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Supplemental Information

Locally secreted BiTEs complement CAR T cells by enhancing killing of antigen heterogeneous solid tumors

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Materials and methods:

Enzyme-linked immunosorbent assay (ELISA)

A standard direct ELISA was performed with DuoSet Ancillary Reagent Kit 2 (R&D systems, Minneapolis, MN). After coating wells with recombinant human EGFR (1μg/mL), EGFRvIII (1μg/mL) and IL13Rα2 (4μg/mL) protein (Sino Biological, Wayne, PA), a 96-well plate was loaded with supernatants which were collected as described above, followed by biotin conjugated goat anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA) detection antibody and horseradish peroxidase conjugated streptavidin. For detecting IFNγ, IL2 and TNFα by ELISA, supernatant was collected from T cells and target cells after 16hrs co-culture at a 1:1 ratio. The detection was performed with DuoSet ELISA kits (R&D Systems, Minneapolis, MN) as the introduction indicated.

Impedance Cytotoxicity Assays

$5x10^4$ target tumor cells or $3x10^4$ target human astrocyte cells were seeded into Axion Biosystems microelectrode-containing 96-well plates (Axion Biosystems, Atlanta, GA). Each impedance plate was prepared prior to experimentation by coating with 0.05% poly-D-lysine hydrobromide (Sigma Aldrich, St. Louis, MO), followed by 20 μg/mL laminin overnight at 37 °C. After coating was complete, wells were rinsed thrice with diH2O and then overlaid with 100 μL of cell culture media. The plate was placed into the Axion Biosystems ZHT analyzer (Axion Biosystems, Atlanta, GA) to record baseline readings of the background impedance without cells present. After baseline was established, the plate was removed from the analyzer and was seeded with 50k target cells in a volume of 200 μL/well. After cell plating, the plate was left in

the cell culture hood for 1h at room temperature to ensure settling and attachment of the cells down to the microelectrodes on the bottom surface. The plate was then returned to the analyzer and data collection began. Data were collected every 1 min for 24 h for cell monolayer growth measurement. For cytotoxicity assessment, the instrument was paused at 24h, and media was exchanged for media containing 1:1 dosages of effector cells or UTD control T cells, or media alone. Changes in impedance are reported as the resistive component of the complex impedance, as described previously. Using AxIS Z software (Axion Biosystems, Atlanta, GA), all data are corrected for "media alone" to remove small changes in media only impedance over time and then normalized to the impedance at the time of addition of effector cells. The % cytolysis calculations utilize the no treatment control and full lysis controls to determine % of target cell cytolysis as follows:

$$\%Cytolysis_{sample}(t) = \left[\frac{Z_{sample}(t) - \overline{Z_{FullLysis}(t)}}{\overline{Z_{TargetOnly}(t)} - \overline{Z_{FullLysis}(t)}}\right] x\ 100\%$$

Immunohistochemistry

Formalin-fixed paraffin-embedded tissue sections, 5µm thick, were stained using antibody against IL13Rα2 (clone E7U7B, Cell Signaling 85677, dilution 1:100, Danvers, MA) and EGFRVIII (clone D6T2Q, Cell Signaling 64952S, dilution 1:100, Danvers, MA), or isotype control (Leica Biosystem PA077, Buffalo Grove, IL). The double staining was done sequentially on a Leica Bond-IIITM instrument using the Bond Polymer Refine DAB Detection System (Leica Microsystems DS9800, Buffalo Grove, IL) and Refine Red Detection System (Leica Microsystems DS9390, Buffalo Grove, IL). Heat-induced epitope retrieval was done in ER2 solution (Leica Bio

systems AR9640) for 20 minutes. The entire experiment was done at room temperature. Slides were washed three times between each step with bond wash buffer or water.

FIGURE AND FIGURE LEGENDS:

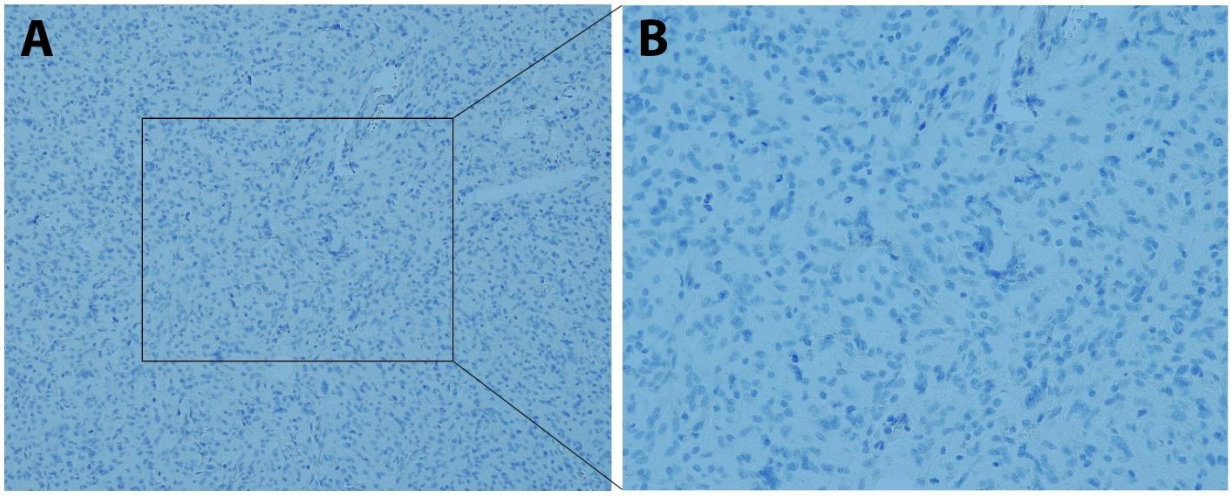


Figure S1. Immunohistochemical stains of IL13Rα2 and EGFRvIII isotype control in resected glioma tissues.

(A) ×100 original magnification. (B) x200 original magnification.

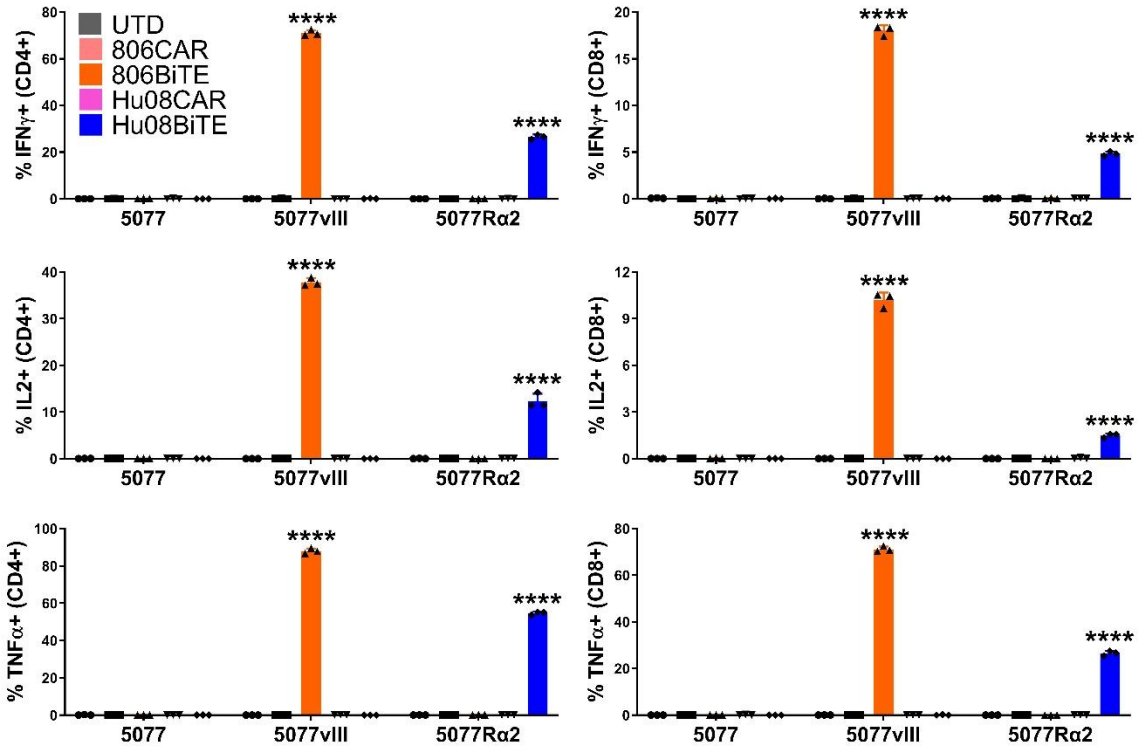


Figure S2. BiTEs significantly responding to target positive glioma cells

Flow based intracellular cytokine (IFNγ, IL2 and TNFα) staining of UTD T cells (UTD) co-cultured with target cells in conditioned media of CAR/BiTE T cells. CD4+ (left) and CD8+ (right) subgroups of T cells were distinguished by human CD8 staining. Statistically significant differences were calculated by one-way ANOVA with post hoc Tukey test. ****$p<0.0001$. Data are presented as means ± SD.

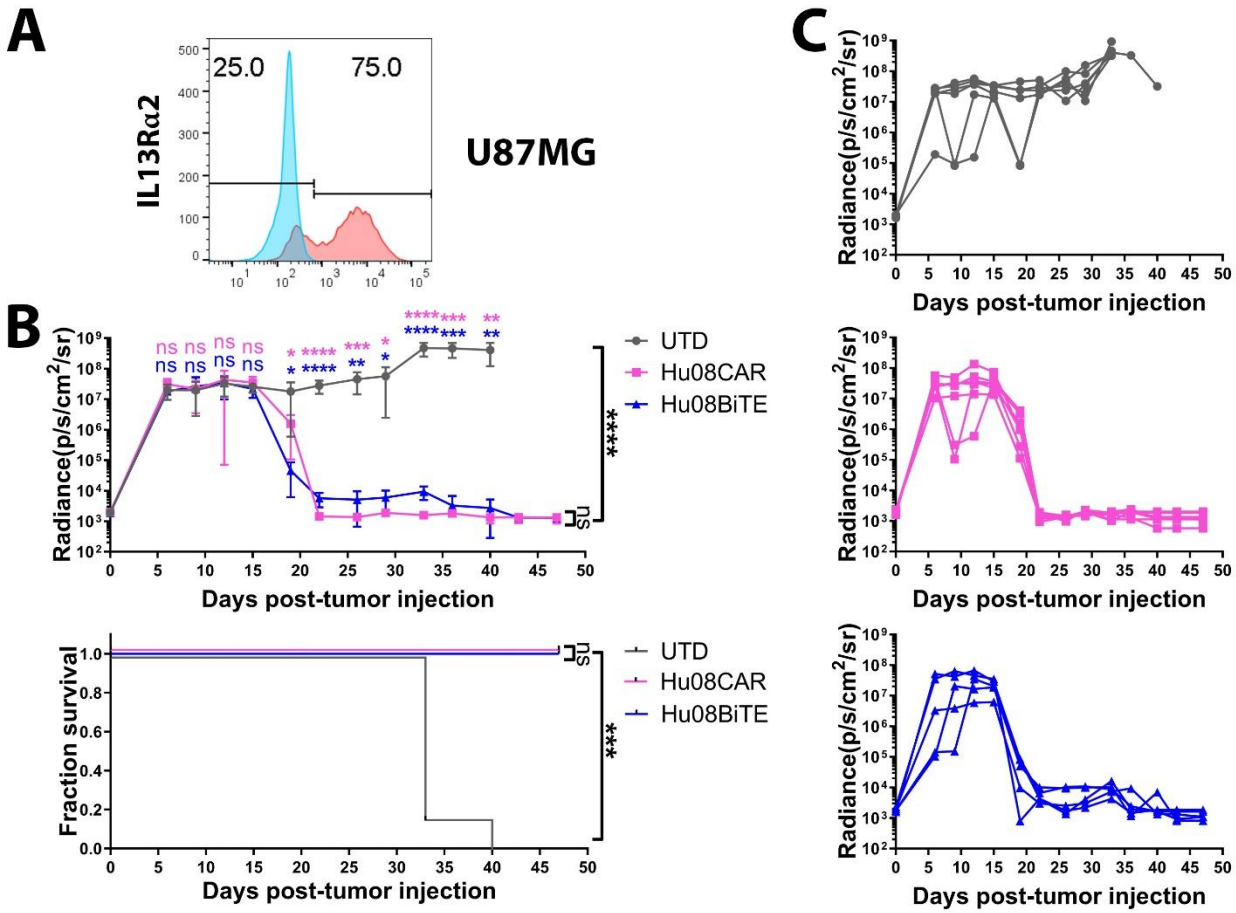


Figure S3. Hu08BiTE T cells significantly inhibited tumor growth in a U87MG orthotopically implanted glioma mouse model.

(A) Flow profile showing the expression of IL13Rα2 (Red) in U87MG cells, with staining control (blue). (B) 800,000 Hu08CAR/BiTE transduced T cells or the same number of un-transduced T cells were given by i.v. infusion in NSG mice orthotopically implanted with the U87MG tumor, 8 days after tumor injection. Bioluminescence imaging were repeated every 3-4 days to evaluate the tumor growth. Endpoint was predefined by the mouse hunch, inability to ambulate as predetermined IACUC approved morbidity endpoint. Statistically significant differences were calculated by one-way ANOVA with post hoc Tukey test. In bioluminescence imaging, the differences were labelled in each time point between experimental groups with un-transduced

T cells infusion group. Linear regression was used to test for significant differences between the experimental groups. Survival based on time to endpoint was plotted using a Kaplan-Meier curve (Prism software). Statistically significant differences were determined using log-rank test. ns, not significant; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Data are presented as means ± SD.

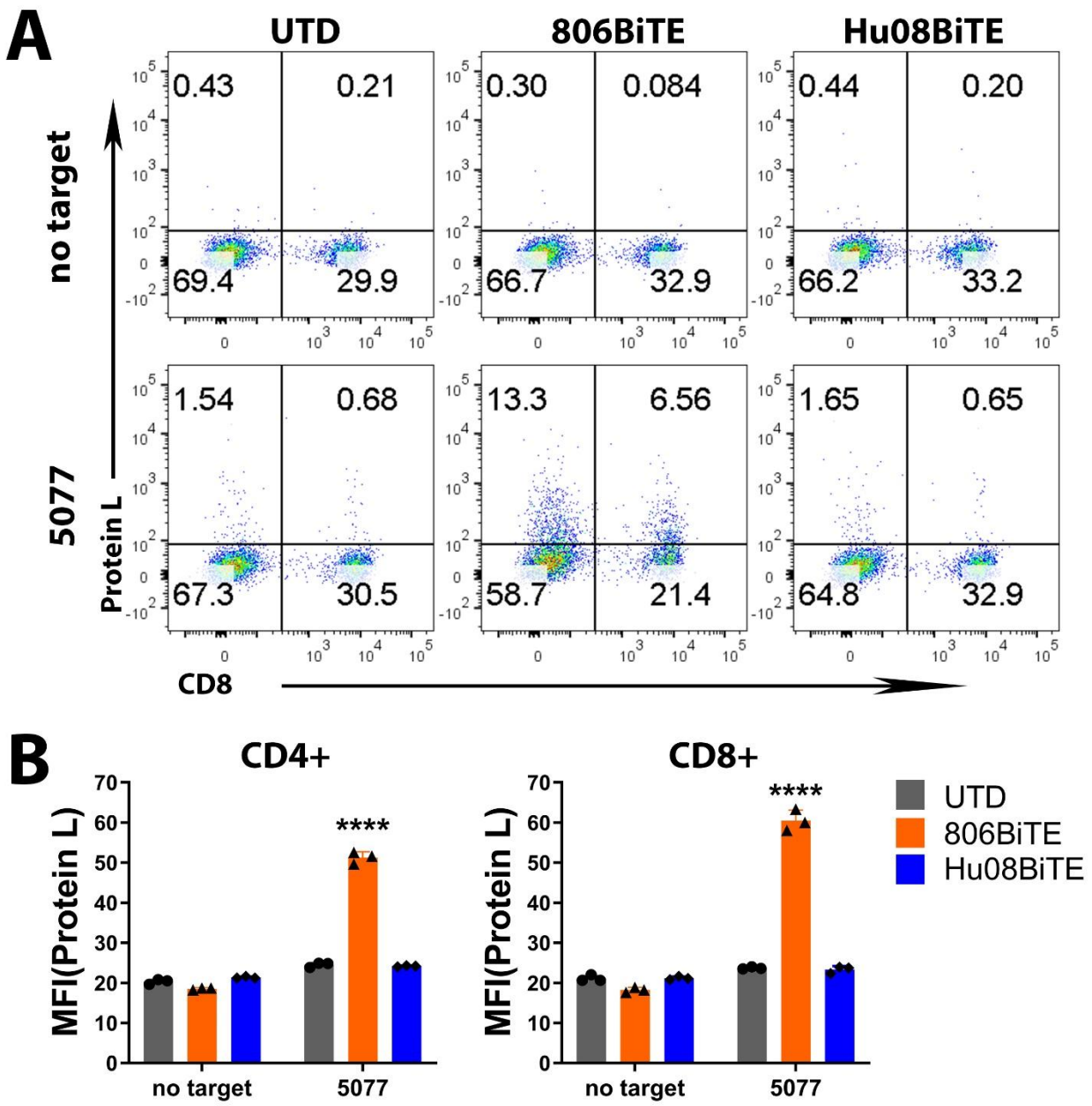
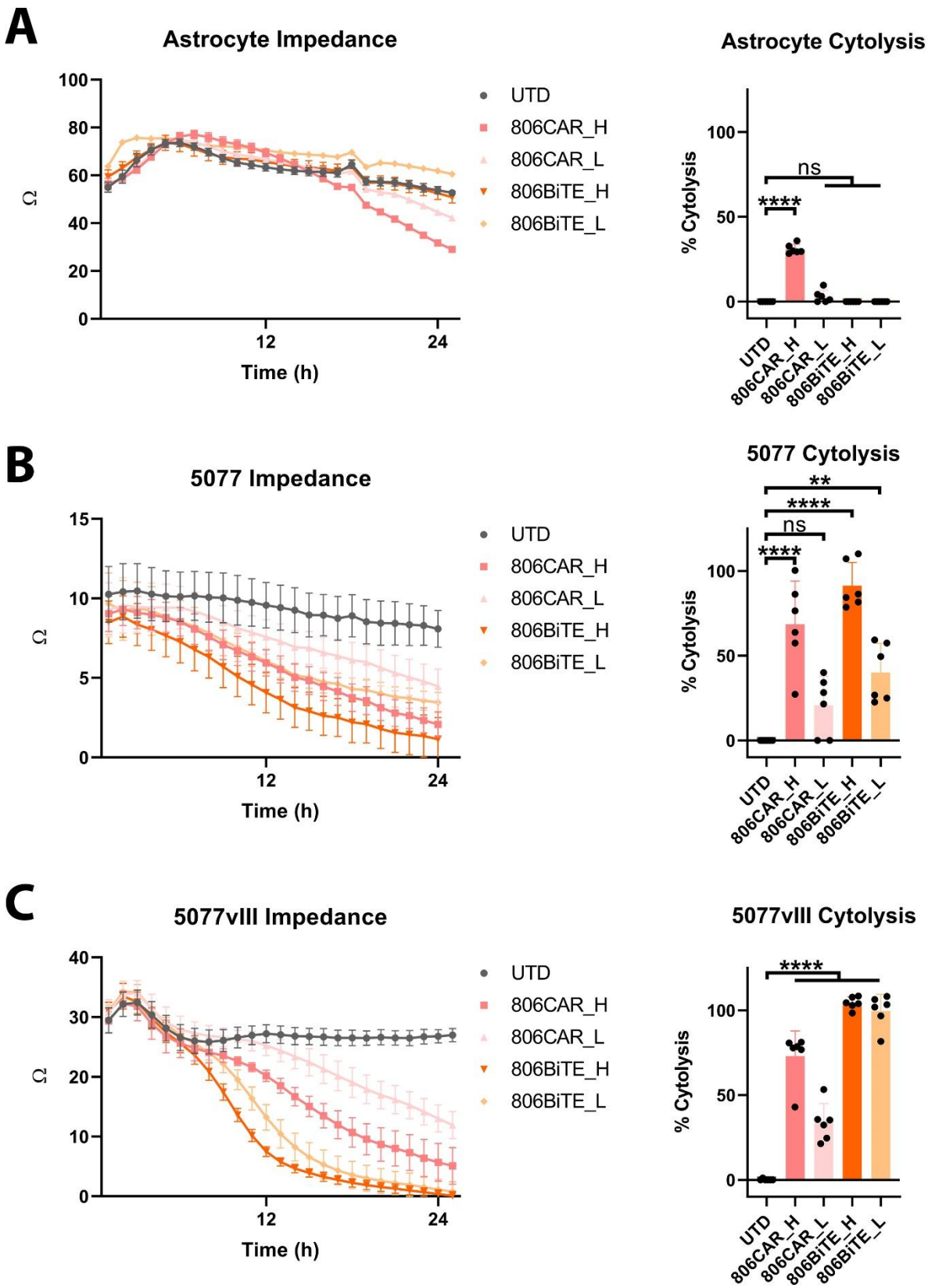


Figure S4. Protein L detected 806BiTE binding on 806BiTE secreting T cells when co-cultured with 5077 cells.

BiTE binding on T cells was detected by biotinylated protein L with secondary streptavidin coupled FITC after 16hrs co-culture. (A) Flow based results of representative samples. CD8 was stained to distinguish the CD4-positive and CD8-positive subgroups of T cells along the x axis. (B)

The median fluorescence intensity (MFI) was quantified on CD4 and CD8 positive T cells. Statistically significant differences were calculated by one-way Analysis of Variance (ANOVA) with post hoc Tukey test. ****$p<0.0001$. Data are presented as means ± SD.


A
Astrocyte Impedance
Ω
Time (h)
UTD
806CAR_H
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B
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5077 Cytolysis
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5077vIII Cytolysis
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Figure S5. 806BiTE T cells minimally responded to physically expressed EGFR on astrocytes and superior responded to EGFR and EGFRvIII expressed on 5077 glioma stem cells in impedance cytotoxicity assays.

CAR/BiTE T cell cytotoxicity assays were performed using cellular impedance recording for monolayers of (A) human astrocytes, (B) 5077 tumor cells, (C) 5077vIII tumor cells over 24 h of co-culture with 1:1 UTD, 806CAR_H, 806CAR_L, 806BiTE_H, or 806BiTE_L CAR T cells. Impedance values (left) were recorded every 60 sec for 24 h. Percent cytolysis values (right) at 24 h shown for all CAR/BiTE T cells compared to UTD T cell groups. N = 6 replicates per treatment. 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 ± SD.

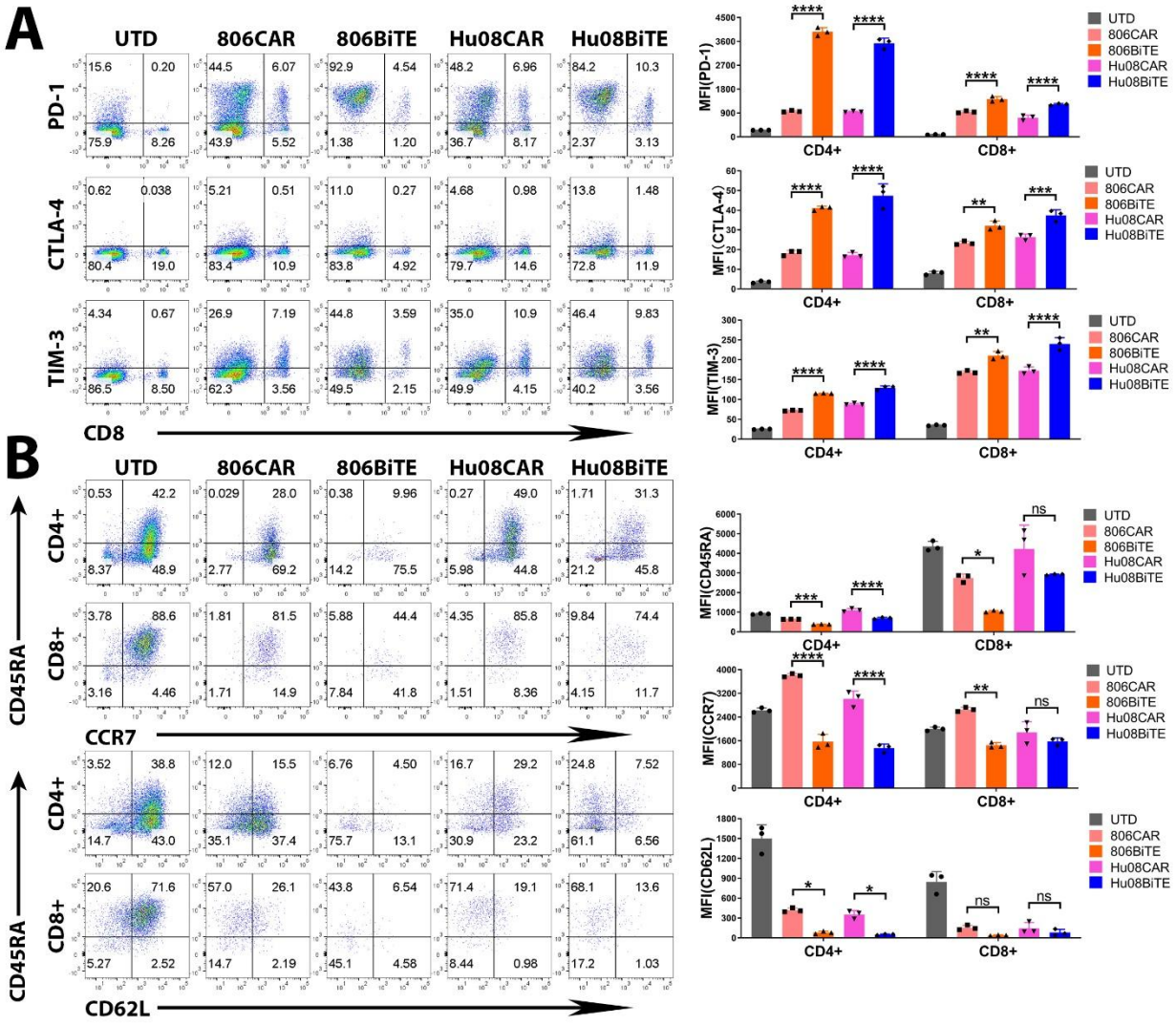

Figure S6. BiTEs significantly upregulated checkpoints and T cell effector subtypes after co-culturing with 5077$^{EGFRvIII+,\ IL13R\alpha2+}$ cells.

(A) The expression of checkpoints (PD-1, CTLA-4 and TIM-3) on the T cells was determined by flow cytometry after overnight co-culturing of CAR/BiTE T cells with 5077$^{EGFRvIII+,\ IL13R\alpha2+}$ cells. Flow based results of representative samples were illustrated, CD8 was stained to distinguish the CD4-positive and CD8-positive subgroups of T cells along the x axis. The median fluorescence intensity (MFI) was quantified and compared between CAR T cells and BiTE T cells. (B) The expression of CD45RA, CCR7 and CD62L on the T cells was determined by flow

cytometry after 4 days co-culturing of CAR/BiTE T cells with 5077$^{EGFRvIII+,\ IL13R\alpha2+}$ cells. Flow based results of representative samples were illustrated. The median fluorescence intensity (MFI) was quantified and compared between CAR T cells and BiTE T cells on CD4-positive and CD8-positive subgroups. 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 ± SD.