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Specialty section:

This article was submitted to
Cancer Immunity
and Immunotherapy,
a section of the journal
Frontiers in Oncology

Received: 04 February 2021

Accepted: 18 August 2021

Published: 10 September 2021

Citation:

Thokala R, Binder ZA, Yin Y, Zhang L,
Zhang JV, Zhang DY, Milone MC,
Ming GL, Song H and O'Rourke DM
(2021) High-Affinity Chimeric
Antigen Receptor With Cross-
Reactive scFv to Clinically Relevant
EGFR Oncogenic Isoforms.
Front. Oncol. 11:664236.
doi: 10.3389/fonc.2021.664236

High-Affinity Chimeric Antigen Receptor With Cross-Reactive scFv to Clinically Relevant EGFR Oncogenic Isoforms

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Tumor heterogeneity is a key reason for therapeutic failure and tumor recurrence in glioblastoma (GBM). Our chimeric antigen receptor (CAR) T cell (2173 CAR T cells) clinical trial (NCT02209376) against epidermal growth factor receptor (EGFR) variant III (EGFRvIII) demonstrated successful trafficking of T cells across the blood–brain barrier into GBM active tumor sites. However, CAR T cell infiltration was associated only with a selective loss of EGFRvIII+ tumor, demonstrating little to no effect on EGFRvIII⁻ tumor cells. Post-CAR T-treated tumor specimens showed continued presence of EGFR amplification and oncogenic EGFR extracellular domain (ECD) missense mutations, despite loss of EGFRvIII. To address tumor escape, we generated an EGFR-specific CAR by fusing monoclonal antibody (mAb) 806 to a 4-1BB co-stimulatory domain. The resulting construct was compared to 2173 CAR T cells in GBM, using *in vitro* and *in vivo* models. 806 CAR T cells specifically lysed tumor cells and secreted cytokines in response to amplified EGFR, EGFRvIII, and EGFR-ECD mutations in U87MG cells, GBM neurosphere-derived cell lines, and patient-derived GBM organoids. 806 CAR T cells did not lyse fetal brain astrocytes or primary keratinocytes to a significant degree. They also exhibited superior antitumor activity *in vivo* when compared to 2173 CAR T cells. The broad specificity of 806 CAR T cells to EGFR alterations gives us the potential to target multiple clones within a tumor and reduce opportunities for tumor escape *via* antigen loss.

Keywords: GBM, glioma, immunotherapy, CAR T cells, adoptive T cell therapy, EGFR

INTRODUCTION

Chimeric antigen receptor (CAR) cells targeting pediatric B cell malignancies have shown unprecedented responses and were the first CAR T cell therapies to receive FDA approval, in 2017 (1–3). The successful application of this therapeutic technology in the treatment of solid tumors, including glioblastoma (GBM), remains a significant challenge; chief among them are tumor heterogeneity, immunosuppressive tumor microenvironment, and antigen escape (4, 5). Successful strategies for overcoming these obstacles are required to advance CAR T therapy in solid tumors.

Epidermal growth factor receptor (EGFR) was one of the first oncogenes identified in GBM and presents an attractive therapeutic target, given its extracellular nature and frequent alterations in GBM. Approximately 60% of GBM specimens contain a mutation, rearrangement, splicing alteration, and/or amplification of EGFR (6). EGFR overexpression, mediated through focal amplification of the EGFR locus as double minute chromosomes, has long been recognized as the most common EGFR alteration, present in 60% of GBM patients (7, 8). Tumor-specific EGFR variant III (EGFRvIII), resulting from deletion of exon 2–7 of wild-type EGFR (wtEGFR), is present in 30% of GBM patients (9). In addition, oncogenic missense mutations EGFR^{A289D/T/V}, EGFR^{R108G/K}, and EGFR^{G598V} have been identified in 12%–13% of cases in the extracellular domain (ECD) of EGFR, independent of EGFRvIII. Missense mutations and EGFRvIII often co-occur with EGFR amplification and activate EGFR receptor independent of its ligand (10). Several of the missense mutations have been shown to have a negative effect on patient survival, driving tumor proliferation and invasion (11).

Our first-in-man CAR T clinical trial (NCT02209376) against EGFRvIII in recurrent GBM demonstrated the safety of a peripheral infusion of CAR T cells and resulted in successful trafficking of the CAR T cells to active tumor sites, across the blood–brain barrier (12). After treatment, CAR T cells infiltrated the GBM tumors rapidly, proliferated *in situ*, and persisted over a prolonged period of time. However, CAR T cell infiltration was associated only with a selective loss of EGFRvIII+ GBM cells. Importantly, post-CAR T-treated tumor specimens showed the continued presence of EGFR amplification and missense mutations, despite the decrease in EGFRvIII target antigen. Persistence of EGFR amplification and ECD missense mutations in the context of loss of EGFRvIII expression suggested that tumor heterogeneity played an essential role for tumor recurrence and continued regrowth.

mAb806, originally raised against EGFRvIII, recognizes a conformationally exposed epitope of wtEGFR when it is overexpressed on tumor cells. The same epitope is not exposed in EGFR expressed on normal non-overexpressing cells (13, 14). ABT-414, an antibody–drug conjugate composed of a humanized mAb806 (ABT-806), showed early efficacy in phase I/II clinical trials with no apparent skin toxicity in treated GBM patients (15). However, a recent Phase III trial was terminated when an interim analysis failed to demonstrate a survival benefit over placebo (16). mAb806 showed an increased binding affinity

for not only EGFRvIII but also EGFR ECD mutations and a low affinity for wtEGFR (11). These findings suggest that mAb806 is a viable therapeutic option for tumors harboring EGFR alterations in addition to EGFRvIII.

In the present study, we have developed EGFR-specific CAR T cells derived from the single-chain fragment variable region (scFv) of 806 mAb, using our standard 4-1BB- ζ construct (17). We then compared 806 CAR T activity with EGFRvIII-specific CAR T cells (2173 CAR T), currently in clinic, for specificity against oncogenic EGFR alterations, including amplified EGFR, EGFRvIII, and extracellular mutations *in vitro* and *in vivo*.

MATERIALS AND METHODS

CAR Constructs

806 scFvs were swapped with scFv of our standard CD19-BB- ζ lentiviral vector described previously to generate 806-BB- ζ CAR (17, 18). Briefly, the nucleotide coding sequences of 806 or C225 scFv with the huCD8 leader were synthesized by GeneArt (Thermo Fisher Scientific, Waltham, MA) with 5' Xba1 and 3' Nhe1 and ligated to Xba1 and Nhe1 sites of CD19-BB- ζ car construct. The C225-BB- ζ CAR was obtained from Dr. Avery Posey's lab at the University of Pennsylvania. The 2173-BB- ζ CAR T construct was obtained from Dr. Laura Johnson's Lab at the University of Pennsylvania (19, 20).

Transduction and Expansion of Primary Human T Lymphocytes

Human primary total T cells (CD4 and CD8) were isolated from normal healthy donors following leukapheresis by negative selection using RosetteSep kits (STEMCELL Technologies, Vancouver, CA, Canada). All specimens were collected with protocol approved by the University Review Board, and written informed consent was obtained from each donor. T cells were cultured in RPMI 1640 (Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (FBS) (VWR, Radnor, PA, USA), 10 mM HEPES (Thermo Fisher Scientific), 100 U/mL penicillin (Thermo Fisher Scientific), and 100 g/ml streptomycin sulfate (Thermo Fisher Scientific) and stimulated with magnetic beads coated with anti-CD3/anti-CD28 (Thermo Fisher Scientific) at the 1:3 T cell-to-bead ratio. Approximately 24 h after activation, T cells were transduced with lentiviral vectors encoding the CAR transgene at an MOI of 3 to 6. On day 5, beads were removed and thereafter cells were counted and fed every 2 days, supplemented with IL 2 150 U/ml until they were either used for functional assays or cryopreserved for future use.

Cell Lines and Cell Culture

The human cell line U87MG was purchased from the American Type Culture Collection (ATCC) and maintained in MEM (Richter's modification) (Thermo Fisher Scientific) with components GlutaMAX-1 (Thermo Fisher Scientific), HEPES pyruvate, and penicillin/streptomycin supplemented with 10% FBS. Primary human keratinocytes were purchased from the Dermatology Core Facility at the University of Pennsylvania. K562 cells were purchased from ATCC and maintained in RPMI

media (Invitrogen, Carlsbad, CA, USA) supplemented with 10% FBS, 20 mM HEPES, and 1% penicillin/streptomycin. Primary astrocytes were purchased (ScienCell Research Laboratories, Carlsbad, CA, USA) and cultured according to the manufacturer's instructions. The cells from early passages were used for cytotoxicity and cytokine experiments. GSC cell lines were cultured in DMEM F-12 media (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 2% B27 without vitamin A (Thermo Fisher Scientific), 20 mM HEPES, and penicillin/streptomycin.

EGFR-Mutant Cell Lines

To produce the overexpressing EGFR cell line (designated as U87MG-EGFR), the lentivirus co-expressing wtEGFR and Cyan Fluorescent Protein (CFP) under the control of the EF-1 α promoter was transduced into the U87MG cell line. On post-transduction day 4, cells were sorted on an Influx cell sorter (BD, Franklin Lakes, NJ, USA) on the basis of high EGFR expression and subsequently expanded. The lentivirus co-expressing CFP and EGFR mutants EGFR^{R108K/G}, EGFR^{A289D/T/V}, or EGFRvIII was transduced into U87MG-EGFR, GSC5077 neurosphere cells (21), and K562 cell lines. CFP-positive cells were sorted by fluorescence-activated cell sorting (FACS). For luciferase killing assays and *in vivo* tracking studies, U87MG and U87MG-EGFR mutant cell lines were transduced with lentivirus click beetle green (CBG) luciferase and green fluorescent protein (GFP). Anti-GFP-positive cells were sorted by FACS.

Cytokine Analysis

CAR T cells and K562 targets expressing EGFR and its variants were cocultured in 1:2 ratio in the R10 medium in a 96-well plate, in triplicate. Plates were incubated at 37°C with 5% CO₂. After 48 h, supernatants were collected and cytokine levels were assessed by ELISA kit (R&D Systems, Minneapolis, MN, USA) for IFN- γ , TNF- α , and IL2 production, according to the manufacturer's instructions.

Chromium Release Assay

The cytolytic efficacy of CAR T cells against K562 cells was evaluated by 4-h chromium release assays using E:T ratios of 5:1, 2.5:1, and 1:1. ⁵¹Cr-labeled target cells were incubated with CAR T cells in complete medium or 0.1% Triton X-100, to determine spontaneous and maximum ⁵¹Cr release respectively, in a V-bottomed 96-well plate. The mean percentage of specific cytolysis of triplicate wells was calculated from the release of ⁵¹Cr using a TopCount NXT (Perkin-Elmer Life and Analytical Sciences, Inc., Waltham, MA) as:

$$100 \times \frac{(\text{experimental release} - \text{spontaneous release})}{(\text{maximal release} - \text{spontaneous release})}$$

Data was reported as mean \pm SD.

Luciferase-Based Cytotoxic Assay

CBG+ target cell lines (U87 variants and GSC5077 variants) were cocultured with CAR T cells at E:T ratios of 10:1, 5:1, and 2.5:1, for 24 h at 37°C. One hundred microliters of the mixture was transferred to a 96-well black luminometer plate, 100 μ l of 66 μ g/ml D-luciferin (GoldBio, St. Louis, MO, USA) was added,

and the luminescence was immediately determined. Results were reported as percent killing based on luciferase activity in wells with tumor cells alone.

CD107a Degranulation

To assess CD107a degranulation, we plated 1×10^5 T cells and 5×10^5 stimulator target cells per well in round-bottom 96-well plates, to a final volume of 200 μ l in complete R10 medium, in triplicates. The CD107a-PE antibody (BD) was added into each well and incubated at 37°C for 4 h, along with surface staining for CD8 (BioLegend, San Diego, CA, USA) and CD3 and then analyzed by flow cytometry.

Flow Cytometry

For CAR detection, cells were stained with biotinylated protein L (GenScript, Piscataway, NJ, USA), goat anti-mouse IgG, and anti-human IgG (Jackson ImmunoResearch Laboratories, West Grove, PA), followed by streptavidin-conjugated allophycocyanin (APC) (BD). The surface expression of EGFR and its mutants was detected by CFP and APC-conjugated cetuximab antibody (Novus Biologicals, Centennial, CO, USA). EGFRvIII expression was detected by anti-EGFRvIII antibody, clone DH8.3 (Santa Cruz Biotechnology, Dallas, TX, USA). Flow analysis done by LSRFortessa (BD) and data were analyzed by FlowJo software (BD).

Animal Experiments

All mouse experiments were conducted according to Institutional Animal Care and Use Committee (IACUC)-approved protocols. NSG mice were injected with 2.5×10^5 U87MG-EGFR/EGFRvIII/Luc+ tumors subcutaneously in 100 μ l of PBS on day 0, seven animals per cohort. Tumor progression was evaluated by luminescence emission on an IVIS Lumina III In Vivo Imaging System (Caliper Life Sciences, Hopkinton, MA, USA) after intraperitoneal D-luciferin injection according to the manufacturer's instructions (GoldBio). Tumor size was measured by calipers in two dimensions and approximated to volume using the following calculation:

$$\text{Volume} = \frac{L \times \pi W^2}{6}$$

Seven days after tumor implantation, mice were treated with 3×10^6 CAR T cells intravenously *via* the tail vein, in 100 μ l of PBS. Survival was followed over time until predetermined IACUC-approved endpoints were reached.

GBM Organoids

GBM organoids (GBOs) were established from primary patient tissue, under a University of Pennsylvania Institutional Review Board-approved protocol and with patient written informed consent, and cocultured with CAR T cells as described previously (22, 23). GBOs were fixed and stained after coculture, using anti-CD3 (BioLegend), anti-cleaved caspase 3 (Cell Signaling Technology, Danvers, MA), anti-EGFR (Thermo Fisher Scientific), anti-EGFRvIII (Cell Signaling Technology), and DAPI (Sigma). To control for tumor heterogeneity, four GBOs per condition were used. Mutational data and variant

allele fractions (VAF) were obtained from the Center for Personalized Diagnostics at the University of Pennsylvania, as described previously (24).

Statistical Analysis

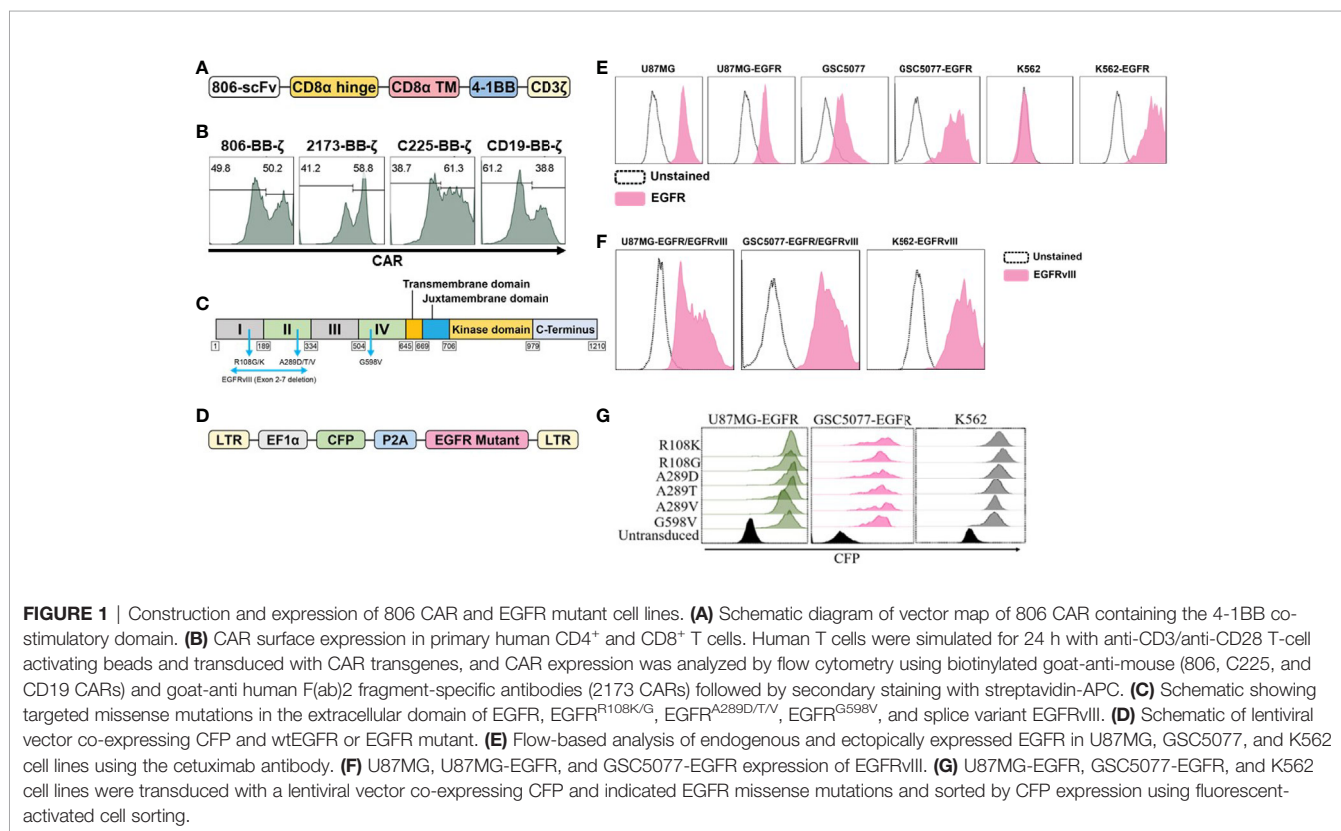
All *in vitro* experiments were performed at least in triplicate. GraphPad Prism 6 software (GraphPad Software, San Diego, CA, USA) was used for statistical analyses. Data were presented as mean \pm standard deviation. The differences between means were tested by appropriate tests. For the mouse experiments, changes in tumor radiance from baseline at each time point were calculated and compared between groups using the t-test or Wilcoxon rank-sum test, as appropriate. Survival determined from the time of T cell injection was analyzed by the Kaplan–Meier method, and differences in survival between groups were compared by the log-rank Mantel–Cox test.

RESULTS

Generation of 806 CARs and Cell Lines Expressing EGFR-Mutated Proteins

In the present study, we have generated CARs that target EGFR and EGFR mutants by fusing the scFv derived from mAb806 to a second-generation CAR construct containing 4-1BB-CD3 ζ signaling 806 CAR, the design of which is shown schematically

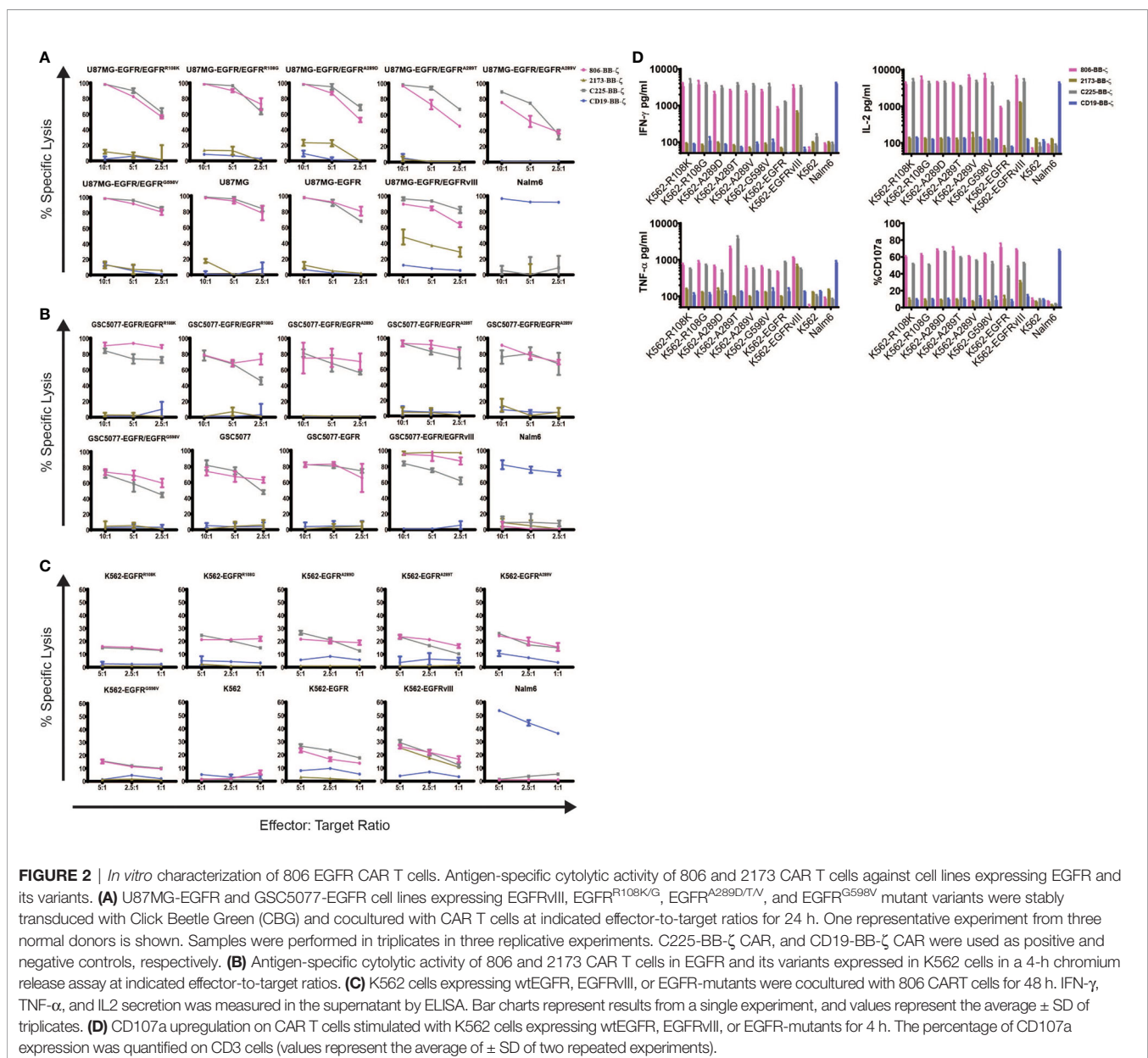
in **Figure 1A**. The EGFRvIII-specific 4-1BB-CD3 ζ -based 2173 CAR used in our clinical trials (NCT02209376 and NCT03726515) was generated for comparative evaluation with 806 CAR. 4-1BB-based cetuximab (C225) and CD19 CARs were used as positive and negative controls. Lentiviral vectors encoding CARs were transduced into a mixture of CD4 and CD8 T cells, and surface expression was confirmed by flow cytometry (**Figure 1B**). We next turned to generating target-positive tumor cell lines, expressing the mutations EGFR^{R108K/G}, EGFR^{A289D/T/V}, EGFR^{G598V}, and EGFRvIII, for testing of our CAR constructs (**Figure 1C**). In order to more faithfully model the EGFR mutations, which are almost always co-expressed with amplified wtEGFR, we transduced the GBM cell line U87MG and patient-derived glioma stem cell line GSC5077 (21), both of which express low levels of wtEGFR, with a lentiviral vector encoding wtEGFR (**Figure 1D**) (resultant lines referred to as U87MG-EGFR and GSC5077-EGFR), as well as K562 chronic myelogenous leukemia (CML) cells that lack endogenous expression of EGFR, with wtEGFR (**Figure 1E**). U87MG-EGFR, GSC5077-EGFR, and K562 cells were also transduced with EGFRvIII lentivirus and expression was then analyzed by an EGFRvIII-specific antibody (**Figure 1F**). A lentiviral vector co-expressing CFP and the targeted EGFR extracellular mutants (**Figure 1D**) was transduced into U87MG-EGFR, GSC5077-EGFR, and K562 cells. The resulting CFP-positive cells were sorted by fluorescence-activated cell sorting to obtain a positively transduced cell population (**Figure 1G**).



In Vitro Characterization of 806 CAR T Cells

To determine the specificity of the 806 and 2173 CARs for overexpressed wtEGFR, EGFRvIII, and the EGFR-ECD mutants, 2173 and 806 EGFR BB- ζ CAR T cells were cocultured with U87MG-EGFR and GSC5077-EGFR cell lines expressing EGFRvIII and extracellular mutants EGFR^{R108K/G}, EGFR^{A289D/T/V}, and EGFR^{G598V}, in 24-h bioluminescence-luciferase based killing assays (Figures 2A, B). While 2173 CAR T cells demonstrated specificity for EGFRvIII alone, 806 CAR T cells efficiently lysed all targets and exhibited similar cytolytic potential as C225 CAR T (Figures 2A, B). Notably, 806 CAR T cells were able to kill U87MG cells, despite expressing only low levels of wtEGFR, at an equal level when compared to overexpressed wtEGFR and EGFRvIII.

Since U87MG-EGFR mutants expressed endogenous and ectopic EGFR, we could not distinguish if the 806 scFv-binding specificity was restricted to the mutant or wtEGFR. To test the exclusive specificity to the mutants, we cocultured 806 and 2173 CAR T cells with the CML cell line K562, transduced to express wtEGFR, EGFRvIII, or EGFR mutants, as K562 does not have any endogenous EGFR (Figure 2B). 806 CAR T cells did not lyse untransduced K562 cells, confirming the lack of EGFR on the parental line. The 806 CAR T cells selectively targeted K562 cells expressing EGFR, EGFRvIII, or EGFR-ECD mutants and demonstrated similar efficacy as C225 CAR T cells. 2173 CAR T cells lysed K562-EGFRvIII cells but did not show any activity against either wtEGFR or the ECD mutants, as expected (Figure 2B). T cell activation was assessed by induction of



surface CD107a expression after coculture of CAR T cells with target-expressing cells (Figure 2C). Antigen-specific effector cytokine production was assessed by coculturing K562 target cells transduced with EGFR and its variants with CAR T cells. The resulting supernatants were analyzed for IFN- γ , TNF- α , and IL2 production (Figure 2D). Untransduced K562 and Nalm6 cells were used as negative controls. 806 and C225 CAR T cells produced similar levels of CD107 degranulation (Figure 2C) and IFN- γ , TNF- α , and IL2 (Figure 2D) in response to EGFRvIII, EGFR-ECD mutants, and EGFR overexpressing cells, while 2173 CAR T cells responded to EGFRvIII alone.

806 CAR T Cells Exhibit Low or No Affinity for EGFR Expressed on Primary Astrocytes and Keratinocytes

Having confirmed the function of the 806 CARs, we next sought to compare the reactivity of 806 and 2173 CAR T cells in response to endogenous levels of EGFR in normal cells, *in vitro*. We cultured primary human keratinocytes and astrocytes, as those cell types express wtEGFR (Figure 3A) and used them to stimulate CAR T cells. We observed production of IFN- γ by C225 CAR T cells, in response to EGFR presented by either astrocytes or keratinocytes, as well as U87MG-EGFR (Figure 3B). In contrast, 2173 CAR T cells produced IFN- γ in response to EGFRvIII antigen alone. 806 CAR T cells exhibited low or no cytotoxicity when cocultured with astrocytes or keratinocytes (Figure 3C), with corresponding low IFN- γ production (Figure 3B).

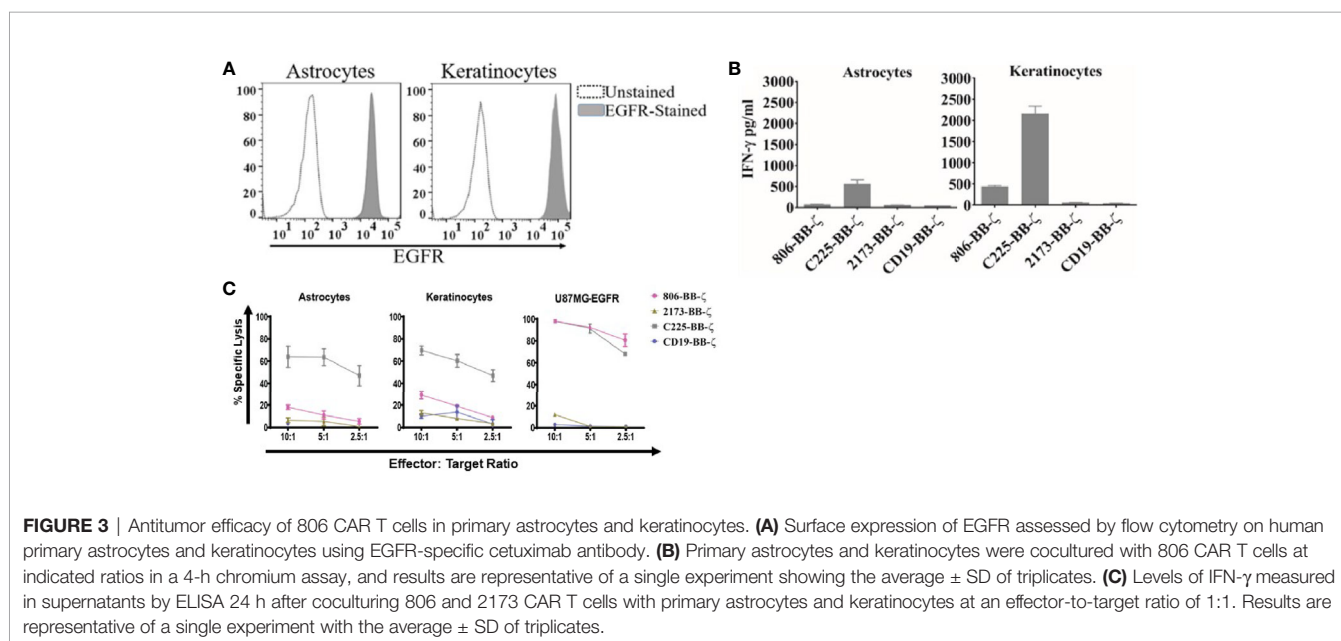
Antitumor Activity of 806 CAR T Cells *In Vivo*

Having compared the antigen-specific effector function of 806 CAR with 2173 CARs, we next sought to confirm its *in vivo* antitumor effects, using immunodeficient NSG mice bearing

human GBM tumors (Figure 4A). On Day 0, U87MG-EGFR/EGFRvIII tumors were implanted subcutaneously, and on Day 5, tumor engraftment was confirmed by bioluminescence imaging (BLI). On Day 7, a single dose of 3×10^6 CAR-positive T cells were infused intravenously ($n = 7$ per cohort). Total bioluminescence (Figure 4B) and individual bioluminescence (Figure 4C) were assessed in 806 and 2173 CAR T cell-treated groups. Animals in the negative control cohort, receiving CD19 CAR T cells, demonstrated rapid tumor growth, with all mice reaching a predetermined humane experimental endpoint by 42 days after initial tumor engraftment. To be noted, all mice in the CD19, 2173, and 806 groups reached experimental endpoint by day 42, 63, and 91, respectively (Figure 4A).

High-Fidelity GBM Organoids Demonstrate Cross-Reactivity of 806 CAR

Given the ability of the 806 CAR to target EGFR alterations beyond EGFRvIII, we turned to patient-derived GBM organoids (GBOs) to demonstrate activity in a heterogeneous model previously characterized to be of high fidelity to human tumors (22, 23). GBOs retain the originating tumor heterogeneity to a high degree out beyond 12 weeks of culturing and maintain the expression of endogenous EGFR and its alterations, providing a valuable model platform for testing therapies aimed at addressing tumor escape. The GBOs selected for coculture experiments contained multiple EGFR mutations (Figure 5A). GBO 9057 had EGFR copy number gain, EGFRvIII, and two missense mutations, EGFR^{G598V} and EGFR^{C595Y}. The missense mutation was found to have a VAF of 24%, while EGFRvIII was identified in less than 10% of the reads, based on next-generation sequencing (NGS). GBO 9066 had EGFR copy number gain, EGFR^{A289V}, and EGFR^{G598V}. Both EGFR^{A289V} and EGFR^{G598V} had a VAF of less than 15%, making determination of co-occurrence impossible through NGS.



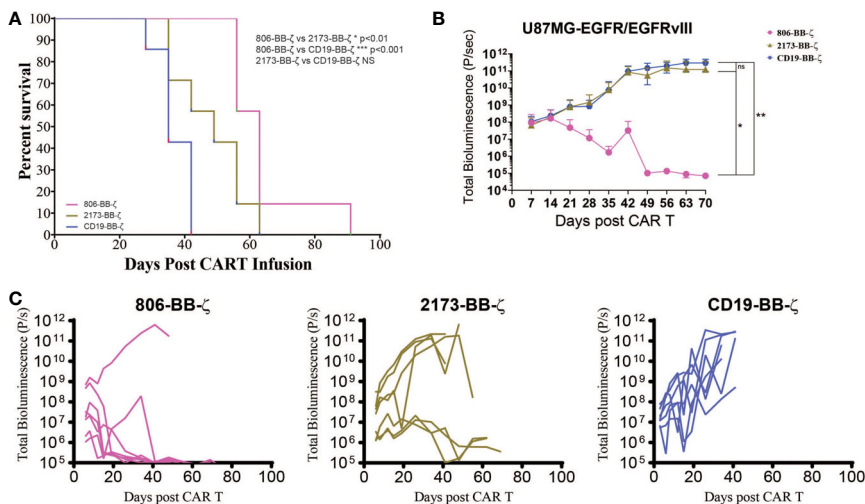


FIGURE 4 | *In vivo* antitumor effect of 806 CAR T cells in NSG mice bearing U87MG-EGFR/EGFRvIII⁺ xenografts. Seven days after 250,000 U87MG-EGFR/EGFRvIII cells were subcutaneously implanted into mice, 3×10^6 T cells were injected intravenously with indicated CAR constructs. **(A)** Survival based on time to endpoint was plotted using a Kaplan–Meier curve and statistically significant differences between CAR groups were determined using the log-rank Mantel-Cox test. Tumor burden was assessed by bioluminescent imaging. Bars indicate means \pm SD ($n = 7$ mice per group). Tumor burden was quantified as total flux **(B)** and in individual mice **(C)** in units of photons/second. Bars indicate means \pm SD ($n = 7$ mice). P = photons. ns, $p > 0.05$; * $p \leq 0.05$; ** $p \leq 0.01$.

GBOs were cocultured with 806, 2173, and CD19 CAR T cells, at a 1:10 E:T ratio, for 72 h before fixation and evaluation. CAR T cell infiltration, as quantified by CD3 staining, was more significant in the 806 CAR T cell population than either the 2173 or CD19 CAR T cell population (Figure 5B). Cleaved caspase 3 (CC3) was used as a measure of cell death and antitumor activity. As with the CD3⁺ cell infiltration, the 806 CAR coculture resulted in higher CC3 levels than either the 2173 or CD19 CAR cocultures (Figure 5C). These results highlighted the broad cross-reactivity of the 806 CAR in a heterogeneous, high-fidelity GBM model. wtEGFR staining in both GBO lines provided additional evidence of the cross-reactive nature of the 806 CAR (Figure 5D). Staining intensity, normalized to CD19 CAR-treated GBOs, showed consistent decreases in 806 CAR-treated GBOs, to a greater degree than the 2173 CAR-treated GBOs (Figure 5E).

DISCUSSION

We have shown broad cross-reactivity of 806 CAR T cells to EGFR mutant proteins resulting in enhanced anti-GBM tumor killing, along with a low on-target, off-tumor effect against both astrocytes and keratinocytes that express wild-type EGFR. Importantly, 806 CAR T cells are able to more effectively control tumor growth in a wtEGFR/EGFRvIII model. 806 CAR T cells also demonstrate greater killing in GBOs with heterogeneity of endogenous EGFR and EGFR mutants, confirming its potential to more effectively treat GBM tumors by limiting the impact of tumor escape due to antigen loss.

With regard to the CAR T trial in recurrent GBM (12), the demonstrated tumor recurrence was likely due to the exclusive

specificity of the scFv employed in the trial. The 2173 construct was chosen for its selective binding to a novel glycine residue formed at the exon 2–7 deletion in EGFRvIII and for a lack of cross-reactivity to wtEGFR (20). However, the binding affinity and target repertoire were of secondary importance. Given the co-occurrence of amplified wtEGFR with EGFRvIII and most ECD missense mutations (11), there is a clinically relevant rationale for targeting multiple EGFR alterations in the GBM population (25). Dual targeting of EGFR and EGFRvIII by CAR T and NK cells has been demonstrated in recent studies using scFvs specific for both antigens (26–29). Our work expands on that, as 806 CAR T cells were able to lyse GBM (U87MG, GSC5077) and non-GBM (K562) cell lines modified to express not only wtEGFR and EGFRvIII but also EGFR extracellular mutations. In comparison, 2173 CAR T cells exhibited specificity for EGFRvIII alone (12, 20). While 806 CAR treated animals did eventually reach experimental endpoints, their loss of weight, patchy hair, and red eyes were suggestive of the development of graft-vs-host disease and not tumor growth. This hypothesis was supported by a lack of palpable or visible tumor at autopsy.

GBM tumors are significantly heterogeneous, both intratumorally (30, 31) and intertumorally (6). Intratumorally, there are mixed cytological subtypes, exhibiting regional differences in gene expression, key genetic mutations, and chromosomal alterations. This polyclonal nature contributes to therapeutic resistance and tumor escape (32). To address intratumoral heterogeneity, relevant targeted therapies would ideally be able to target larger tumor cell populations within the entire tumor bulk. Given the co-occurrence of wtEGFR amplification seen with EGFR mutations and splice variants (24), the cross-reactive EGFR-targeting 806 scFv should provide greater tumor cell coverage, resulting in better tumor control. The potential

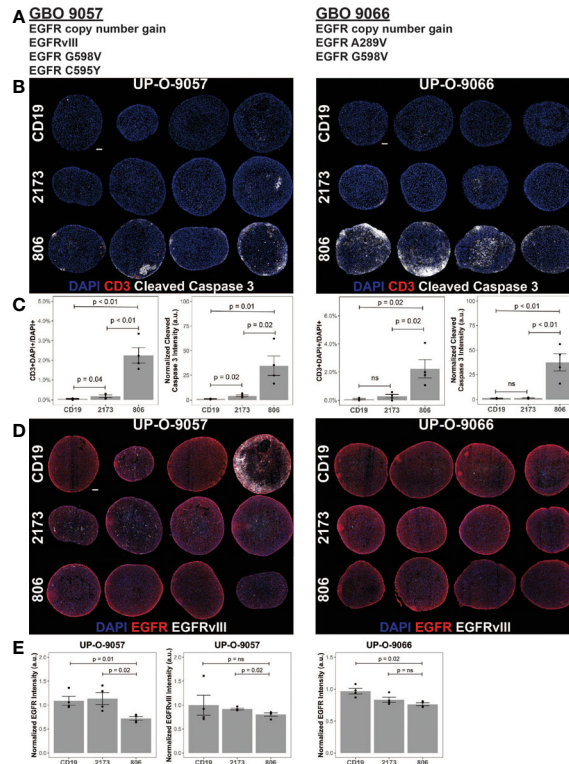


FIGURE 5 | 806 CAR T activity in heterogeneous GBOs highlights cross-reactivity of 806 binder against oncogenic EGFRs. CAR T coculture with GBOs was used to demonstrate anti-EGFR activity. **(A)** EGFR alterations identified in each GBO line. **(B)** Immunofluorescence images of CAR T cells engrafted GBOs, for four organoids per condition, 9057 (left) and 9066 (right). Blue = DAPI; red = CD3⁺; white = cleaved caspase 3⁺ (CC3⁺), scale bar = 100 μ m. **(C)** Quantification of CD3⁺ cells (left) and CC3⁺ cells (right) showing antitumor activity from the 806 CAR T cells. **(D)** Immunofluorescence images of CAR T cell targets in GBOs, for 9057 (left) and 9066 (right). Blue = DAPI; red = EGFR⁺; white = EGFRvIII⁺, scale bar = 100 μ m. **(E)** Quantification of EGFR⁺ (left) and EGFRvIII⁺ signals (right) showing antitumor activity from the 806 CAR T cells. Error bars are \pm standard error. ns, $p > 0.05$.

for broader tumor control was demonstrated through the high-fidelity, heterogeneous GBO model (22). GBOs have retained the originating tumor heterogeneity to a high degree, as assessed by both mRNA and protein levels. These “mini-tumors” provided the opportunity to test 806 CAR T cells against a target-heterogeneous tumor and showcase its ability to exert antitumor activity against a greater portion of the tumor than the compared 2173 CAR and CD19 CAR T cells. While the VAFs associated with the originating tumors of the GBOs allow for hypothesizing of independent EGFR mutant tumor populations, one caveat is that the NGS methods used do not allow for concrete determination of subpopulations. The data were subject to bias from tumor viability and number of reads of the sample. Additionally, the heterogeneity of the EGFR variants on amplified alleles is complicated by the mechanisms of amplification of EGFR. GBMs frequently harbor double minutes, extrachromosomal sequences of DNA that are acentric and lead to asymmetric distribution to daughter cells (33). This causes increased cell-to-cell heterogeneity of EGFR alterations in GBM.

Intertumoral variation, from patient to patient, reduces the applicable population for targeted therapies. However, there are gene families frequently found altered across GBM (6). In particular, EGFR amplification is found in up to 60% of

GBMs. Concurrently with amplification, 30%–40% of GBM tumors express the constitutively active mutant variant, EGFRvIII (34). Combined with the intratumoral expression of EGFR variants, these data suggest that targeting the EGFR family of tumor-specific alterations may successfully address both inter- and intratumoral heterogeneity.

Several EGFRvIII-targeted agents are currently in development or in clinical trials for the treatment of GBM. Although the preclinical data from experimental studies evaluating these therapies have been promising, their efficacy in the clinic has yet to be conclusively demonstrated (35–37). In a vaccination approach to target the EGFRvIII in GBM patients, a phase III trial for newly diagnosed glioblastoma failed to show overall efficacy despite 60%–80% of recurrent tumors showing complete loss of EGFRvIII positive cells (38). Additional trials targeting EGFRvIII demonstrated similar loss of EGFRvIII concurrent with tumor recurrence (12, 39, 40). Similarly, the EGFRvIII-targeting CAR T trial illustrated the continued presence of EGFR amplification and oncogenic EGFR ECD missense mutations despite EGFRvIII antigen loss in posttreatment tumor specimens (12). These results confirm the need to target multiple EGFR alterations simultaneously.

Despite preclinical efficacy, the success of wtEGFR targeting mAbs cetuximab and panitumumab has been associated with on-target, off-tumor toxicity in other tumor types, due to their significant binding to EGFR expressed on normal tissues (41, 42). Their clinical activity in GBM has yet to be successfully demonstrated in large-scale studies. Coculture of 806 CAR T cells with basal physiologic EGFR-expressing normal tissue cell lines did not lead to significant cell killing in our work. Previous work has suggested that the 806 epitope is exposed on both mutated EGFR (EGFRvIII, EGFR^{R108G/K}, EGFR^{A289D/T/V}) as well as amplified wtEGFR found on tumors, but not accessible on wtEGFR found on normal tissue (43). The wtEGFR differences have been proposed to be due to different posttranslational mannose modifications and kinetics of EGFR trafficking in tumors compared to normal tissue (44). Multiple clinical trials with humanized mAb 806 conjugated to a microtubule inhibitor (ABT-414) have demonstrated only low levels of cutaneous toxicity (45–47). The therapeutic window of CAR T cells for tumor-associated antigens relies on the quantitative difference between antigen-overexpressing tumor and antigen-low normal tissue. Preclinical studies targeting EGFR and erBB2 with affinity-lowered CAR T cells have demonstrated potent antitumor effects against high antigen density while sparing low antigen density normal tissue (48–50). The demonstrated cross-reactivity of 806 CAR T cells for EGFR alterations, including amplified wtEGFR, EGFRvIII, and ECD missense mutations, suggests that 806 CAR T cells may be a more efficacious therapeutic strategy to achieve tumor control and prevent tumor escape *via* target antigen loss.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

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ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the University of Pennsylvania Institutional Review Board. The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by University of Pennsylvania Institutional Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

RT and ZB conceived and carried out the experiments, with contributions from YY, LZ, JZ, and DZ for specific assays. MM, GLM, HS, and DO'R supervised the project. RT and ZB wrote the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

The described work was funded by the GBM Translational Center of Excellence, the Templeton Family Initiative in Neuro-Oncology, The Maria and Gabriele Troiano Brain Cancer Immunotherapy Fund, and NIH (R35NS116843 to HS and R35NS097370 to GLM).

ACKNOWLEDGMENTS

The authors thank the Human Immunology Core at the University of Pennsylvania for providing leukocytes for the described work, the Stem Cell and Xenograft Core at the University of Pennsylvania for assistance with the animal work, and the Small Animal Imaging Facility at the University of Pennsylvania for the bioluminescence imaging.

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