Supplemental Figure Legends

Online Supplemental Figure S1. Characterization of T cell products during manufacturing. (A) Left, Viability of cells throughout the manufacturing timeline, pooled for 4 donors. Right, Cell counts throughout the manufacture calendar, pooled for 4 donors. VFC-CAR (blue) N=36; RV-CAR (green) N=27; VFC-Ctrl (gray) N=25. (B) Left, Percent of CAR+ cells as measured by flow cytometry when electroporated on day 2 or day 3 post-isolation. Right, Percent of TCR- cells as measured by flow cytometry when electroporated on day 2 or day 3 post-isolation. * indicates p<=0.05.

Online Supplemental Figure S2. Immunophenotyping gating strategy. Cells were assayed by spectral cytometry with a 21-color immunophenotyping panel on day 10 of manufacture. (A) Cells were gated for lymphocytes, singlets, live cells, and CD45+ cells. (B) Subsequent gates for transgene+ cells were determined using fluorescence-minus-one (FMO) controls for each marker (left of each panel), with a representative full stain shown at right for each color. (C) Gating strategy for downstream immunophenotyping analysis of memory and effector states. Gates for each color were established using FMO controls, at left for each panel. All panels in blue boxes show representative data from one replicate of VFC-CAR T cells. Panel in green shows representative data from one replicate of RV-CAR T cells. Panel in grey shows representative data from one replicate of VFC-Ctrl T cells. All antibodies were titrated at 5 different concentrations to determine optimal staining conditions. SSC-A, side scatter area. FSC-A, forward scatter area. SSC-H, side scatter height. FSC-H, forward scatter height.

Online Supplemental Figure S3. CAR T cell immunophenotypes. Cells were assayed by spectral cytometry with a 21-color immunophenotyping panel on day 10 of manufacture. (A) Additional markers are shown in **figure 4**. Gating strategies are shown in online supplemental **figure S3**. VFC-CAR (blue) N=7; RV-CAR (green) N=7; VFC-Ctrl (gray) N=8 across two donors. Significance was determined by ordinary one-way ANOVA; * indicates p≤0.05; ** indicates p≤0.01; *** indicates p≤0.001; **** indicates p≤0.0001.

Online Supplemental Figure S4. Transcriptional signatures of single CAR T cells. (A) tSNE projection of single cell RNA-seq data from 15 samples of manufactured cell products, both pre- and post-antigen exposure. Gross clustering patterns indicate similar cell populations across donors; each tSNE plot shows aggregate cells for each cell type both prior to and after antigen exposure, separated by donor. (B) Violin plots show expression of the pan-T cell markers CD3E, CD8A, and CD4. Clusters 15, 19, 20, and 21 showed decreased expressed of all three markers and were determined to be contaminating cancer cells from co-culture samples; these clusters are not shown on the tSNE plots and are excluded from all downstream analyses. Clusters 0, 5, 6, 8, 9, 13, and 17 were determined to contain CD8+ cytotoxic T cells; clusters 2, 3, 4, 7, 10, 12, and 14 were determined to contain CD4+ helper T cells. Clusters 1, 16 and 18 were determined to have mixed or low CD4/CD8 expression and were not annotated; they are designated as "other" in **figure 5B** and **C**. Expression level refers to log normalized data.

Online Supplemental Figure S5. Memory-associated gene expression in single cell RNA-sequencing. (A) Violin plots show expression of six markers of early/stem cell memory phenotypes (LEF1, TCF7, IL7R, FOXP1, ID3, and BCL2). (B) Violin plots show expression of

three markers of central memory T cells (CD62L, CCR7, and CD27). (C) Violin plots show expression of four markers of effector memory T cells (ID2, T-bet, Blimp-1, and GNLY). Clusters 0, 3, and 4 were determined to predominantly express early/stem-like memory markers. Clusters 5, 7, and 12 were determined to express a mix of stem-like and central memory markers. Clusters 6, 8, and 9 were determined to express a mix of stem-like, central, and effector memory markers. Cluster 10 was determined to express a predominantly Th2 memory phenotype. Clusters 2 and 14 were determined to express a Th2 effector phenotype. Clusters 11, 13, and 17 were determined to express an effector phenotype along with some exhaustion markers. Clusters 1, 16, and 18 showed low or mixed CD4/CD8 expression and were not annotated. Clusters 15, 19, 20 and 21 were determined to be cancer cells and were not included in downstream analyses. Expression level refers to log normalized data.

Online Supplemental Figure S6. T helper 2 (Th2) and terminal effector gene expression in single cell RNA-sequencing. (A) Violin plots show expression of eight markers of Th2 helper T cells (IL4, IL5, IL13, GATA3, STAT6, GFI1, MAF, and IRF4). (B) (B) Violin plots show expression of three markers of terminal effector T cells (GZMB, PRF1, and CXCR3). Clusters 0, 3, and 4 were determined to predominantly express early/stem-like memory markers. Clusters 5, 7, and 12 were determined to express a mix of stem-like and central memory markers. Clusters 6, 8, and 9 were determined to express a mix of stem-like, central, and effector memory markers. Cluster 10 was determined to express a predominantly Th2 memory phenotype. Clusters 2 and 14 were determined to express a Th2 effector phenotype. Clusters 11, 13, and 17 were determined to express an effector phenotype along with some exhaustion markers. Clusters 1, 16, and 18 showed low or mixed CD4/CD8 expression and were not annotated. Clusters 15, 19, 20 and 21 were determined to be cancer cells and were not included in downstream analyses. Expression level refers to log normalized data.

Online Supplemental Figure S7. Exhaustion-associated gene expression in single cell RNA-sequencing. (A) Violin plots show expression of fifteen markers of exhaustion in T cells (PD1, LAG3, TIM3, CD39, BATF, CTLA4, BTLA, HAVCR1, IRF4, NFATC1, NFATC2, EOMES, T-bet, TIGIT, ADORA2A). Clusters 0, 3, and 4 were determined to predominantly express early/stem-like memory markers. Clusters 5, 7, and 12 were determined to express a mix of stem-like and central memory markers. Clusters 6, 8, and 9 were determined to express a mix of stem-like, central, and effector memory markers. Cluster 10 was determined to express a predominantly Th2 memory phenotype. Clusters 2 and 14 were determined to express a Th2 effector phenotype. Clusters 11, 13, and 17 were determined to express an effector phenotype along with some exhaustion markers. Clusters 1, 16, and 18 showed low or mixed CD4/CD8 expression and were not annotated. Clusters 15, 19, 20 and 21 were determined to be cancer cells and were not included in downstream analyses. Expression level refers to log normalized data.

Online Supplemental Figure S8. Single Cell RNA sequencing immunophenotyping. tSNE plots as shown in **figure 5**, colored for expression levels of all markers assayed for protein-level expression (figures 4 and S4). (A) Feature plots show expression of the pan T cell markers CD45, CD3E and TRAC. (B) Feature plots show expression of the memory and differentiation markers CD95, CD62L, CCR7, CD27, and CD28. (C) Feature plots show expression of the exhaustion markers PD1, LAG3, TIM3, TIGIT, and CD39. (D) Feature plot shows expression of the activation marker HLA-DR. (E) Feature plot shows expression of the T cell

trafficking/inflammatory marker CXCR3. (F) Feature plot shows expression of the senescence marker CD57.

Online Supplemental Figure S9. Transcriptional signatures of single CAR T cells prior to and after target antigen exposure. (A) Proportion of transgene+ cells from all pre-antigen samples within each annotated cluster. (B) Proportion of transgene+ cells from all post-antigen samples within each annotated cluster. Each color represents a different cluster, shown in **figure 5A**. Purple clusters are memory-associated; yellow clusters are effector-associated; grey clusters could not be identified as pure T cell clusters due to a mix or lack of robust CD4/CD8 expression. Bar charts are the same as those shown in **figure 5B** and **C**, separated by CD8 and CD4-specific clusters. Similar patterns of memory vs. effector formation were observed in both cytotoxic and helper T cells, with VFC-CAR and VFC-Ctrl cells skewing towards a memory phenotype prior to antigen exposure, and VFC-CAR and RV-CAR T cells acquiring an effector phenotype after antigen exposure.

Online Supplemental Figure S10. VFC-CAR T cells induce robust regression of GD2+ neuroblastoma solid tumors *in vivo* with high persistence. (A) Schematic of the *in vivo* mouse dosing strategy using NSG mice harboring GD2-positive CHLA20 neuroblastoma tumors. (B) Representative IVIS images of NSG mice with CHLA20 tumors that were treated with either 10 million VFC-CAR, RV-CAR, or VFC-Ctrl T cells. (C) Kaplan-Meyer survival curve for mice. VFC-CAR (blue) N=10; RV-CAR (green) N=8; VFC-Ctrl (gray) N=7. (D) Box plots show presence of CAR+CD45+ human T cells in mouse spleens, as measured by flow cytometry. (E) Flow cytometry plots show that human CD45+CAR+ VFC-CAR and RV-CAR T cells are found in tumors, but VFC-Ctrl cells are not.

Online Supplemental Figure S11. Bioluminescence, tumor growth, weight gains, T cell persistence, and memory formation after T cell treatment *in vivo*. (A) Flux measurements for individual luciferase-positive tumors for all mouse experiments. VFC-CAR, N=10. RV-CAR, N=8. VFC-Ctrl, N=7. (B) Left, individual mouse percent weight change throughout the experiment. Right, average percent weight change in mice per treatment condition. (C) Flow cytometric gating strategy used to assay mouse spleens for human T cells and CAR-positive cells. (D) Boxplots showing the expression levels of naïve (T_n), stem cell memory (T_{scm}) and central memory (T_{cm}) markers on human T cells found in mouse spleens. For immunophenotyping: RV-CAR, N=6. VFC-Car, N=7. VFC-Ctrl, N=6.

Online Supplemental Figure S12. Effect of *TRAC* knockout CAR T cell phenotype A) *In vitro* immunophenotyping of exhaustion markers and the memory marker CD62L. Flow cytometry was performed on day 10, concurrently with infusion into mice. (B) *In vivo* immunophenotyping of exhaustion markers and the memory marker CD62L. Flow cytometry was performed on T cells isolated from spleens, collected 20 days after initial infusion of CAR T products. *indicates $p \leq 0.05$; ** indicates $p \leq 0.01$; *** indicates $p \leq 0.001$; **** indicates $p \leq 0.0001$.