# Neuro-Oncology Advances

# Tandem chimeric antigen receptor (CAR) T cells targeting EGFRvIII and IL-13Rα2 are effective against heterogeneous glioblastoma

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**Abstract**
**Background.** Chimeric antigen receptor (CAR)T cells have achieved remarkable responses in patients with hematological malignancies; however, the potential of this therapeutic platform for solid tumors like glioblastoma (GBM) has been limited, due in large part to the targeting of single antigens in a heterogeneous disease. Strategies that allow CART cells to engage multiple antigens concomitantly may broaden therapeutic responses and mitigate the effects of immune escape.
**Methods.** Here we have developed a novel, dual-specific, tandem CART (TanCART) cell with the ability to simultaneously target both EGFRvIII and IL-13Rα2, two well-characterized tumor antigens that are frequently found on the surface of GBM cells but completely absent from normal brain tissues. We employed both standard immunological assays and multiple orthotopic preclinical models including patient-derived xenograft to demonstrate efficacy of this approach against heterogeneous tumors.
**Results.** Tandem CAR T cells displayed enhanced cytotoxicity *in vitro* against heterogeneous GBM populations, including patient-derived brain tumor cultures ($P < .05$). Compared to CART cells targeting single antigens, dual antigen engagement through the tandem construct was necessary to achieve long-term, complete, and durable responses in orthotopic murine models of heterogeneous GBM, including patient-derived xenografts ($P < .05$).
**Conclusions.** We demonstrate that TanCART is effective against heterogeneous tumors in the brain. These data lend further credence to the development of multi-specific CART cells in the treatment of GBM and other cancers.

**Key Points**
- Tandem CART (TanCART) cells were efficiently generated using a single lentiviral construct at high transduction efficiencies.
- Simultaneous, dual-antigen targeting withTanCART cells achieved complete and durable cures in murine models of heterogeneous GBM.

Glioblastoma (GBM) is the most common and most aggressive primary malignant brain tumor, remaining uniformly lethal despite multimodal therapy including maximal resection, radiation therapy, and chemotherapy.[1] Immune-based therapy including checkpoint blockade with antibodies has demonstrated proven clinical benefit across several different liquid and solid cancer

## Importance of the Study

The most common primary malignant brain tumor, GBM, is also the most lethal, with few effective treatment options. Prior studies have shown that CAR T cells targeting the tumor-specific mutation of epidermal growth factor receptor (CART-EGFRvIII) have the capacity to accumulate in and target EGFRvIII-expressing glioma. However, CARs against single antigens are hampered by tumor heterogeneity, leading to eventual immune escape and progression. To mitigate these effects, we modified CART-EGFRvIII to simultaneously target IL-13αR2, another frequently expressed glioma-associated antigen, using a novel tandem CAR (TanCAR) that incorporates antigen-recognition moieties already shown to be safe in Phase I clinical trials. TanCART cells had superior activity compared to monospecific CARs and were able to achieve complete and durable tumor responses in murine models of heterogeneous GBM. This strategy warrants further investigation in patients and could be generalized to target alternative antigen combinations for both GBM and other solid tumors.

types, but has been less successful in GBM, which may be due to inadequate priming of T cells and a local immunosuppressive microenvironment.[2]

One way to bypass the limited T cell receptor (TCR) repertoire and to greatly enhance the activity of antitumor lymphocytes is to engineer T cells to express chimeric antigen receptors (CARs) and to infuse these cells as adoptive immunotherapy. Several CARs targeting different antigens in GBM have been recently described (eg, EGFRvIII,[3] IL-13Rα2,[4] HER2,[5] EphA2[6,7]), some of which are in early clinical development. Importantly, CAR T cells have been shown to localize to the brain and, in at least one case, were able to mediate the regression of late-stage, multifocal, bulky tumors. However, despite initial responses, CAR T cells against single antigens have been associated with disease recurrence, target-antigen loss, and escape in both GBM[3,4] and in hematologic malignancies in which CD19 was targeted.[8]

Novel CAR T cells that target multiple antigens simultaneously have the potential to mitigate the impact of antigen heterogeneity and have emerged as a promising strategy for antigen-loss variants in hematological cancers.[9] Several combinations of target antigens have been proposed with the goal of offsetting immune escape in GBM. However, prior efforts have involved targets with substantial expression in healthy tissues (eg, HER2),[10] an approach that has been shown carry the potential risk of fatal autoimmune toxicity.[11]

Unlike other GBM-associated surface antigens, EGFRvIII and IL-13Rα2 are unique in that they are frequently present on the surface of GBM[12] but completely absent or expressed at negligible levels in somatic tissues, thereby minimizing the risks of on-target, off-tumor toxicity. Recent work has also revealed that EGFRvIII and IL-13Rα2 can be co-expressed in the same cell and that intracellular crosstalk between these two molecules specifically confers a growth advantage to tumors,[13] making them an especially favorable combination for concomitant targeting.

Here, we report preclinical testing and development of a novel tandem CART cell (TanCART) with dual specificity for EGFRvIII and IL-13Rα2, housed in a single transgene construct. We describe the design and the functional capacity of this therapy, demonstrating superiority of TanCART compared to monospecific CART cells in models of heterogeneous tumors and patient-derived GBM.

## Materials and Methods

### Cell Lines

The human glioma cell line U87MG and the chronic myeloid leukemia line K562 were purchased from the American Type Culture Collection (ATCC). The patient-derived glioblastoma neurosphere cell line, BT74, was provided by Dr. Santosh Kesari.[14] This cell line was transduced to constitutively express green fluorescent protein. To isolate IL-13Rα2-positive cells, endogenous IL-13Rα2$^{high}$ U87MG cells were selected by flow cytometry. To create EGFRvIII-positive, IL-13Rα2-negative cells, the U87MG cell line was genetically modified by knocking out IL-13Rα2 through CRISPR-Cas9 with a single-guide RNA (sgRNA) from the Brunello library. This line was then engineered to express EGFRvIII by lentiviral transduction.[15] The K562 cell line was genetically modified to express either IL-13Rα2 or EGFRvIII by lentiviral transduction. Cells were then sorted to collect a clonal population of either IL-13Rα2-negative, EGFRvIII-positive or IL-13Rα2-positive, EGFRvIII-negative. U87MG cells were cultured in Eagle's Minimum Essential Medium (EMEM), supplemented with 10% heat-inactivated fetal bovine serum (FBS) as well as penicillin and streptomycin. K562 cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 (1X) + GlutaMAX, supplemented with 10% FBS as well as penicillin and streptomycin. BT74 cells were cultured in Neurobasal medium, supplemented with L-Glutamine, B27 supplement, N2 supplement, heparin, penicillin, streptomycin, amphotericin B, human recombinant epidermal growth factor, and human recombinant fibroblast growth factor, as previously described.[14]

### CAR T Cell Constructs

All CAR constructs incorporated a second-generation design containing either a hGM-CSF or CD8 leader sequence, a CD8 transmembrane (TM) domain, a 4-1BB costimulatory

domain, and a CD3-zeta signaling domain. In addition, an mCherry reporter, separated by a T2A cleavage site, was inserted to allow for measurement of the transduction efficiency of the transduced cells. All experiments were performed with donor-matched activated untransduced (UTD) T cells, which served as a negative control. The CAR T cell targeting IL-13Rα2 is based on a previously described construct from Dr. Christine Brown and colleagues,[16] which consists of a membrane tethered IL-13 ligand mutated at E13Y, which allows the zetakine to bind more specifically to IL-13Rα2 relative to the more widely expressed IL-13Rα1/IL-4Rα complex. CART-EGFRvIII contains a humanized scFv that is specific for EGFRvIII over wild-type EGFR.[17] The tandem CAR construct was constructed using both the IL-13 zetakine and the humanized EGFRvIII scFv as described above.

## CAR T Cell Production

Healthy donor leukopaks were purchased from the Massachusetts General Hospital blood bank under an IRB-approved protocol. Primary human T cells (Stem Cell Technologies, Cat. #15061) were purified and cryopreserved. To generate CAR T cells, primary T cells were thawed and activated using anti-CD3/CD28 Dynabeads (Life Technologies) at a 3:1 Dynabeads:T cells ratio on Day 0. On Day 1, activated T cells were transduced with lentivirus at a multiplicity of infection of 5 or 10. CART cells were de-beaded on Day 6–7 to allow for further expansion. Transduction efficiency was measured on Day 9–10 and cells were stored in liquid nitrogen on Day 10–15. Cells were cultured in RPMI 1640 (1X) + GlutaMAX with 10% FBS, penicillin, streptomycin, and supplemented with 20 IU ml$^{-1}$ recombinant human IL-2. CART cells expressing different CAR constructs were normalized for CAR expression by adding UTD cells before cryopreservation and thawed prior to functional assays. All reported numbers of CAR T cell in the manuscript are based on CAR positivity.

## T Cell Activation and Proliferation Assays

We conducted T cell activation assays where UTD cells, CART-EGFRvIII, CART-IL-13Rα2, or TanCART cells were co-cultured with K562 cells expressing either IL-13Rα2 or EGFRvIII for 16 h. The cells were then subjected to flow cytometry and assessed for CD69 expression. Long-term proliferation assays were performed using two iterations of irradiated (ie, 10 000 Rads in a Cesium-137 irradiator) K562 expressing the aforementioned targets of interest. Irradiated K562 cells were co-cultured with effector cells from three healthy donors at a 1:1 ratio. Media was replenished on Day 3–4. Every week, cells were counted to measure population doubling. Following counting, CAR T cells were re-stimulated with irradiated K562.

## Cytotoxicity Assays

We measured cytotoxicity for the U87MG using the xCelligence Acea Biosciences RTCA Multi Plate reader (ACEA Biosciences). This instrument uses non-invasive electrical impedance monitoring to detect cell attachment in real-time. E-Plate View 96 plates were washed twice and plated with 20 000 U87MG cells per well. Cell index was measured for 27 h before the addition of effector cells. The assay was recorded over 96 h and measurements were taken every 15 min. Percentage specific lysis was calculated using the following equation: Percentage = [(cell index of target cells – cell index of CAR T cells)/(cell index of target cells)] × 100. For the BT74 neurosphere line, cytotoxicity was measured by IncuCyte Live Cell Analysis. This instrument measures cytotoxicity through the measurements of total green-fluorescent area. Proliferation of effector cells was measured by quantifying the total red fluorescence area. BT74 cells were cultured at 10 000 cells per well for three days prior to the addition of effector cells.

## Flow Cytometry

The following antibody clones were used for each target: EGFRvIII (L8A4, Absolute Antibody), IL-13Rα2 (SHM38, BioLegend), CD69 (FN50, BioLegend), CD3 (UCHT1, BD Biosciences), CD4 (SK3, BD Biosciences), and CD8 (SK1, BD Pharmingen). DAPI was used to distinguish live cells from the dead. Cells were stained in the dark for 15 min at room temperature, washed twice in PBS with 2% FBS, and acquired on a Fortessa X-20 (BD Biosciences).

## Animal Models

The mice used in these experiments were NOD.Cg-Prkdc$^{scid}$IL2rg$^{tm1Wjl}$/SzJ (NSG) purchased from the Jackson Laboratory. They were bred under pathogen-free conditions and in line with the protocols approved by the Massachusetts General Hospital Institutional Animal Care and Use Committee (IACUC). Mice were randomized to intraventricularly receive either UTD, CART-EGFRvIII, CART-IL-13Rα2, or TanCART cells suspended in PBS. For all intracranial implantations, tumor cells were loaded in a 10 µl syringe with a detachable 31 gauge needle (Hamilton). With the assistance of a stereotactic frame, tumor cells were implanted at 2 mm lateral to the bregma and a depth of 4 mm from the surface of the skull at the coronal suture. CAR T cells were loaded in an Ultra-Fine Insulin Syringe (3/10 mL 30G × 12.7 mm, BD Biosciences) and delivered intraventricularly, contralateral to the tumor implantation, infused at 2 mm to the left of and 0.3 mm anterior to bregma at a depth of 3 mm, as previously described.[18] For all experiments, mice were treated with 1 × 10$^6$ CAR T cells, which has been previously reported to be in a typical "low" dose range for murine studies.[19] Mice were monitored by trained, dedicated research personnel responsible for observing and evaluating animals. Euthanasia was determined by humane endpoints defined by deteriorating body condition, weight loss, inability to rise or ambulate, dehydration, and the presence of ulcerated, necrotic or infected tumors. Post-hoc power calculations were performed between TanCART-treated mice versus those treated with the UTD control utilizing log transformed bioluminescence measurements of tumor growth (BT74) as a continuous variable at day 70 post-treatment. Such an analysis with three mice per

group had a 97.2% power to detect a difference between groups assuming α = 0.05.[20]

## Statistical Analysis

All analyses were performed with GraphPad Prism 7.0c software. Data were presented as means ± SD or SEM with statistically significant differences determined by tests as indicated in figure legends. All data presented in the manuscript, both *in vitro* and *in vivo*, reflect the results of multiple experiments (at least two), in some cases across several different model systems.

# Results

## Design and Generation of CAR T Cells With Single and Dual Antigen Specificity

To target GBM, we designed several second-generation CAR constructs with single and dual specificity for IL-13Rα2 and EGFRvIII. We used two monospecific CAR T cells, CART-EGFRvIII and CART-IL-13Rα2, which employed the humanized anti-EGFRvIII scFv and IL-13 zetakine respectively, both of which have established safety profiles in prior clinical trials.[3,16] In addition, we designed a TanCART construct with dual specificity consisting of both the anti-EGFRvIII scFv and IL-13 zetakine translated in tandem separated by a flexible glycine-serine linker (Figure 1A and B). We generated and tested the two possible orientations regarding the order of antigen-recognition domains (ie, humanized EGFRvIII scFv proximal to the membrane with IL-13 zetakine distal, and *vice versa*). The construct consisting of a proximal IL-13 zetakine and distal EGFRvIII scFv resulted in unforeseen effects on lentivirus production and yielded prohibitively low titers, precluding T cell transduction. Regarding the flexible linker, we selected a standard $(Gly_4Ser)_4$ sequence that we have optimized and used with success in our previously published work.[21] In addition, this 20 amino acid Gly-Ser linker was employed in the original manuscript describing TanCAR, which also informed our design.[22]

All CAR T cells were generated *via* lentiviral transduction into primary human lymphocytes isolated from three healthy donors and manufactured according to the timeline depicted in Figure 1C. Using a third-generation, self-inactivating lentiviral vector system, comparable transduction efficiencies and mean fluorescence intensities (MFI) were achieved across all constructs as measured by flow cytometry for the mCherry reporter gene (Figure 1D, Supplementary Figure 1). Surface CAR expression using polyhistidine-tagged cognate antigen did not yield significantly different proportional expression when compared to mCherry alone (data not shown).

## Single-antigen Engagement is Sufficient to Activate and Induce Proliferation of TanCART Cells

We studied the activation of TanCART cells compared to corresponding single-antigen specific CARs in the presence
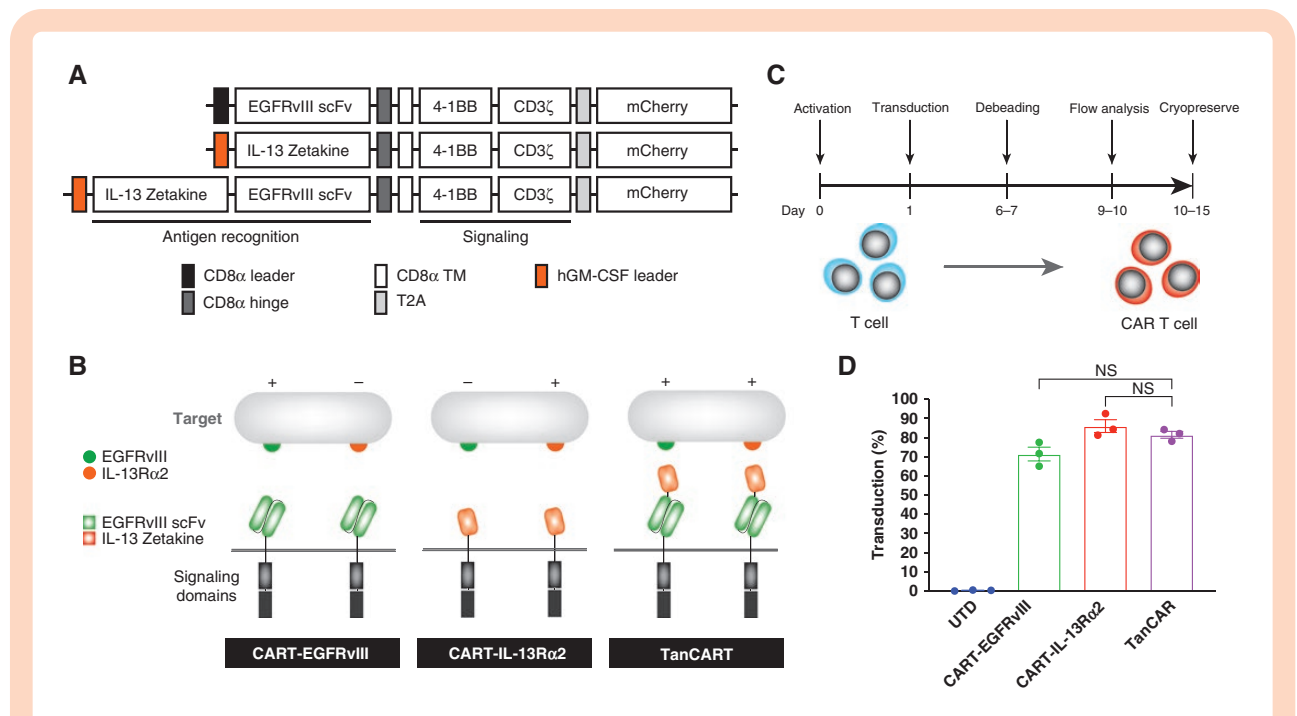


**Figure 1. Design and generation of TanCART cells.** (A) Single- and dual-specific CARs were designed to target EGFRvIII and IL-13Rα2. scFv, single-chain variable fragment; TM, transmembrane domain; T2A, 2A self-cleaving peptide. (B) Illustration of the second-generation constructs for CART-EGFRvIII, CART-IL-13Rα2, and TanCART. (C) Schematic depicting the timeline for production of CAR T cells. (D) Average CAR transduction efficiencies in primary human T cells from 3 healthy donors. Data are shown as mean ± SD.

of either the EGFRvIII or IL-13Rα2 antigen. In response to stimulation with cells expressing EGFRvIII, we found that T cells transduced with TanCART and CART-EGFRvIII strongly upregulated CD69 surface expression in contrast to agnostic cells transduced with CART-IL-13Rα2 or otherwise untransduced (UTD) cells (Figure 2A). Conversely, in the presence of IL-13Rα2-expressing targets, only cells transduced with either TanCART or CART-IL-13Rα2 upregulated CD69 (Figure 2B). A more detailed analysis demonstrated that TanCART upregulated exhaustion markers (eg, PD-1, TIM-3, and LAG-3) and produced Th1 proflammatory cytokines (eg, IL-2, IFNγ, and TNFα) in response to both single-positive, pooled, and double-positive target cell lines in a similar fashion compared to their monospecific counterparts (Supplementary Figure 2). However, only TanCART cells responded as such in the presence of both single-antigen positive lines, unlike monospecific CAR T cells which did so only in the presence of their respective cognate antigen.

Next, we investigated the proliferative capacity of TanCART in response to single-antigen stimulation. We performed long-term growth cultures using irradiated target cells. Upon stimulation with irradiated K562$^{EGFRvIII}$ we found that TanCART cells demonstrated logarithmic growth over three weeks. The difference between TanCART and the monospecific CART-EGFRvIII in this setting was not significant (Figure 2C). Likewise, when stimulated with

K562$^{IL-13Rα2}$, TanCART cells also proliferated with growth kinetics that were not significantly different when compared to monospecific CART-IL-13Rα2 T cells throughout the same interval (Figure 2D). Thus, we found that single-antigen engagement is sufficient to activate and induce proliferation of TanCART cells, and that these parameters do not appear to be significantly different when compared to monospecific CART cells targeted at each antigen in isolation.

## TanCART Cells Demonstrate Superior Cytotoxicity Against Heterogeneous Glioma

To model heterogeneous glioma and the effects of antigen escape, we generated GBM cell lines with expression of either EGFRvIII or IL-13Rα2 (ie, either EGFRvIII$^+$/IL-13Rα2$^-$ U87MG or EGFRvIII$^-$/IL-13Rα2$^+$ U87MG). Using these newly generated GBM cell lines, we produced mixed target cell populations at different proportions. We used target ratios of 10:90 and 90:10, as this has been described previously to recapitulate heterogeneous EGFRvIII expression in preclinical models, and to provide a relatively stringent model that allowed us to test efficacy of the TanCART against monospecific CARs, even when the vast majority of the tumor expressed the monospecific target of interest. We evaluated the cytotoxicity of TanCART cells against these mixed cell populations using an impedance-based
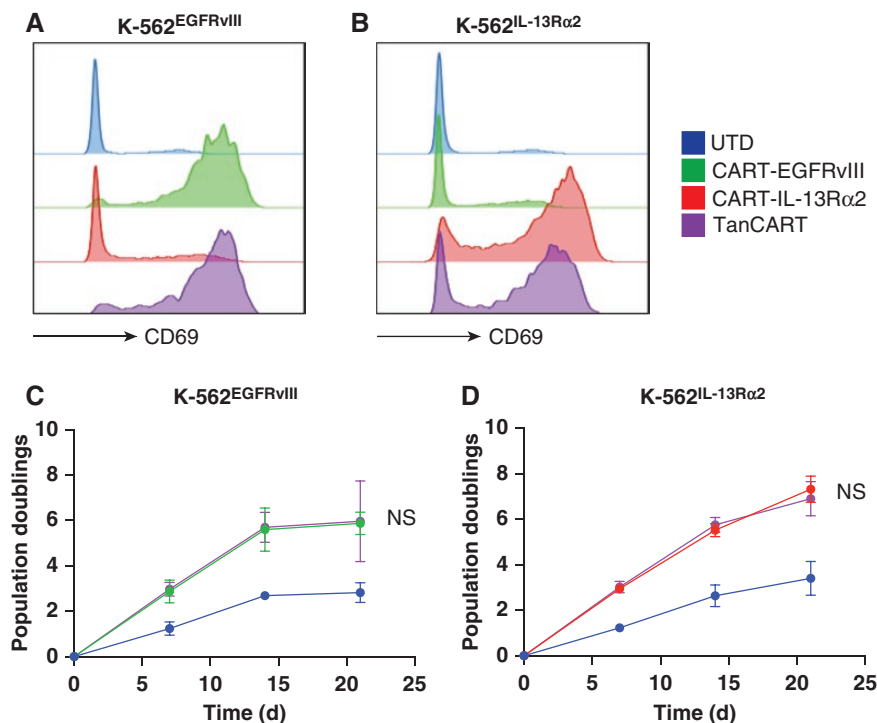


**Figure 2.** **TanCART cells activate and proliferate upon single antigen encounter.** (A) Activation of UTD, CART-EGFRvIII, CART-IL-13Rα2, and TanCART cells in response to stimulation with K562-EGFRvIII and (B) K562-IL-13Rα2. CD69 expression was measured following 16 hours of co-culture at an effector-to-target ratio of 1:1. (C) Long-term proliferation of UTD, CART-EGFRvIII, CART-IL-13Rα2, and TanCART cells during weekly stimulations with irradiated K562-EGFRvIII and (D) K562-IL-13Rα2. Data are shown as mean total cell counts ± SD of three healthy donors. Statistical significance was calculated using unpaired two-tailed t-tests, NS = not significant.

assay at various effector-to-target ratios. Using a predominantly EGFRvIII-positive target population (Figure 3A), real-time measurements demonstrated that while wells containing monospecific CAR T cells yielded only partial elimination of the mixed target cell population, TanCART cells mediated rapid tumor cytolysis with enhanced kinetics and potency (Figure 3B, Supplementary Figure 3). When displayed as percentage cytotoxicity at several time points, TanCART cells were more efficacious against mixed glioma populations, even at lower effector-to-target ratios (Supplementary Figure 3), compared to their monospecific counterparts (Figures 3C and D). These results were consistent in tests against a predominantly IL-13Rα2-positive population, wherein TanCART also exhibited more rapid and complete cytotoxicity when compared to either monospecific CAR T cell alone (Figure 3E–H). TanCART did not exhibit increased off-target activity against target cells compared to monospecific CAR T cells targeting EGFRvIII (Supplementary Figure 4).

The GBM patient-derived xenograft (PDX) BT74 has been previously described as an intrinsically heterogeneous cell line that maintains physiologically relevant levels of EGFRvIII and faithfully recapitulates characteristics of the primary disease.[23] BT74 is somewhat unique given that, in a study of more than 11 established GBM PDX neurospheres available to us, only one (ie, BT74, formerly GBM6) demonstrated suitable EGFRvIII expression for our purposes.[23]

We found that BT74 neurospheres express endogenous and heterogeneous levels of both EGFRvIII and IL-13Rα2, consistent with previous reports from human studies of GBM (Figure 4A).[24] We used an image-based, live-cell assay to assess the antitumor effects of TanCART. We observed that TanCART cells tended to cluster and proliferate in response to BT74 neurospheres in culture (Figure 4B). We also demonstrated specific antitumor activity of TanCART against BT74 (Figure 4C). Additional evaluation revealed concomitant proliferation of TanCART cells that was enhanced compared to wells containing monospecific CAR or UTD T cell controls (Figure 4D).
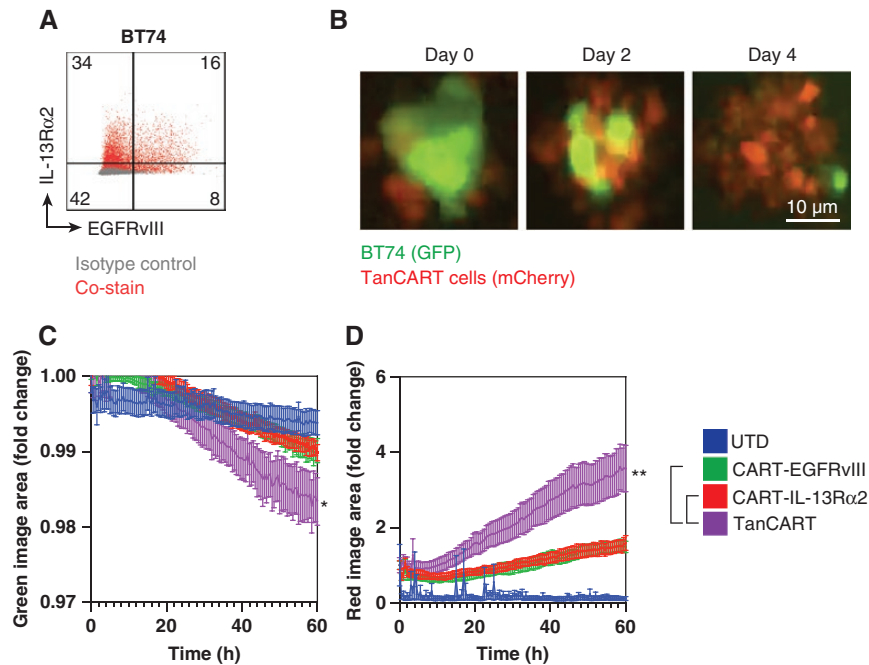
## TanCART Cells are Efficacious Against Heterogeneous Glioma in an Orthotopic Mouse Model

We assessed the anti-tumor activity of TanCART cells against mixed human glioma (U87MG) populations orthotopically implanted into the brains of NSG mice. Using this model, we demonstrated complete and durable treatment responses with universal long-term survival in all mice treated with TanCART cells. Conversely, mice treated with monospecific CAR T cells displayed incomplete responses and shorter survival times comparable to those receiving UTD T-cell control (Figure 5A–D).

Having demonstrated effective killing of an engineered model of heterogeneity, we extended our findings to test
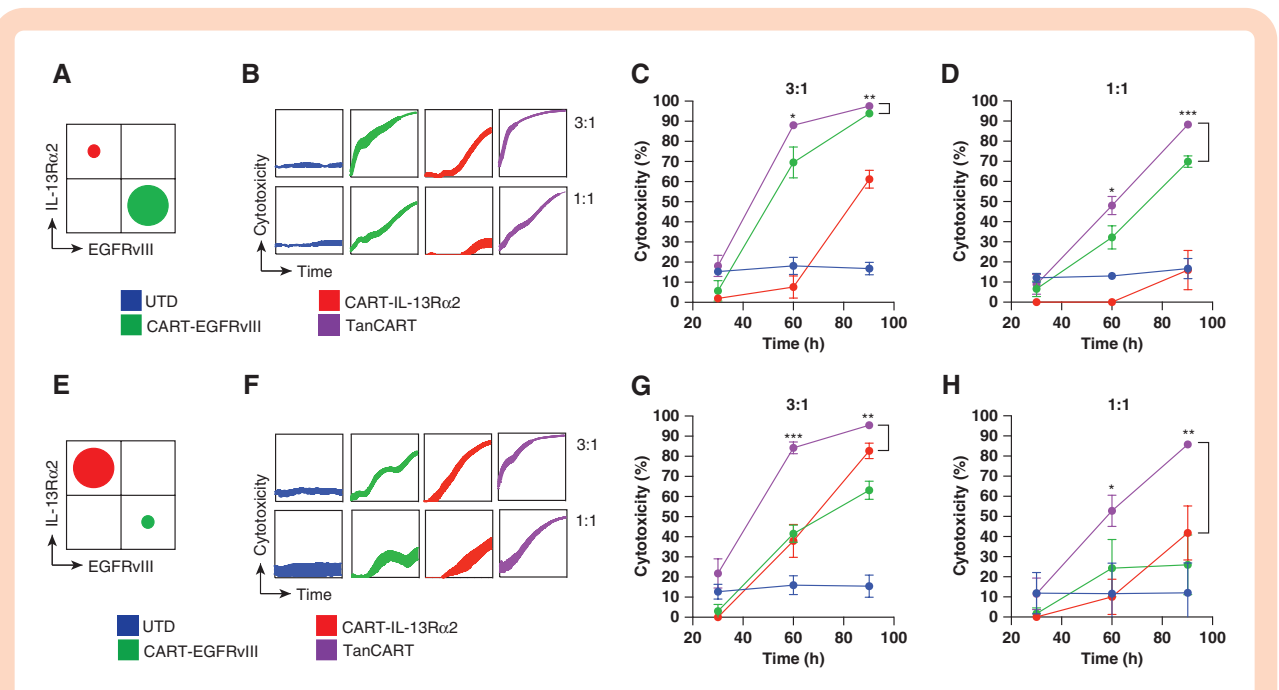


**Figure 3.** **TanCART cells exhibit *in vitro* cytotoxicity against heterogeneous GBM cell populations.** (A) Co-cultures of mixed target U87MG cells at 90:10 (EGFRvIII:IL-13Rα2) were utilized at effector-to-target ratios of 3:1 and 1:1. (B) Impedance-based cytotoxicity assays of UTD, CART-EGFRvIII, CART-IL-13Rα2, and TanCART cells against mixed U87MG target populations were performed. Effector cells were added to plated target cells at 28 h. (C) Individual time points were recorded at 30-, 60- and 90-hour timepoints at effector-to-target ratios of 3:1 and (D) 1:1. (E) Co-cultures of mixed target U87MG cells at 10:90 (EGFRvIII:IL-13Rα2) were also tested using (F) impedance-based cytotoxicity, again at ratios of (G) 3:1 and (H) 1:1. Data were measured in triplicates and are shown as mean ± SD. Statistical significance was calculated using unpaired two-tailed t-tests, $*P \leq .05$, $**P \leq .01$, $***P \leq .001$, and represent differences between bracketed groups.

**Figure 4.** **TanCART cells are effective against heterogeneous GBM PDX and proliferate in longitudinal assays *in vitro*.** (A) Baseline surface expression levels of EGFRvIII and IL-13Rα2 in the GBM PDX, BT74. (B) Representative images from co-cultures with BT74 neuropsheres with TanCART cells during a cytotoxicity assay at an effector-to-target ratio of 1:1. (C) Live-cell analysis of BT74 neurosphere lysis by UTD, CART-EGFRvIII, CART-IL-13Rα2, and TanCART cells by quantification of total green image area. (D) Proliferation of effector cells measured by total red image area. Data were measured in triplicates and are shown as mean ± SEM. Experiments were repeated with at least two different normal donors showing similar results. Statistical significance at the 60-hour time point was calculated using unpaired two-tailed t-tests, *$P \leq$ .05.

the *in vivo* treatment of the highly physiologic PDX, BT74. Mice were engrafted intracerebrally with BT74 cells and treated with effector cells by a single intraventricular infusion (Figure 5E). Compared to BT74-bearing mice treated with monospecific CAR T cells, only the group treated with TanCART cells exhibited universal, complete, and durable responses (Figure 5F). Brains from surviving mice treated with TanCART did not have detectable tumors by gross or histopathological examination. Mixed tumors (eg, either U87MG-based or BT74 PDX) maintained heterogeneous expression of both target antigens following engraftment. However, in mice treated with monospecific CAR T cells, treatment failure corresponded with loss of expression of the corresponding tumor antigen of interest (Supplementary Figure 5).

## Discussion

CAR T cell therapy holds great promise for patients with GBM, but early clinical trials suggest that treating heterogeneous tumors using a single antigen approach may be limited by immune escape and tumor progression. Targeting multiple antigens simultaneously with a tandem CAR T cell has the potential to mitigate this effect. In the current study, we applied this strategy to generate a novel CAR that targets two GBM antigens (eg, EGFRvIII and IL-13Rα2). This CAR incorporates antigen-recognition domains identical to

those that have been demonstrated to be safe in humans at the amino-acid level. An additional advance is the specific combination of antigens we used, which allows TanCART cells to target gliomas while minimizing on-target toxicity or cross-reactivity with normal somatic tissues. Finally, we found that TanCART was able to mediate complete and durable responses in murine models of GBM including PDX, with activity superior to that of their monospecific CAR counterparts against heterogeneous tumors.

Identifying suitable combinations of surface CAR targets can be difficult since most antigens associated with GBM are also widely expressed throughout the human body. Underscoring this challenge, dual-specific[10] and trivalent[25] CAR T cells for GBM consisting of a scFv against HER2[10] and intracellular CD28 costimulatory domains have been previously described; however, HER2 is known to be broadly expressed across several epithelial tissues, and CAR T cells targeting HER2 have resulted in the development of severe autoimmunity in humans—in one patient leading to fulminant and fatal on-target toxicity.[11] A dual-specific CAR T cell targeting EphA2 has been recently described as well, although this target is also known to be widely expressed in healthy tissues.[26] By contrast, we selected EGFRvIII for the TanCART platform based on our previous clinical experience demonstrating safety of CAR T cells targeting this mutation, and because it represents one of the few truly tumor-specific GBM-associated surface markers that has been characterized. IL-13Rα2 is also
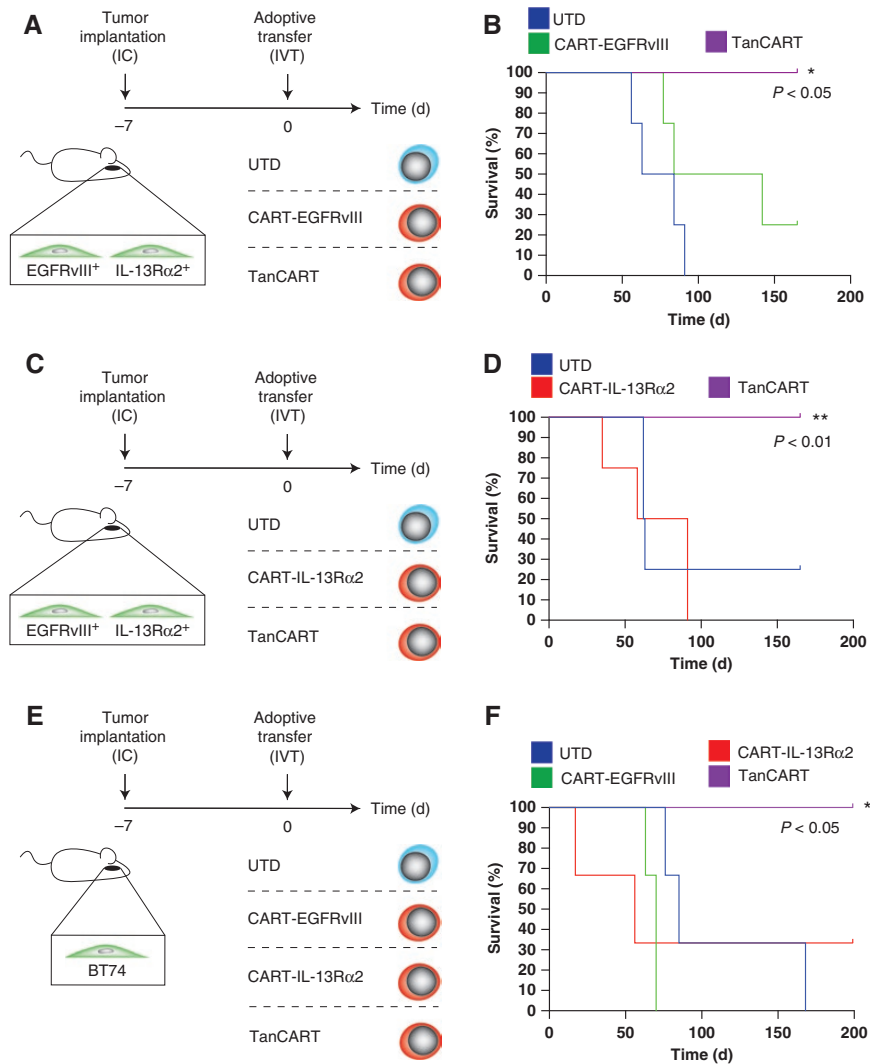
**Figure 5.**  **TanCART cells achieve complete and durable antitumor responses against heterogeneous brain tumors in an orthotopic animal model of GBM.** (A) Schematic of the experimental design where mice were intracerebrally implanted with a mixed population of U87MG and treated with $1 \times 10^6$ effector cells intraventricularly seven days later ($N = 4$). (B) Kaplan-Meier survival analysis of the aforementioned experiment in which the tumor consisted primarily of IL-13Rα2-positive cells (10:90, EGFRvIII:IL-13Rα2). (C) Schematic of the experimental design where mice were alternatively engrafted with tumors consisting primarily of EGFRvIII-positive cells (90:10, EGFRvIII:IL-13Rα2) and (D) treated with monospecific or TanCART cells. (E) Mice were also engrafted intracerebrally with the GBM PDX, BT74 ($5 \times 10^5$ cells) and treated with a single dose of $1 \times 10^6$ effector cells intraventricularly 7 days later ($N = 3$). (F) Kaplan-Meier survival analysis demonstrating survival of TanCART-treated animals compared to UTD and negative control, monospecific CAR T cell groups. Statistical significance was calculated by the log-rank test between TanCART and monospecific CAR T cell groups, $*P \leq .05$.

frequently detected in GBM and has been targeted safely by CAR T cells in humans, with negligible expression in normal brain and peripheral organs.[27] It has been recently shown that expression of IL-13Rα2 and EGFRvIII in glioma tissues is significantly heterogeneous, suggesting that strategies to target these two antigens simultaneously could provide broader activity within individual tumors. In one study, five out of five EGFRvIII-positive tumors had detectable expression of IL-13Rα2, and two out of five of these tumors had levels of expression greater than 75%.[28] We designed several second-generation CAR constructs with single and dual specificity for EGFRvIII and IL-13Rα2.

We used monospecific CAR T cells directed against EGFRvIII (CART-EGFRvIII), which employed a humanized anti-EGFRvIII scFv and had been previously optimized for high specificity against EGFRvIII over wild-type EGFR.[29] In addition, we used monospecific CAR T cells directed against IL-13Rα2 (CART-IL-13Rα2), which were constructed from a previously described zetakine binder, a membrane-tethered IL-13 ligand mutated at E13Y, to confer higher specificity for IL-13Rα2 relative to the more widely expressed IL-13Rα1/IL-4Rα complex.[30,31] Importantly, both monospecific CAR T cells, CART-EGFRvIII and CART-IL-13Rα2, have previously established safety in prior early-phase clinical

trials.[3,32] Together, these CARs formed the basis of the TanCART construct and design presented here.

In our study, all mice in the TanCART group had durable and complete responses despite the fact that a sizeable portion of BT74 cells in culture was not found to express high levels of either antigen. The explanation for this finding is unclear, but may either stem from artifact associated with minor HLA mismatch between tumor and donor T cells, or may be due to fluctuations in target antigen expression within tumors themselves. We know, for instance that tumor antigen expressed naturally by gliomas—as opposed to transduced or modified cell lines—can change over time. It has been described, for instance that EGFRvlll present in primary tumor specimens may be absent at later times such as at recurrence.[33] In the same vein, in a previous clinical trial of the IL-13Rα2 CAR T cell for glioblastoma—despite heterogeneous target antigen expression prior to treatment—complete responses were initially observed after administration of a monospecific CAR, although this was followed by eventual disease recurrence.[4] The explanation for this outcome is currently unclear and is the subject of further investigation.

In a recent study, we reported that CAR T cells designed to secrete bispecific T cell engagers (BiTEs) against a broadly expressed antigen at very low concentrations could effectively circumvent antigen escape without detectable systemic toxicity.[34] Both the TanCART and CART.BiTE platforms could potentially address shortcomings observed in our previous clinical trials of CART-EGFRvlll—namely the successful targeting of EGFRvlll but subsequent outgrowth and progression of tumors with underlying heterogeneity.[3] Notably, rather than competing with each other, TanCART and bispecific antibodies are likely compatible, and combining these technologies could represent an opportunity to further expand the multivalent capacity of the fundamental CART cell approach.

Similarly, an alternative strategy to targeting multiple antigens simultaneously includes the concomitant infusion of monospecific CARs, which could ultimately mediate antitumor activity against heterogeneous tumors in a similar fashion to the TanCART approach. However, one of the main advantages of TanCART is the ability to manufacture cell therapy using a single genetic modification; that is to say, with insertion of a single transgene and infusion of a single gene-modified cell product, which greatly enhances its translational potential. This is especially true given that producing autologous CAR T cells for patients remains a financially challenging and technically laborious process.

The TanCART design creates a Boolean OR argument, which allows T cells to engage both single and double-positive populations. In the setting of a Boolean OR function, it might be hypothesized that the ability to engage both antigens, and the relative abundance of either target antigen, might lead to activation below the recognition threshold that would be expected when either antigen is encountered in isolation. CAR T cell potency has indeed been shown to be highly dependent on target antigen expression. This differentiates CAR T cells from native T cell receptors (TCR).[35,36] That is to say, whereas a single peptide-MHC complex has been shown to be sufficient to elicit a cytolytic response, CARs often require a certain minimal threshold of target antigen expression before a meaningful antitumor response can be achieved. This was evidenced, for example,

by immune escape related to the emergence of antigen low variants in a clinical trial of CD22 CART cells in patients with B-ALL.[37] As aforementioned, the tandem CAR approach provides a Boolean OR argument, which, at least in theory, allows for target cell recognition at a threshold of antigen expression reflected by the sum of both target antigens combined. Thus, one advantage of tandem CARs may be that they widen a therapeutic window for an otherwise weakly expressed target antigen considered in isolation.

In our group, we typically administer CAR T cells directly into the cerebrospinal fluid to achieve high effector-to-target ratios in the intracranial space. Evidence from preclinical and clinical studies suggests that locoregional delivery may enhance treatment of tumors in the central nervous system, which are thought to be separated from the periphery to some degree by the blood-brain barrier.[4,38,39] Administering cell therapies directly into cerebrospinal fluid may represent a feasible approach to maximizing localization of cell therapies to gliomas, since access into the lateral ventricle is performed routinely by neurosurgeons with ease, and because a substantial portion of cerebrospinal fluid is known to regularly cycle to reach the interstitial spaces of the brain parenchyma, where intrinsic tumors like GBM are located.[40]

We have consistently found intraventricular delivery of CAR T cells to be feasible and superior to systemic delivery when treating tumors in the brain in our preclinical models.[31,41–43] This was also observed in a clinical trial for IL-13Rα2 CAR T cells where a complete response was observed only after repeated intraventricular infusion, as opposed to prior intracavitary administration in the same individual.[4] Whether CAR T cells in the CSF simply traffic through CNS compartments by bulk flow or alternatively *via* regulated processes at the blood-cerebrospinal fluid barrier is currently unclear and requires further investigation. Ultimately, the potential mechanisms underlying survival and trafficking of CAR T cells in this setting may be best addressed in syngeneic models or even humans, which have the capacity to capture appropriate physiological interactions among infused cells, a replete immune system, and other endogenous tissues.

There is currently a relative dearth of adequate preclinical animal models that precisely reflect both the intact immunity and the profile of target antigen expression that would be encountered in patients with GBM. We chose the NSG mouse model as it permits the direct evaluation of human T cells in a format that is also translatable in terms of assessing efficacy against human glioma. A disadvantage of this system is the absence of a representative tumor microenvironment and endogenous immune system, which may modulate anti-tumor responses[44] or could reveal additional relevant toxicities. Optimizing immune-competent models may greatly enhance future studies seeking to test novel constructs with maximum fidelity in the preclinical setting. To date, these models are unfortunately still not fully predictive of human toxicities from novel immunotherapies. If and when TanCART cells are translated to Phase I clinical trials, the treating physicians will be required to discuss potential toxicities as part of the informed consent process.

A limitation of our study is that certain cell lines were transduced to overexpress antigens of interest, apart from the BT74 neurospheres. It is likely that the BT74 PDX tumor

line most closely recapitulated endogenous target expression in GBM, as the cells remained unmodified and had naturally heterogeneous patterns of expression.

To our knowledge, this is the first report of a tandem CAR T cell targeting EGFRvIII and IL-13Rα2 simultaneously. The results obtained with this construct address prior shortcomings in clinical trials of both EGFRvIII- and IL-13Rα2-targeted CAR T cells and warrant further investigation in patients with GBM.

## Supplementary Material

Supplementary material is available online at *Neuro-Oncology Advances* online.

## Keywords

chimeric antigen receptor | glioblastoma | immunotherapy.

## Authorship statement

A.S., B.D.C., and M.V.M. designed the research. A.S., A.A.S., R.R., S.R.B., A.A.B., M.J.F., M.C.K., R.C.L., M.B.L., L.G.R., I.S., and J.S. performed experiments. D.P.C., B.S.C., W.T.C., G.P.D., E.R.G., B.V.N., H.W., M.V.M, and B.D.C. contributed reagents and analytic tools. A.S., A.A.S., J.Y.G., M.V.M., and B.D.C. analyzed and interpreted data. A.S., A.A.S., M.V.M., and B.D.C. wrote the paper.

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