSTEM DISORDERS	Anemia	1	1
	CARDIAC DISORDERS	Sinus tachycardia		1
	GASTROINTESTINAL DISORDERS	Nausea		2
		Vomiting		2
	GENERAL DISORDERS AND ADMINISTRATION SITE CONDITIONS	Fatigue		1
		Fever		1
	INFECTIONS AND INFESTATIONS	Covid-19		1
	INVESTIGATIONS	Lymphocyte count decreased		2
		Neutrophil count decreased	1	2
		White blood cell decreased	2	2
	METABOLISM AND NUTRITION DISORDERS	Hypokalemia		1
	NERVOUS SYSTEM DISORDERS	Dizziness		1
		Headache	1	2

Subject	Body System Organ Class	Toxicity Type	Baseline Grade (if present at pre-Cr1W1 dose administration)	Maximum Grade Post CAR T
01659-S014	BLOOD AND LYMPHATIC SYSTEM DISORDERS	Anemia		2
		Eosinophilia	1	1
	GASTROINTESTINAL DISORDERS	Abdominal pain		2
		Cheilitis		2
		Dysphagia		1
		Nausea		2
		Vomiting		1
	GENERAL DISORDERS AND ADMINISTRATION SITE CONDITIONS	Fatigue		2
		Fever		2
		Gait disturbance		1
		Malaise		2
		Pain		2
	INVESTIGATIONS	Neutrophil count decreased		1
		White blood cell decreased		1
	METABOLISM AND NUTRITION DISORDERS	Anorexia		2
	MUSCULOSKELETAL AND CONNECTIVE TISSUE DISORDERS	Chest wall pain		1
		Muscle weakness upper limb		2
		Neck pain		2
	NERVOUS SYSTEM DISORDERS	Akathisia		1
		Facial nerve disorder		1
		Headache		2
		Muscle weakness right-sided		2
		Nystagmus		2
		Somnolence		1
	PSYCHIATRIC DISORDERS	Irritability		1
	RENAL AND URINARY DISORDERS	Urinary incontinence		2
		Urinary retention		1
	RESPIRATORY, THORACIC AND MEDIASTINAL DISORDERS	Epistaxis		1
	SKIN AND SUBCUTANEOUS TISSUE DISORDERS	Angular cheilitis		2
		Rash maculo-papular		1
		Skin atrophy		1
		Urticaria		1
	VASCULAR DISORDERS	Hypertension		2
		Hypotension		1



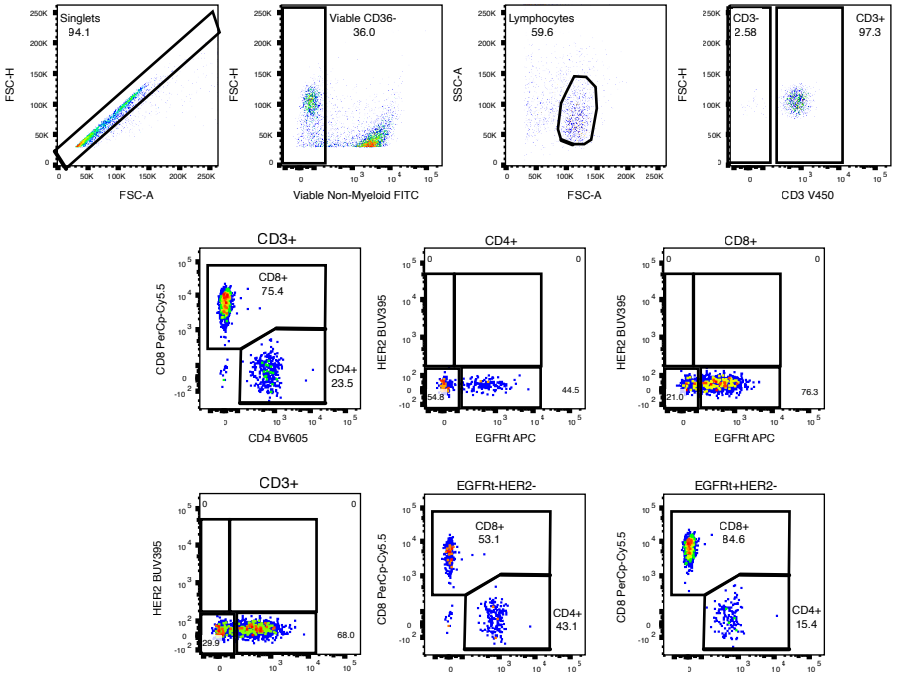
Supplementary Figure 10. Intent-to-treat survival post initial CAR T cell infusion. Swimmer's plot of all subjects with DIPG enrolled on BrainChild-03 Arm C Dose Regimen 1 that received a CAR T dose. S005, S006, and S014 enrolled following progression (black triangle), while S008 enrolled prior to progression. Blue dash marks above each line demarcate CAR T cell infusions, while red triangles below each line correspond to MRI images displayed in Figure 3.

Supplementary Figure 11



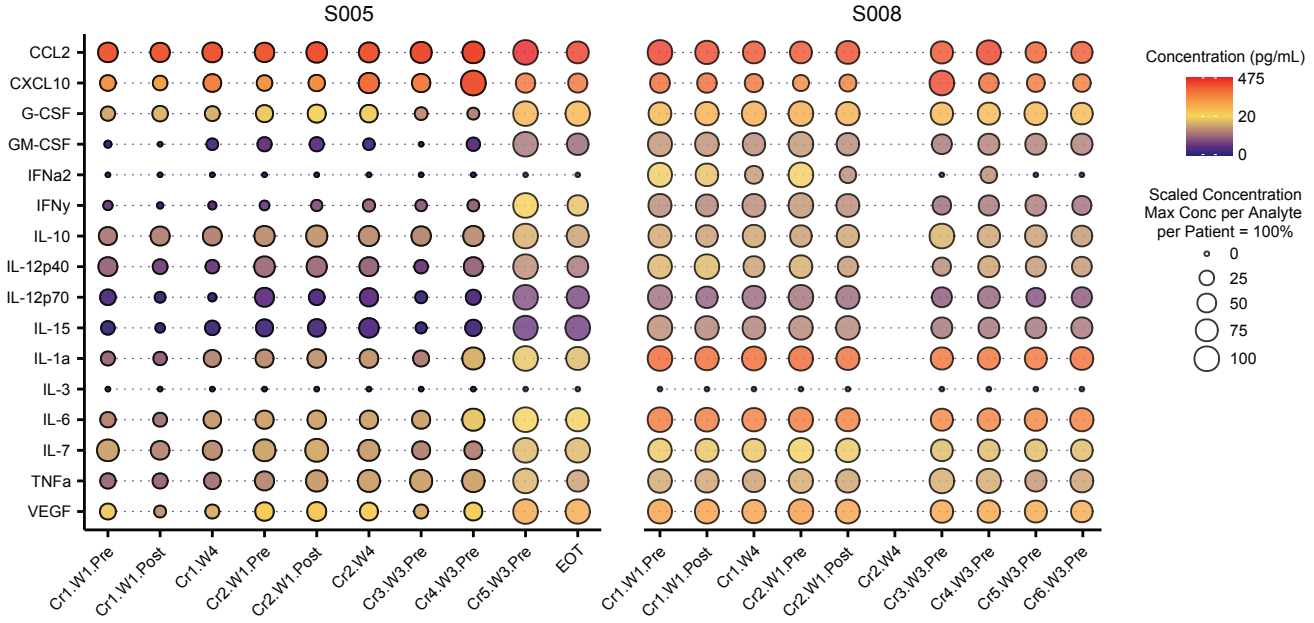
Supplementary Figure 11. CSF CAR T cells in patient S005. Using the gating strategy shown in Supplemental Figure 12, (a) the total number of Lymphocytes collected in each CSF sample acquisition on the flow cytometer were plotted for S005 (green bars, left axis). Overlayed on the plot (right axis) is the percentage of lymphocytes expressing CD3 (open circles) or EGFRt CAR tag (filled circles). Samples with less than 100 lymphocytes, the limit of quantitation (LOQ) requirement for the flow assay, were excluded from T and CAR T cell reporting. The peak engraftment timepoint for S005 is highlighted (red box). (b) Flow plots displaying CAR (EGFRt+) T cell detection and the CD4+/CD8+ expression in the EGFRt+ population at peak engraftment is shown.

Supplementary Figure 12



Supplementary Figure 12. Representative flow gating strategy for CAR T cell detection. Gating strategy used for flow-based CAR detection in CSF. Selection of the singlet, viable, CD36- lymphocyte population was performed prior to T cell gating and examination of the EGFRt+ CAR T cells and CD4/CD8 expression.

Supplementary Figure 13



Supplementary Figure 13. Few chemokine and cytokine fluctuations detected in serum samples post treatment. Cytokine concentrations in serum across both patients were converted to log2 scale and represented with circles filled with color. Red color corresponds to the highest log2 concentration measured across both patients. Yellow and blue colors correspond to 50% and 0% of the log2 concentration, respectively. Additionally, concentrations relative to the maximum concentration observed for a given patient and analyte are represented by the size of the circles, to highlight the fluctuation in cytokine concentrations throughout a patient's CAR T cell treatment.

Supplementary Table 2. Timepoints of post infusion correlative study collections.

Subject S005		
Visit	Days Post Initial Treatment	Days Post "Pre" Visit
Cr1.W1.Pre	0	N/A
Cr1.W1.Post	3	3
Cr1.W3.Pre	14	N/A
Cr1.W3.Post	17	3
Cr1.W4	19	N/A
Cr2.W1.Pre	33	N/A
Cr2.W1.Post	38	5
Cr2.W3.Pre	48	N/A
Cr2.W3.Post	52	4
Cr2.W4	54	N/A
Cr3.W1.Pre	61	N/A
Cr3.W4	81	N/A
Cr4.W1.Pre	90	N/A
Cr4.W4	110	N/A
Cr5.W1.Pre	117	N/A
EOT	138	N/A

Subject S006		
Visit	Days Post Initial Treatment	Days Post "Pre" Visit
Cr1.W1.Pre	0	N/A
Cr1.W1.Post	2	2

Subject S008		
Visit	Days Post Initial Treatment	Days Post "Pre" Visit
Cr1.W1.Pre	0	N/A
Cr1.W1.Post	2	2
Cr1.W3.Pre	15	N/A
Cr1.W3.Post	20	5
Cr1.W4	22	N/A
Cr2.W1.Pre	29	N/A
Cr2.W1.Post	34	5
Cr2.W3.Pre	43	N/A
Cr2.W3.Post	48	5
Cr2.W4	50	N/A
Cr3.W1.Pre	57	N/A

Cr3.W4	78	N/A
Cr4.W1.Pre	85	N/A
Cr4.W4	106	N/A
Cr5.W1.Pre	113	N/A
Cr6.W1.Pre	141	N/A
Cr6.W4	162	N/A
Cr7.W1.Pre	169	N/A
Cr8.W1.Pre	217	N/A
Cr9.W1.Pre	251	N/A
Cr10.W1.Pre	280	N/A
Cr11.W1.Pre	308	N/A
Cr12.W1.Pre	335	N/A
EOT	392	N/A

Subject S014		
Visit	Days Post Initial Treatment	Days Post "Pre" Visit
Cr2.W3.Pre	42	N/A
Cr3.W1.Pre	63	N/A
Cr4.W1.Pre	105	N/A
Cr5.W1.Pre	147	N/A

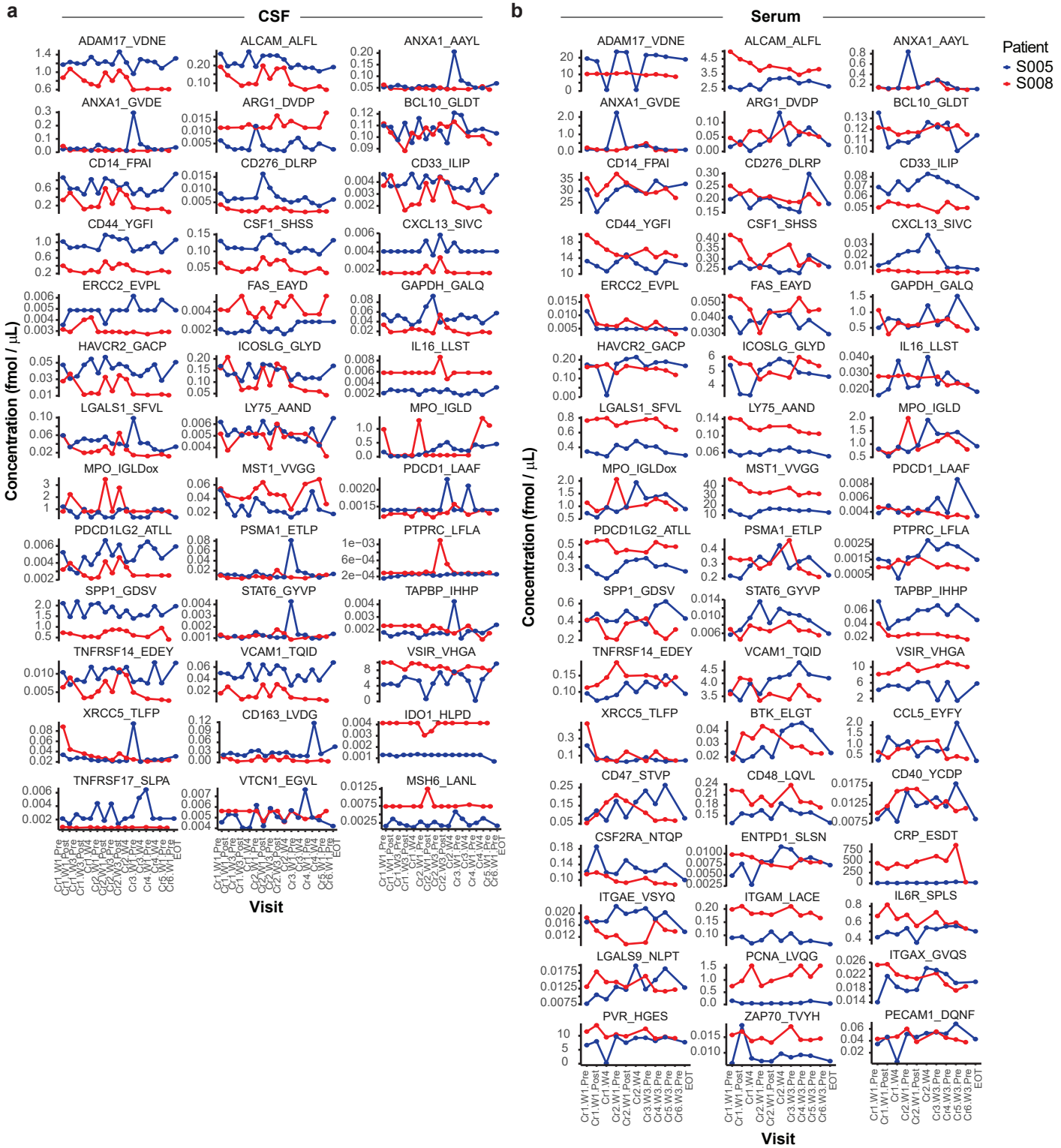
Supplementary Table 3. No CAR T cell DNA was detected in the peripheral blood of any patient. FLAP-EF1 qPCR was performed on DNA isolated from S005, S008, and S014's peripheral blood mononuclear cells at the timepoints indicated, including at the time of apheresis (PreA) through their End of Therapy (EOT) timepoint. No CAR T cell DNA was detected at any timepoint tested.

Subject S005		
Timepoint	Assay	Engineered Cell DNA Detected? (Yes/No)
PreA	FLAP-EF1 qPCR	No
Cr1.W1.Pre	FLAP-EF1 qPCR	No
Cr1.W1.Post	FLAP-EF1 qPCR	No
Cr1.W4	FLAP-EF1 qPCR	No
Cr2.W1.Pre	FLAP-EF1 qPCR	No
Cr2.W1.Post	FLAP-EF1 qPCR	No
Cr2.W4	FLAP-EF1 qPCR	No
Cr3.W3.Pre	FLAP-EF1 qPCR	No
Cr4.W3.Pre	FLAP-EF1 qPCR	No
Cr5.W3.Pre	FLAP-EF1 qPCR	No
EOT	FLAP-EF1 qPCR	No

Subject S008		
Timepoint	Assay	Engineered Cell DNA Detected? (Yes/No)
PreA	FLAP-EF1 qPCR	No
Cr1.W1.Pre	FLAP-EF1 qPCR	No
Cr1.W1.Post	FLAP-EF1 qPCR	No
Cr1.W4	FLAP-EF1 qPCR	No
Cr2.W1.Pre	FLAP-EF1 qPCR	No
Cr2.W1.Post	FLAP-EF1 qPCR	No
Cr3.W3.Pre	FLAP-EF1 qPCR	No
Cr4.W3.Pre	FLAP-EF1 qPCR	No
Cr5.W3.Pre	FLAP-EF1 qPCR	No
Cr6.W4.Pre	FLAP-EF1 qPCR	No
EOT	FLAP-EF1 qPCR	No

Subject S014		
Timepoint	Assay	Engineered Cell DNA Detected? (Yes/No)
PreA	FLAP-EF1 qPCR	No
Cr1.W1.Pre	FLAP-EF1 qPCR	No
Cr1.W1.Post	FLAP-EF1 qPCR	No
Cr1.W4	FLAP-EF1 qPCR	No
Cr2.W1.Pre	FLAP-EF1 qPCR	No
Cr2.W1.Post	FLAP-EF1 qPCR	No
Cr2.W4	FLAP-EF1 qPCR	No
Cr3.W3.Pre	FLAP-EF1 qPCR	Not-Available
Cr4.W3.Pre	FLAP-EF1 qPCR	No
Cr5.W3.Pre	FLAP-EF1 qPCR	No
Cr6.W3.Pre	FLAP-EF1 qPCR	Not-Available

Supplementary Figure 14



Supplementary Figure 14. Targeted proteomics during intracranial B7-H3CAR T cell therapy. Targeted immuno-MRM peptide concentrations in CSF (a) and serum (b) plotted versus visit for patients S005 (blue) and S008 (red). Analyte name is represented by gene symbol followed by the first four amino acids of the target peptide. Only peptides detected above the lower limit of quantification in at least one visit from both patients were plotted.