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EphA3 CAR T cells are effective against glioblastoma in preclinical models

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ABSTRACT

Background Adoptive T-cell therapy targeting antigens expressed in glioblastoma has emerged as a potential therapeutic strategy to prevent or delay recurrence and prolong overall survival in this aggressive disease setting. Ephrin receptor A3 (EphA3), which is highly expressed in glioblastoma; in particular, on the tumor vasculature and brain cancer stem cells, is an ideal target for immune-based therapies.

Methods We have designed an EphA3-targeted chimeric antigen receptor (CAR) using the single chain variable fragment of a novel monoclonal antibody, and assessed its therapeutic potential against EphA3-expressing patient-derived glioblastoma neurospheres, organoids and xenografted glioblastoma tumors in immunodeficient mice.

Results In vitro expanded EphA3 CAR T cells from healthy individuals efficiently recognize and kill EphA3-positive glioblastoma cells in vitro. Furthermore, these effector cells demonstrated curative efficacy in an orthotopic xenograft model of glioblastoma. EphA3 CAR T cells were equally effective in targeting patient-derived neurospheres and infiltrate, disaggregate, and induce apoptosis in glioblastoma-derived organoids.

Conclusions This study provides compelling evidence supporting the therapeutic potential of EphA3 CAR T-cell therapy against glioblastoma by targeting EphA3 associated with brain cancer stem cells and the tumor vasculature. The ability to target patient-derived glioblastoma underscores the translational significance of this EphA3 CAR T-cell therapy in the pursuit of effective and targeted glioblastoma treatment strategies.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Over the last two decades, there has been minimal improvement in the overall survival of patients with glioblastoma. Previous attempts to develop an effective immunotherapy for glioblastoma have largely failed. Recent studies have shown that erythropoietin-producing hepatoma type-A receptor 3 (EphA3) is overexpressed in glioblastoma and is associated with cancer stem cells and newly formed tumor microvasculature.

WHAT THIS STUDY ADDS

⇒ We have designed a novel EphA3-targeted chimeric antigen receptor (CAR) T-cell therapy and demonstrate that these CAR T cells can kill glioblastoma patient-derived cell lines, organoids and block the outgrowth of glioblastoma in vivo.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ Results from this study suggest that EphA3 targeting can disrupt critical structures for tumor progression and resistance. This approach may offer improved survival of patients with glioblastoma.

BACKGROUND

Glioblastoma is the most common type of primary brain cancer in adults. Despite increasingly aggressive treatments incorporating surgical resection followed by concomitant radiotherapy and chemotherapy, survival rates for patients with glioblastoma have only seen modest improvements over the past decades. No cure is available and, if untreated, glioblastoma can result in death in 6 months or less.[1 2] Such poor prognosis has prompted the development of advanced therapies to supplement the current standards of care. In recent years, immunotherapy using T cells engineered to express chimeric antigen receptors (CARs) have revolutionized the treatment of many cancers, increasing the hope for glioblastoma therapy. CD19-directed CAR T-cell therapy has shown remarkable success in treating B-cell acute lymphoblastic leukemia and certain subtypes of B-cell non-Hodgkin's lymphomas.[3 4] CAR19 therapy's success may partly be due to the uniform expression of CD19 in B-cell malignancies and its restricted expression to mature B cells, B-cell precursors and plasma cells in normal tissues. Furthermore, CAR19 treatment-induced B-cell aplasia can be tolerated by patients due to effective clinical management. In solid tumors, the lack of an ideal, or appropriate target has complicated the development of CAR T-cell therapies.

In this study, we describe a CAR targeting erythropoietin-producing hepatoma (Eph) type-A receptor 3 (EphA3). Eph receptors are highly expressed during embryonic

development and are found in almost all tissues during embryogenesis.[5] EphA3 is well established for its role during embryogenesis where it drives developmental processes such as cell adhesion, cell migration, and tissue boundary formation. In the brain, directly after birth, its expression is typically downregulated and is minimally expressed in the brain,[6] and lowly expressed in healthy tissues.[7 8] In cancer cells, these proteins are overexpressed and they contribute to cancer pathogenesis.[9 10] EphA3 expression has been reported in a number of solid and hematological cancers, including in glioblastoma.[11–15] In glioblastoma cells, EphA3 is highly expressed in mesenchymal stromal cells of the microvasculature and self-renewing glioblastoma stem cells (GSCs), where it has a functional role in survival and self-renewal.[13 16 17] More recently EphA3 was shown to be significantly elevated in recurrent post-treatment versus primary disease[18 19] suggesting a role in treatment resistance and tumor relapse. These findings establish this receptor as an attractive tumor-specific target for glioblastoma therapy. We show that targeting EphA3-positive tumor with CAR T cells induces a potent antitumor response, and provide preclinical proof-of-principal that EphA3 CAR T cells may be a potentially effective therapeutic strategy for the treatment of glioblastoma.

RESULTS

Isolation and characterization of EphA3-specific monoclonal antibody

To generate the EphA3-specific monoclonal antibody, mice were immunized with recombinant EphA3 protein, as outlined in the methods section. Following in vitro assessment for EphA3-specific reactivity, 10 unique hybridoma clones were expanded and then assessed for antigen-specific reactivity using serially diluted EphA3 antibody. Clone 3C3-1 showed the highest functional avidity for EphA3 (figure 1A) and was selected for further

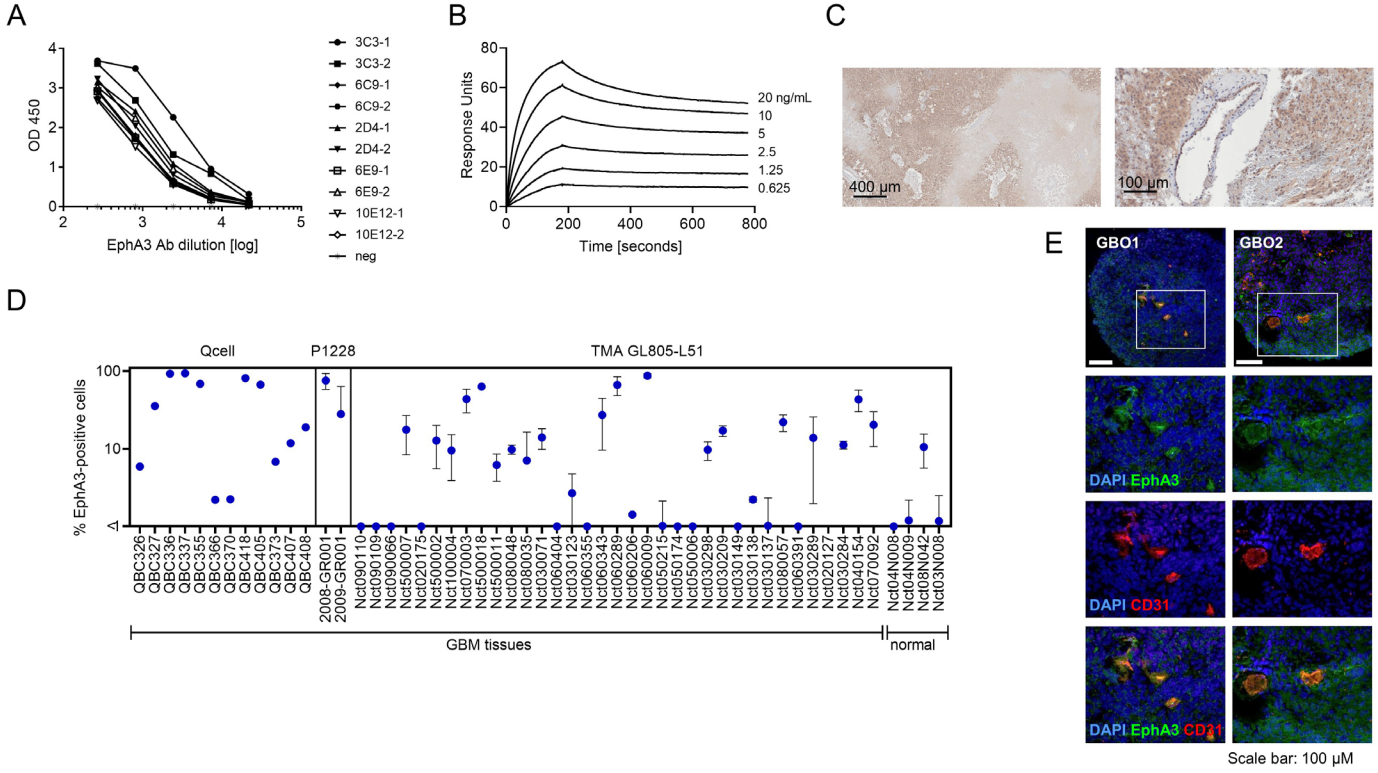

Figure 1 Novel 3C3-1 anti-EphA3 monoclonal antibody has a high affinity for human EphA3 which is expressed on glioblastoma (A) ELISA titration curve of 10 EphA3-specific monoclonal antibody clones. Monoclonal antibody 3C3-1 was selected for further analysis. (B) Surface plasmon resonance sensorgram of clone 3C3-1 binding to EphA3 protein. Kinetic data of antibody binding to EphA3: association (Ka) $2.22\times10^{8}\pm1.59\times10^{7}$ (1\Ms), dissociation (Kd) $3.85\times10^{-5}\pm2.79\times10^{-5}$ (1/s) and equilibrium (KD) $1.14\times10^{-13}\pm7.54\times10^{-14}$ (M). The reported values are the mean±SD of at least two independent experiments (C) immunohistochemistry staining of EphA3 in glioblastoma tissue. Sections were stained with anti-EphA3 3C3-1 antibody and brown areas are positive reactions stained by DAB. Images capture the spatial distribution of EphA3 within the tissue, highlighting tumor heterogeneity. (D) EphA3 protein quantification. The percentage of EphA3-positive cells per section on patient sample was calculated from tissue sections from QCell (n=1 section per patient), clinical trial P1228-QGBM01 and TMA GL805-L51 (n=1–3 section(s) per patient). (E) Two GBO cryosections generated from patient QBC420. Organoids were co-stained with anti-EphA3 (green) and the endothelial cell marker CD31 (red). DAPI (blue) was used for nuclear staining. Images with an enlarged area from each tumor are shown. EphA3, erythropoietin-producing hepatoma type-A receptor 3; GBO, glioblastoma organoid; TMA, tumor micro array; GBM, glioblastoma; DAPI, 4′,6-diamidino-2-phenylindole; DAB, 3,3′-Diaminobenzidine.

characterization. Using surface plasmon resonance (SPR) the purified 3C3-1 antibody showed high binding affinity (K_D of 0.114 pM) to sensor-bound EphA3 protein (figure 1B). In the following experiments, we used the 3C3-1 antibody to assess expression of EphA3 in glioblastoma tissue sections. Representative immunohistochemistry staining images for EphA3 in a biopsy from a patient with recurrent glioblastoma (P1228–GR001) is shown in figure 1C. EphA3 expression was detected in 35/49 (71%; >1% EphA3-positive cells) of glioblastoma biopsies, although this expression was highly variable within individual tissues, and also across multiple biopsies with low to negligible expression in normal tissues (figure 1D and online supplemental figure 1). Further analysis of patient-derived glioblastoma organoids (GBOs) revealed disseminated EphA3 expression throughout the organoid and, importantly, co-localization of EphA3 with CD31-positive cells which are associated with the tumor vasculature, a niche for GSCs[13 20] (figure 1E).

EphA3 CAR design and functional characterization of EphA3 CAR T cells

A second-generation EphA3-targeted CAR expression vector with an antigen-binding domain incorporating sequences of the variable heavy (V_H) and light (V_L) chains of 3C3-1 monoclonal antibody was synthetically designed. This CAR included a CD8 transmembrane region, either CD28 or 4-1BB costimulatory domains, and an activation signal via CD3ζ (online supplemental figure 3A). A CD19 CAR, based on the FMC-63 antibody sequence, was used as a control. T cells expressing EphA3 CAR with either CD28 or 4-1BB costimulatory domains showed comparable in vitro expansion, cytokine expression and cytotoxic function (online supplemental figure 3B–D). However, these CAR T cells showed differential gene expression suggesting a potential impact of costimulatory domains on the maturation of these effector cells (online supplemental figure 3E). CAR T cells with 4-1BB costimulatory domain showed higher expression of *SCOCS3, ITK, MYB and TNFSF10* genes, while T cells with CD28 costimulatory domain showed increased expression of *ITCH, CD27. FOXP1, PTGER2, PRKCH, XIAP* and *PVR* genes which are associated with improved survival and proliferation (online supplemental figure 3F). Based on these observations we selected EphA3 CAR with a CD28 costimulatory domain for further studies (figure 2A).

Next, we validated the in vitro expansion capability and functional characterization of EphA3 CAR T-expressing cells. Peripheral blood mononuclear cells (PBMCs) from four healthy individuals were stimulated with T-cell TransAct followed by transduction with a lentivirus vector encoding the EphA3 CAR. These T cells were expanded for 14 days and then assessed for cellular and phenotypic composition. Data presented in figure 2B–D shows that these in vitro expanded EphA3 CAR T cells predominantly included a combination of central memory, effector memory and effector memory CD45RA$^+$ T cells, while a small proportion of naïve T cells were also detected. Furthermore, these EphA3 CAR T cells specifically recognized and killed EphA3$^+$ U251 cells while EphA3$^-$ U87 cells were not lysed (figure 2E,F and online supplemental figure 2).

In vivo therapeutic assessment of EphA3 CAR T cells

In the first set of experiments, we assessed the specificity of EphA3 CAR T cells using an in vivo xenograft murine model bearing EphA3$^+$ U251 and EphA3$^-$ U87 glioma tumors (figure 3A). Data presented in figure 3B shows that adoptive immunotherapy with EphA3 CAR T cells reversed tumor outgrowth of U251, while the outgrowth of U87 tumors was not impacted. Notably, both U87 tumors treated with EphA3-CAR T cells and U251 tumors treated with non-transduced cells reached similar sizes exceeding 800 mm^3 by day 35. Furthermore, CD19 CAR T cells or non-transduced control T cells, showed no impact on the outgrowth of U251 tumors (figure 3C).

We next assessed the in vivo therapeutic potential of the EphA3 CAR T cells using an orthotopical xenograft model. EphA3$^+$ U251 cells were transplanted using a stereotactic device, at 3 mm depth, into the right cerebral hemisphere of NRG mice. These animals were treated with either EphA3 CAR T cells or CD19 CAR T cells, intravenously as outlined in figure 4A. Adoptive immunotherapy with EphA3 CAR T cells blocked the growth of EphA3$^+$ U251 tumors, while CD19 CAR T cells had minimal impact on tumor growth and the majority of the animals had to be sacrificed due to increasing tumor burden (figure 4B,C and online supplemental figure 4). In the group of animals treated with EphA3 CAR T cells (n=17; two different experiments), one mouse relapsed with a metastatic EphA3$^+$ spinal tumor at day 70 and two mice from the same group were sacrificed due to weight loss. Autopsy revealed signs of splenomegaly and thymic hyperplasia, consistent with graft-versus-host disease. Analysis of blood at weekly intervals (for 4 weeks) after treatment revealed that animals treated with EphA3 CAR T cells maintained circulating human EphA3 CAR$^+$ CD3$^+$ T cells in the peripheral blood compared with those treated with CD19 CAR T cells (figure 4D).

Immune recognition of primary glioblastoma cells and organoids by EphA3 CAR T cells

In the next set of experiments, we used five patient-derived glioblastoma cell lines FPW1, SJH1, WK1, PB1 and JK2 (obtained from the QCell bank) and assessed immune recognition by EphA3 CAR T cells. These cells demonstrated variable expression of EphA3 protein on their cell surface (figure 5A). Flow cytometry analysis showed that primary glioblastoma FPW1 and SJH1 cells were homogenously positive for EphA3, while the expression of EphA3 in WK1, PB1, and JK2 was restricted to a subset of cells (figure 5A). Next, we exposed these tumor cells to EphA3 CAR T cells and assessed expression of tumor necrosis factor (TNF) in effector cells using an intracellular cytokine assay. Data presented in figure 5B shows that four out of five primary glioblastoma cells (FPW1, SJH1, WK1

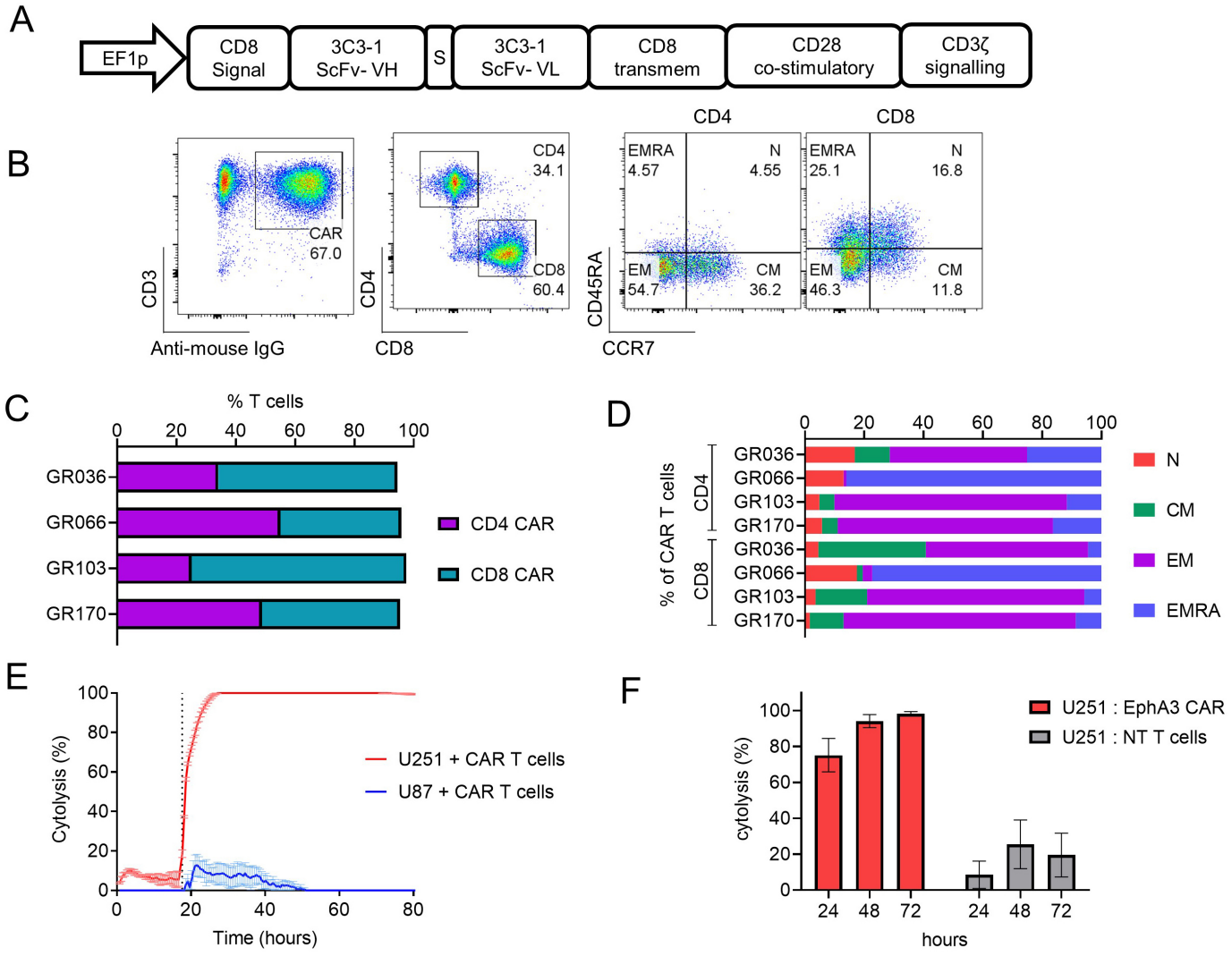

Figure 2 In vitro therapeutic assessment of EphA3 CAR T cells (A) schematic of EphA3 CAR construct. The EF1 promotor drives transcription of the CAR containing the 3C3-1 scFv and CD28 derived co-stimulatory domain followed by the CD3ζ activation sequence. (B) Peripheral blood mononuclear cells from four healthy participants (GR036, GR066, GR103, and GR170) were stimulated with anti-CD3/CD28, transduced with EphA3-CAR encoding lentivirus and cultured for 14 days. Cells were stained with anti-CD3, anti-CD4 anti-CD8, and anti-mouse IgG antibodies, to detect surface CAR protein. Representative FACS plots of CAR T-cell fraction, CAR-CD4 and CAR-CD8 subsets, and memory populations as determined using CCR7 and CD45RA subgating. (C) Proportion of CD4 and CD8 EphA3-CARs and (D) T-cell memory subsets defined as naïve (N: $CCR7^{+}CD45RA^{+}$), central memory (CM: $CCR7^{-}CD45RA^{+}$), effector memory (EM: $CCR7^{-}CD45RA^{-}$), and effector memory RA^{+} (EMRA: $CCR7^{-}CD45RA^{+}$). EphA3-CAR T cells demonstrated cytotoxic activity against glioblastoma cell line U251 (EphA3-expressing glioblastoma cell line). (E) In a real-time killing assay, EphA3 CAR T cells were added to monolayers of U251 or U87 cells at a 5:1 effector-to-target ratio, and the percentage cytolysis was recorded over time. The average cytolysis of three triplicates±SD is shown. (F) Cytolytic potential of EphA3-CAR T cells from four donors was tested against U251 cells. Targets were incubated at a 1:5 effector-to-target ratio. Show are mean percentage cytolysis±SD (n=2–3 replicates) at 24, 48 and 72 hours post addition of effectors. CAR, chimeric antigen receptor; EphA3, erythropoietin-producing hepatoma type-A receptor 3; V_H, variable heavy; V_L, variable light; ScFv, single chain variable fragment.

and PB1) stimulated strong expression of TNF in EphA3 CAR T cells, while a low level of TNF was detected in CAR T cells following exposure to JK2 cells.

Next, we evaluated the killing potential of the EphA3 CAR T cells against these primary glioblastoma cells. EphA3 CAR T cells demonstrated exceptional efficacy in killing glioblastoma monolayers derived from SJH1 and FPW1, achieving a half-maximal killing time (KT50) of <50 hours (figure 5C,D). Interestingly, despite their heterogeneous EphA3 expression, WK1, PB1 and JK2 cells were also efficiently killed by EphA3 CAR T cells with a KT50 value of approximately 50 hours. We extended this analysis by culturing these five primary glioblastoma cells at low densities to promote the formation of neurospheres. After 2 weeks, most of the primary glioblastoma cells formed neurospheres, with the exception of FPW1 cells which maintained monolayer cell growth. These neurospheres were co-cultured with EphA3 CAR T cells

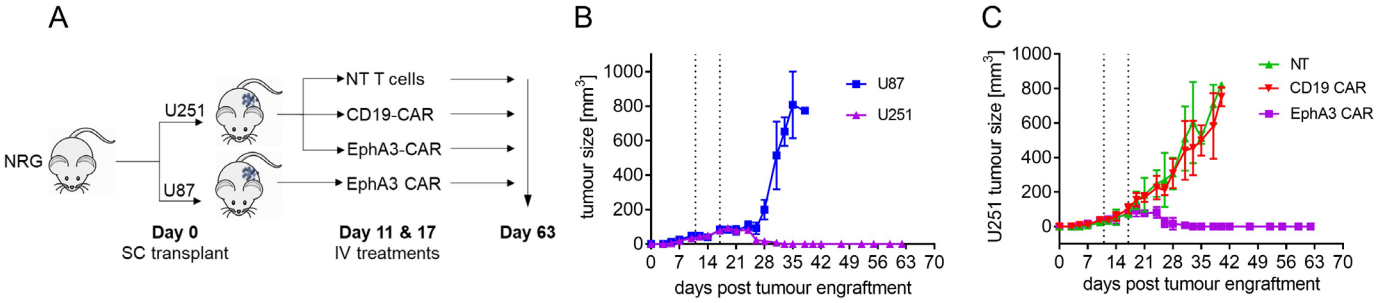

Figure 3 EphA3 CAR T cells demonstrate selective EphA3-targetted killing in vivo. (A) Schematic representation of in vivo experiment. NRG mice were transplanted with luciferase-expressing U251 (EphA3$^+$) or U87 (EphA3$^-$) cells subcutaneously in the flank. Mice were treated once tumors reached approximately 60 mm^3. NT (non-transduced) T cells, CD19-CAR (non-glioblastoma specific CAR T cells), or EphA3-CAR T cells were administered intravenously on days 11 and 17 (dotted lines) post-engraftment with two doses of 20×10^{6}T cells (CAR-positive cells ranged between 22% and 36% of products). (A&C) Tumor size was measured over the course of the experiment. Data points represent the mean±SD of one experiment (n=5 mice per group). CAR, chimeric antigen receptor; EphA3, erythropoietin-producing hepatoma type-A receptor 3; IV, intravenous; SC, subcutaneous.

or CD19 CARs, in the presence of fluorescein isothiocyanate (FITC)-labeled annexin V, a marker of apoptosis. By day 4, apoptotic cells were observed in all primary glioblastoma neurospheres co-incubated with EphA3-CAR T cells (figure 5E and online supplemental videos 1 and 2; online supplemental videos 3 and 4; and online supplemental videos 5 and 6).

To further validate the capacity of EphA3 CARs to target primary glioblastoma, we generated GBOs from three patients undergoing glioblastoma resection surgery

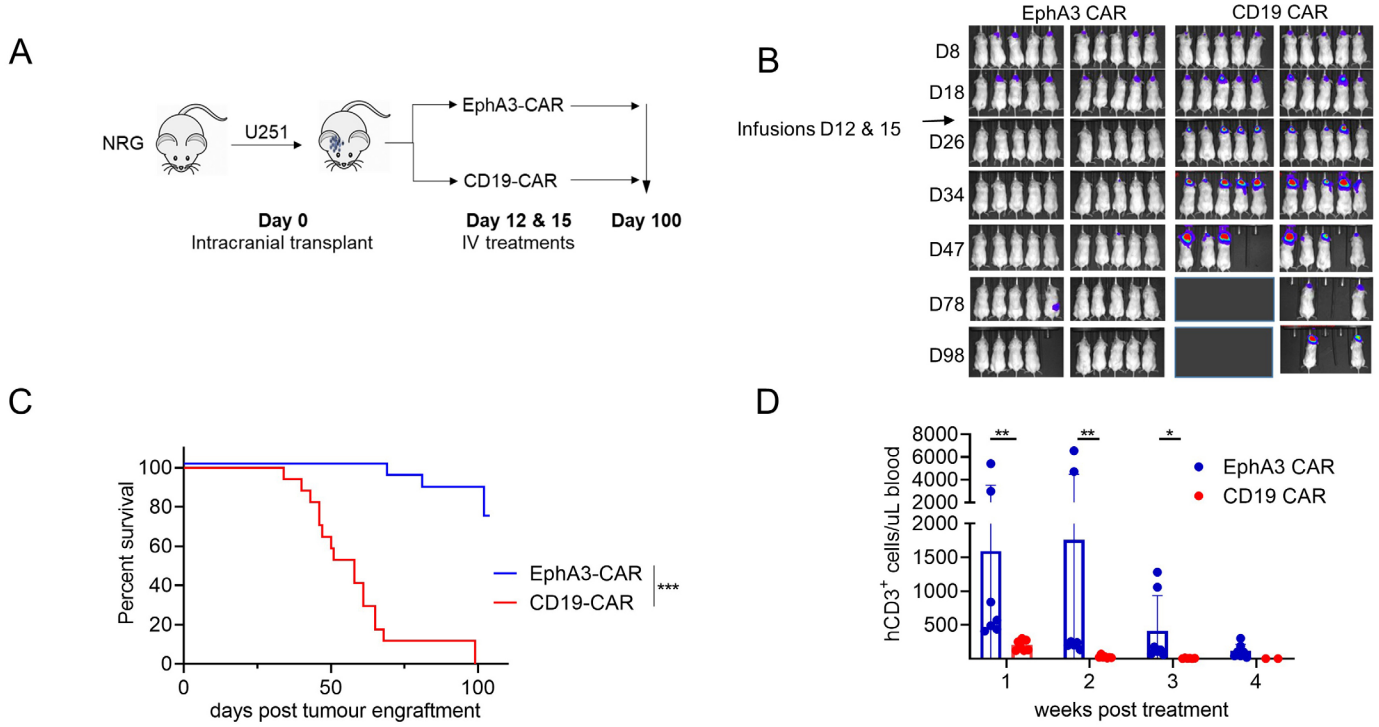

Figure 4 EphA3-CAR T cells prevent the growth of glioblastoma tumors in an orthotopic xenograft mode. (A) Luciferase-expressing cells U251 were orthotopically transplanted in the right cerebral hemisphere of NRG mice. Once tumor had established mice were regrouped so as to normalize the average starting tumor burden per group. Mice received two IV infusions of EphA3-CAR T cells as treatment, and CD19-CAR T cells were administered to control groups. (B) Tumor formation was monitored by bioluminescence imaging of mice up to 100 days post tumor engraftment. Representative image of two independent experiments. (C) Kaplan-Meier survival curve of the mice of two independent experiments (n=17 mice per group). Mice were sacrificed in accordance with institutional ethical guidelines, and not limited to symptoms related to tumor growth. Log-rank (Mantel-Cox) test. (D) Flow cytometry analysis of peripheral blood of mice identifying absolute cell number of circulating human CD45$^+$CD3$^+$ cells. Shown is mean±SD, each symbol is representative of one mouse (n=7 from one experiment). Statistical analysis by the Wilcoxon matched-pairs signed rank test. ***p<0.001, **p<0.01, *p<0.05. CAR, chimeric antigen receptor; EphA3, erythropoietin-producing hepatoma type-A receptor 3; IV, intravenous.

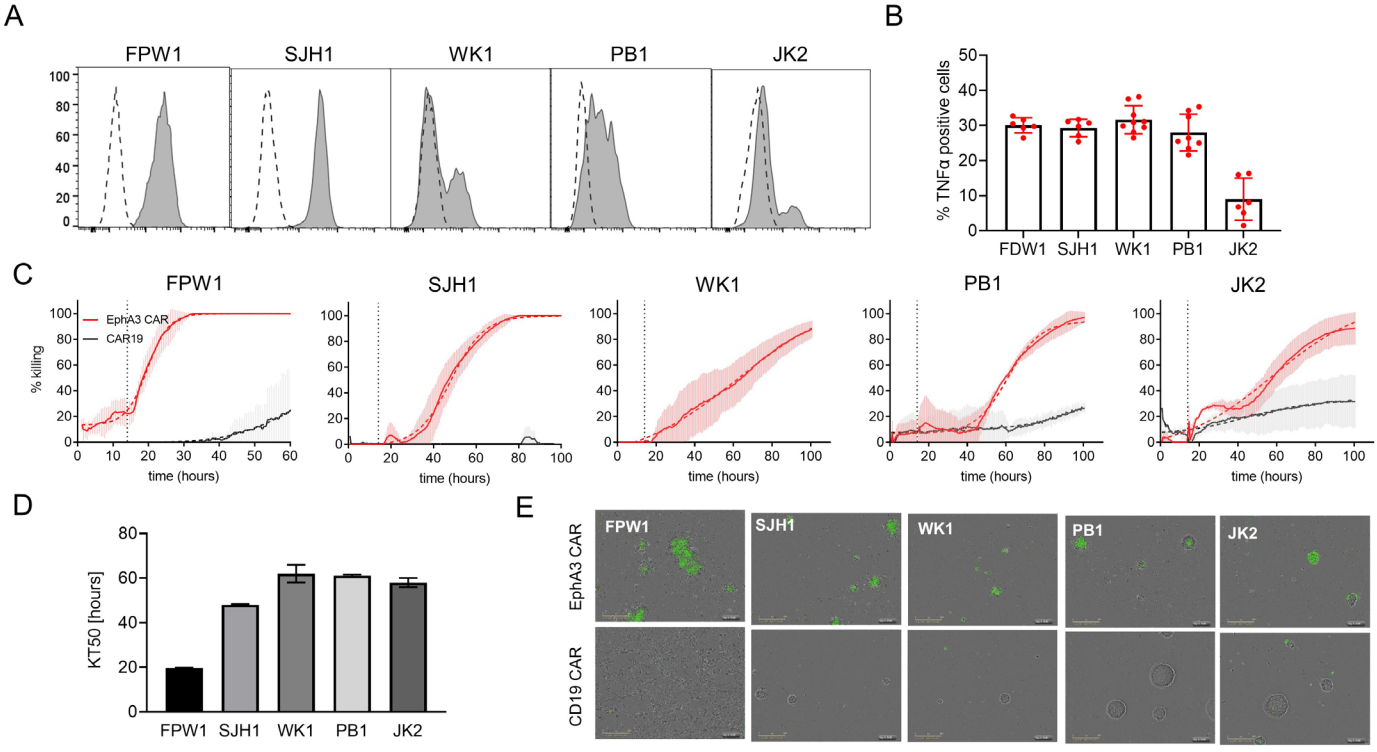

Figure 5 EphA3-CAR T cells target and kill primary glioblastoma cells. (A) The 3C3-1 monoclonal antibody was used to evaluate, by flow cytometry, EphA3-expression of patient-derived glioblastoma primary cell cultures from the QCell bank. Histograms depict the percentage of viable EphA3-positive cells stained with clone 3C3-1 compared with secondary anti-IgG antibody alone (dotted line), from two independent experiments. (B) EphA3-CAR T-cell cultures were co-incubated overnight with the patient-derived glioblastoma samples at a 1:1 ratio (T cell: glioblastoma target). Frequency of total $CD4^+TNF^+$ and polyfunctional $CD4^+TNF^+$interferon-γ^+ cells in response to glioblastoma targets. Shown is the mean±SD of at least two independent experiments with triplicates. (C) Glioblastoma cells were cultured as monolayers on laminin-coated E-plates for xCELLignece. Glioblastoma cells were co-cultured with EphA3-CAR T cells, or control CD19-CAR T cells. Data represent the mean percentage cytolysis±SD (n=2 replicates). (D) A sigmoidal dose-response curve (variable slope) was extrapolated from each killing data curve, and the half-maximal killing time (KT50) was determined. Data represent KT50 average±SE (n=2 replicates). (E) Glioblastoma primary cultures were plated at low density and propagated as floating aggregates. Glioblastoma spheroids were established from all cultures except FPW1 which grew as a monolayer. Glioblastoma cells were co-cultured with EphA3-CAR T cells or CD19 CAR T. Cytotoxicity was measured kinetically using the IncuCyte platform over 4 days, and cell death indicated by uptake of FITC-conjugated annexin V. Shown are representative merged bright field and fluorescent microscopy images of cultures at day 4. Green represents annexin V uptake by the neurospheres. CAR, chimeric antigen receptor; EphA3, erythropoietin-producing hepatoma type-A receptor 3; TNF, Tumor necrosis factor; FITC, Fluorescein isothiocyanate.

(QBC417, 418, and 420). Although EphA3 expression was detected in all GBOs, levels and distribution varied considerably highlighting tumor heterogeneity of this receptor (figure 6A). GBOs were co-cultured with 30,000 EphA3-CAR T cells equating to approximately 10 GBO cells per T cell. On day 2 there was a notable disruption of the integrity of the organoids cultured with EphA3 CAR, in comparison to those incubated with non-transduced T cells or untreated controls (figure 6B and online supplemental figure 5A,B). TNF and interferon (IFN)-γ was detected in the culture supernatants of EphA3 CAR T cell-treated wells suggesting activation of the CAR T cells (online supplemental figure 5C). GBO immunohistochemistry (IHC) at 15, 30 and 48 hours post-treatment revealed appreciable EphA3-CAR T-cell infiltration into the GBO (online supplemental figure 5D). After 2 days, the EphA3 CAR T cells had infiltrated the GBO and induced apoptosis as shown by cleaved caspase 3 staining of surrounding cells (figure 6C,D). Interestingly, the level of $CD3^+$ T-cell infiltration and apoptosis was proportional to the level of EphA3 expression in the organoids. There was significant infiltration observed in high EphA3-expressing GBOs QBC417/18, while only peripheral infiltration was observed in QBC420, which exhibited low EphA3-expression. Taken together, these observations demonstrate that EphA3 CAR T cells can effectively infiltrate and target EphA3-positive glioblastoma.

DISCUSSION

The development of CAR T-cell therapy has revolutionized cancer therapy by leveraging the immune system's capacity to identify and eliminate tumor cells. Glioblastoma-specific CAR T cells have been engineered

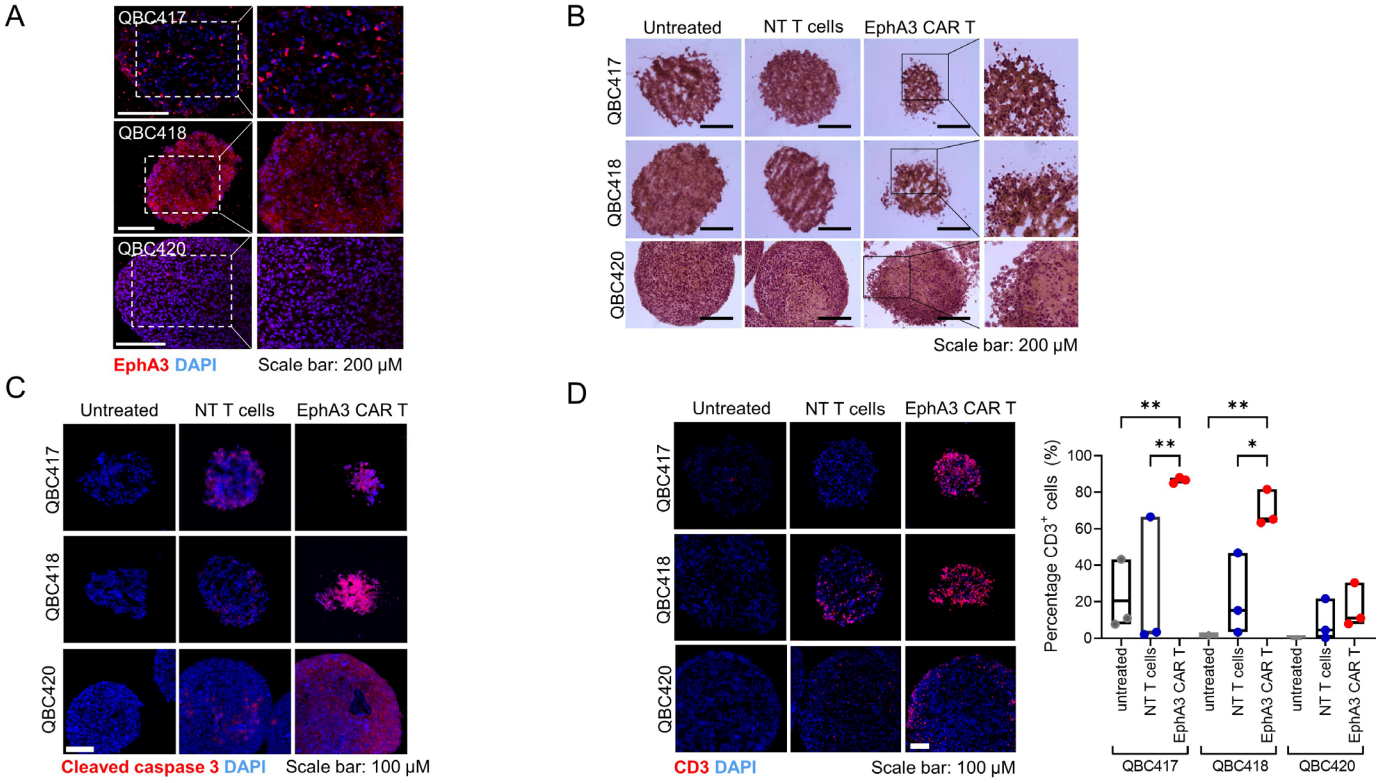

Figure 6 EphA3-CAR T cells infiltrate glioblastoma. (A) Immunofluorescence staining of EphA3 (red) in GBOs was established from three patients undergoing glioblastoma resection surgery. (B) H&E images of untreated, non-transduced (NT) and EphA3-CAR T cell treated GBOs of all three samples, after 48 hours incubation. (C) Immunofluorescence staining of cleaved caspase 3 (red) in untreated, NT control and EphA3-CAR T cell treated GBOs of all three samples, after 48 hours incubation. (D) Immunofluorescence staining and quantification of CD3 (red) in untreated, NT control and EphA3-CAR T cell treated GBOs of all three samples, after 48 hours incubation. Statistical analysis by multiple comparisons of an ordinary one-way analysis of variance, **p<0.01, *p<0.05. CAR, chimeric antigen receptor; EphA3, erythropoietin-producing hepatoma type-A receptor 3; GBO, glioblastoma organoid; DAPI, 4′,6-diamidino-2-phenylindole.

for various target antigens. CAR T-cell targets such as B7-H3, chlorotoxin, NKG2D, EphA2, CD133 and others, are in trials and currently recruiting patients.[21] EGFRvIII, HER2, and IL13Rα2 have completed trials with varying degrees of success.[22–25] However, achieving durable responses remains elusive. Eph receptors overexpression has been associated with various cancers. EphA2 has indeed been explored as a target for various clinical trials.[18] However, some concerns were recently raised when EphA2-directed antibody-drug conjugate candidate ATRC-301 showed toxicity in non-human primates. The study revealed safety signals, including bleeding, that led the disbanding of the development of EphA2 antibody drug conjugate.[26] Previous studies from our group had identified EphA3 as a potential target in glioblastoma since this protein is overexpressed in >40% of glioblastoma clinical.[13] While the EphA3 protein is highly expressed during fetal development however, directly after birth, its expression drops and is minimally expressed in the healthy adult brain.[5 7 10] We have corroborated these findings by using an in-house anti-EphA3 antibody (clone 3C3-1) for EphA3 histology on a panel of glioblastoma, and normal brain tissue samples, as well as demonstrated EphA3 expression within the vascular niche of glioblastoma. Targeting EphA3 in glioblastoma holds significant importance due to its involvement in multiple aspects of glioblastoma biology. EphA3 expression is associated with the mesenchymal cell-state, known for its aggressive and infiltrating nature. EphA3 is associated with GSC, particularly in the perivascular regions and is restricted to newly formed tumor microvasculature, and not in normal endothelium.[11 13] The vascular niche plays a crucial role in tumor microenvironment modulation by supplying nutrients and oxygen, but also by regulating immune cell infiltration, cytokine levels and extracellular matrix composition. Targeting the vasculature can modulate the tumor microenvironment to favor anti-tumor immune responses and inhibit tumor-promoting signals.[27 28] Furthermore, targeting the newly formed tumor microvasculature is a strategy that aims to disrupt the blood supply of tumors, thereby starving them of oxygen and nutrients and further hindering their growth and expansion by disrupting angiogenesis. EphA3 has been shown to co-localize with stem cell markers in glioblastoma, suggesting it might play a role in maintaining the cancer stem cell population. Targeting EphA3 could potentially eliminate these cancer stem cells, leading to a more durable therapeutic effect.

Targeting EphA3 has emerged as a promising strategy with an encouraging safety profile. EphA3 also shows higher and more restricted expression in glioblastoma cells compared with EphA2, which is also expressed in normal tissues.[29] This potentially reduces the risk of off-target effects with EphA3-targeted CAR T cells. In fact, a Phase I clinical trial in leukemia targeting EphA3 with the monoclonal antibody (KB004) showed this treatment to be safe with minimal adverse events.[30] Similarly, a trial using the EphA3 monoclonal antibody ifabotuzumab conjugated to ^{89}Zr showed clear uptake of the antibody in glioblastoma tumor, and no uptake in the normal brain Phase I clinical trials in patients with leukemia and brain cancer targeting EphA3 using monoclonal antibody (KB004) demonstrate its safety and tolerability, with minimal adverse events reported.[31] Based on its target and safety profile, EphA3 emerges as a compelling candidate for CAR T-cell therapy.

Data presented in this manuscript shows that EphA3-targeted CAR T cells expanded from healthy individuals exhibit remarkable efficacy in specifically killing EphA3-positive glioblastoma cells. Previous studies have demonstrated that adoptive immunotherapy or antibody-drug conjugation with monoclonal therapy targeting EphA3 (IIIA4 clone) is safe with no systemic toxicity, further reinforcing its safety profile with high specificity on-target and minimal or no off-tumor effects.[16 30] Our preclinical studies show that adoptive transfer of EphA3 CAR T cells in tumor-bearing immunodeficient animals can proliferate, and traverse the mouse blood-brain-barrier and penetrate and eliminate the U251 xenografts within the mouse brain. This therapeutic benefit was sustained for >100 days post tumor engraftment and <6% animal showing relapse of the disease. These observations correlated with in vivo proliferation of EphA3 CAR T cells which has been typically associated with enhanced overall survival in patients receiving CAR T-cell therapy.[32] While we acknowledge that this approach does not provide a complete picture of trafficking of the T cells through other organs, our preclinical studies indicate that the CAR T cells can proliferate, traverse the blood brain barrier (BBB), and functionally eliminate the tumor cells within the brain.

While preclinical in vivo studies with glioblastoma tumor cell lines provided encouraging results, previous studies have shown that promising preclinical findings have not translated when examined in clinical trials.[33] It is now well established that the glioblastoma microenvironment is marked by both cellular and histological heterogeneity, encompassing distinct cell types and variations in cell density and vascularization across different regions, and EphA3 protein expression varies, contingent on the cell type.[34] We extended our assessment of EphA3 CAR T cells using patient-derived primary tumor cells and organoids since these closely mimic the heterogeneity observed in tumors, with regards to EphA3 expression. EphA3 CAR T cells efficiently recognized primary glioblastoma cells expressing varying levels of EphA3 expression demonstrating the ability to target heterogeneous EphA3-expressing tumors. We hypothesize that these EphA3-positive subpopulations are the initial targets for the CAR T cells. Once activated, the CAR T cells may target neighboring EphA3-negative cells through bystander killing. This phenomenon has been observed in CAR T-cell therapy and can be mediated by the Fas/FasL signaling pathway or through soluble factors released by activated T cells.[35 36] Bystander killing is associated with eliminating neighboring cells and is distinct from off-target effects of CARs. While the exact mechanism driving bystander killing in this model is intriguing, it falls outside the scope of the current manuscript.

We further extended these observations using patient-derived explant GBOs which reflect the complex tumor microenvironment more accurately. Among the three organoids, QBC417, derived from recurrent tumor, displayed, in scattered cells, the highest level of EphA3 expression, consistent with findings in recurrent glioblastoma.[18 19] EphA3 CAR T cells manufactured from healthy individuals were able to penetrate into GBOs and dissociated, to varying extents, all three organoids. These observations were coincident with significant infiltration of CAR T cells and cleaved caspase 3 expression within the organoids, which directly correlated with the level of EphA3 expression. These finding underscores the ability of the EphA3 CAR to penetrate the intricate architecture of patient tumors and induce apoptosis of glioblastoma cells, highlighting a particularly promising feature of these cells and their target.

As we move forward, several key components will be crucial for optimizing the EphA3 CAR T-cell therapy. Streamlining the EphA3 CAR T cells manufacturing process, defining the optimal dosing schedule, and investigating potential combinatorial approaches are essential considerations. The recent glioblastoma trials offer valuable insights. While the EGFRvIII CAR T-cell combination with pembrolizumab showed limited efficacy, this trial established the safety of this approach.[37] The trial published by Bagley *et al* underscores the importance of the route of administration and dosage optimization.[38] Additionally, Lin and colleagues with GD2-targeted CAR T cells and interleukin (IL)-7 receptor integration highlights strategies for enhancing CAR T-cell persistence. Learning from these trials will inform the design of more effective future trials.[39]

Development of allogeneic EphA3 CAR T cells offers a unique opportunity to rapidly offer this as an “off-the-shelf” adoptive immunotherapy for wider patient access and affordability. Third-party human leukocyte antigen (HLA)-matched T-cell therapy has been successfully used for the treatment of virus-associated complications, including virus-associated cancers.[40 41] A thorough safety assessments and monitoring for potential adverse effects will be paramount for the successful progression of this therapeutic approach.

In conclusion, our study establishes EphA3 as a promising target for CAR T-cell therapy in glioblastoma and

shows that these effector cells can be used as a potential immunotherapy for these patients. The efficacy demonstrated across various models, including patient-derived organoids, positions the EphA3 CAR T cells immunotherapy approach as a compelling candidate for further development as a novel therapeutic option for patients with glioblastoma.

METHODS

Generation of monoclonal anti-EphA3 antibody

DNA sequence of the extracellular domain of EphA3 (P29320 amino acids 21–541) with a polypetide histidine tag was cloned into the mammalian expression vector pcDNA3.4 and the EphA3 protein was expressed in Expi293F cells. EphA3 protein was purified from the cell culture supernatant by a one-step purification by HisTrap FF Crude. BALB/c (n=3) and C57BL6 (n=3) mice were immunized with EphA3 protein subcutaneously with 50 µg/animal, followed by three boost immunizations with EphA3 protein 25 µg/animal on days 14 and 28, and intravenously at day 50±7. Cell fusion and screening was performed 4 days after the final boost. Primary binder screening was performed by ELISA with EphA3 protein and a histidine-tagged irrelevant protein. Relevant parental clones were subcloned and screened for EphA3-specific reactivity using ELISA. Briefly, 96-well Nunc plates were precoated with 50 µL of recombinant EphA3-His protein 1 µg/mL in phosphate buffer saline (PBS) and incubated at 4°C overnight. Plates were washed with PBS containing 0.05% Tween 20 (PBST) and blocked with 5% skim milk. Serially diluted serum samples were added to the wells and incubated for 2 hours at room temperature. After washing with PBST, plates were incubated with HRP-conjugated sheep anti-mouse Ig antibody (SouthernBiotech) for 1 hour. Plates were washed and incubated with 3,3',5,5'-Tetramethylbenzidine substrate solution for 10 min and then color development was stopped by adding 1N HCl. OD at 450 nm was measured using an ELISA plate reader.

Surface plasmon resonance

SPR experiments were performed on a Biacore T200 instrument (Cytiva). The EphA3 protein was immobilized via amine coupling to the surface of an HBS-EP (10 mM HEPES, 150 mM NaCl, pH 7.4 0.05% Tween20, 3 mM EDTA) pre-equilibrated CM5 chip. Biacore multicycle kinetics was performed to determine the binding affinity of 3C3-1. The antibody was diluted from 20 ng/mL to 0.625 ng/mL (4.167 × 10 – 12 – 1.333 × 10 – 10 M) in the running buffer HBS-EP and flowed over the surface at 40 µL/min with a contact time of 180 s and a dissociation time of 600 s. The surface was regenerated with a 30 µL injection of 10 mM glycine pH 2.5 at 30 µL/min over all four flow cells. The experiment was performed in duplicate to yield minimum triplicate data, with analysis temperature of 25°C. Data processing and analysis was performed using Biacore T00 Evaluation Software (Cytiva). Double-reference subtraction was performed for all data sets. Kinetic data was obtained by fitting a 1:1 binding model.

Variable domain sequencing of the 3C3-1 hybridoma

Total RNA was isolated from 3C3-1 hybridoma cells following the technical manual of TRIzol reagent. Total RNA was reverse-transcribed into complementary DNA (cDNA) using universal primers following the technical manual of PrimeScript first Strand cDNA Synthesis Kit. Antibody fragments of heavy and light chains were amplified by rapid amplification of cDNA ends. Amplified antibody fragments were cloned into a standard cloning vector separately and colony PCR was performed to screen for clones with inserts of correct sizes.

Glioblastoma cell lines and primary cells

Primary glioblastoma cells were obtained from QCell, a glioblastoma patient-derived cell line resource from QIMR Berghofer.[42–44] FPW1, SJH1, WK1, PB1, and JK2 cells were maintained as adherent GSC cultures on laminin-coated plates, or as tumorspheres. Cells were cultured in StemPro NSC SFM (Invitrogen) supplemented with EGF (20 ng/mL) and FGF (10 ng/mL) as per the manufacture's guidelines. Cell characterization, genome and RNA sequencing data is available from: https://www.qimrberghofer.edu.au/commercial-collaborations/partner-with-us/qcell. U251-MG and U87-MG cells were originally obtained from American Tissue Culture Collection (ATCC) (Manassas, Virginia, USA) and transduced with Firefly Luciferase Lentifect (Gene Copoeia, Rockville, Maryland, USA) as described previously.

EphA3 tissue immunohistochemistry

Immunohistochemistry staining was performed on 3–4 µM sections from formalin-fixed, paraffin-embedded tissue sections from patients (QCell bank or P2282), or from tumor microarray GL805-L51 (US Biomax). After deparaffinization in xylene, the slides were rehydrated. Antigen retrieval was performed at 125°C for 5 hours at pH 6. Specimen was incubated with EphA3-specific monoclonal antibody (clone 3C3-1; 6 µg) for 2 hours at 4°C. Sample was washed with 0.1% Tween 20/PBS and then incubated with horse raddish peroxidase (HRP)-labeled anti-mouse antibodies and DAPI nuclear counterstain. 4′,6-diamidino-2-phenylindole (DAB) staining was used to visualize EphA3 using the Aperio ScanScope AT Turbo and ImageScope software (Leica). QuPath software's positive cell detection algorithm was used to enumerate EphA3-positive cells as a percentage of total cells in the tumor section. Parameters: nucleus area=10–400 µm^2 (DAPI channel), cell expansion=2 µm including cell nucleus; intensity parameters: threshold=0.1, maximum background intensity=2 (DAB channel).

Flow cytometry for CAR characterization and EphA3 expression

Cell surface expression of EphA3 was analyzed using 3C3-1 monoclonal antibody. Glioblastoma primary cells

or cell lines, were incubated with 3C3-1 at 0.02 mg/mL for 30 min at 4°C in PBS containing 2% fetal calf serum (FCS), washed and incubated with secondary Alexa Fluor 488-conjugated anti-mouse IgG antibody (Thermo Fisher) for a further 30 min at 4°C in the dark. For T-cell population analysis, cells were labeled with fluorescent antibodies against human Live/Dead fixable near-IR Dead Cell stain (Molecular Probes), CD45RA-FITC, CD8-PerCP-Cy5.5, CD3-APC, CD27-APC-R700, CD95-BV421, CD28-BV480, CD57-BV605, CD4-BV786, CCR7-PE-Cy7. Different T-cell subpopulations and memory populations were determined. For CAR expression analysis, goat anti-mouse IgG (H+L) cross-adsorbed AF546 (Thermo Fisher) was used. Cells were incubated for 30 min at 4°C and washed. Cells were subsequently stained with anti-CD4 and CD8 antibodies as described above. Fluorescence was examined using an LSRFortessa Flow Cytometer (BD) and acquired data analyzed using FlowJo software (TreeStar).

CAR T-cell expansion

Blood was collected in 9 mL Vacuette tubes with K3 EDTA (Greiner). PBMCs were isolated in a 50 mL SepMate tube (STEMCELL Technologies) according to the manufacturer's instructions within 2 hours of blood collection. After cell counting, PBMCs were cryopreserved at 5–10×10^6 cells per 1 mL in freeze media (RPMI basal media containing 20% FCS (Gibco), 10% DMSO (Merck) and placed inside a Nalgene Mr Frosty Cryo Freezing Container (Thermo Fisher Scientific) at −80°C for at least 4 hours. Cells were later transferred to vapor phase nitrogen until required. To generate CAR T cells, PBMC were stimulated with T-cell TransAct (Miltenyi Biotec) according to the supplier's instructions. Cells were transduced with EphA3 CAR-encoding lentivirus (multiplicity of infection [MOI]=20) in G-Rex 24 wells (Wilson Wolf) at 1×10^6 cells/cm^2 in 500 µL transduction media (RPMI basal media containing 10% FCS (Gibco), 300 IU recombinant IL-2 and 1× LentiBOOST). Cultures were maintained at 37°C and 5% CO_2 in culture media containing 120 IU IL-2. Cultures were either Fluorescence-activated cell sorting (FACS) was carried out on days 7–10 using anti-mouse IgG (Thermo Fisher) and cultured to day 17; or cultured, without sorting, up to day 14. Mock-transduced T cells were generated by stimulating and culturing cells as described above, without lentivirus addition. These cells were used as non-transduced controls.

Glioblastoma xenograft studies

Glioblastoma xenograft models were initiated in 6–8-week-old NSG (NOD-scidIL2Rgnull) mice. For subcutaneous tumor xenografts, 5×10^6 luciferase-expressing glioblastoma cell lines (U251 or U87) were injected into the right flank of mice and tumors were allowed to establish. CAR T-cell treatment was initiated once tumors reached 60 mm^3. These animals were adoptively treated with 20×10^6 EphA3 CAR T cells intravenously and monitored for weight and physical condition. In vivo bioluminescence imaging was performed using a Xenogen IVIS 100 as previously described. Tumors were measured using vernier calipers and mice euthanized when tumors reached approximately 1 cm^3. For the orthotopic xenograft model, tumor cells were implanted intracranially as previously described.[16] Briefly, 2×10^4 luciferase-expressing U251 cells were transplanted, using a stereotactic device, into the right forebrain of NRG mice. Tumor formation was monitored using in vivo bioluminescence imaging. To achieve consistent initial tumor burden across all groups, following tumor detection, mice were tumor-matched based on bioluminescence imaging results to create groups with similar average tumor burden prior to treatment. Animals were either treated with EphA3 CAR T cells, CD19 CAR T cells or non-transduced T cells for controls. Mouse peripheral blood was collected weekly by retro-orbital bleeds and stained with LIVE/DEAD Near-IR Dead cell stain, and anti-human CD8-PerCP-Cy5.5, CD3-APC, CD4-AF700, CD45 V500; and anti-mouse CD45 V450, for 30 min at 4°C. FACS lyse solution was used to lyse red blood cells (RBCs). Cells were run with Precision Count Beads (BioLegend) in order to quantify $hCD3^+$ cells. Mice were monitored for tumor size and signs of distress as per approved ethics and animals were euthanized at the ethical endpoint.

Intracellular cytokine assay

U251 cells were co-incubated with EphA3 CAR T cells overnight in the presence of GolgiPlug and GolgiStop (BD Biosciences). Cells were washed and stained with anti-CD8-PerCP-Cy5.5 (eBioscience), anti-CD4-Pacific Blue (BD Biosciences), and LIVE/DEAD Near-IR Dead Cell stain. Cells were fixed and permeabilized with Cytofix/Cytoperm (BD Biosciences), washed again and stained with anti-IFN-γ-Alexa Fluor 700 and anti-TNF-Allophycocyanin (APC) (both from BD Biosciences). Cells were washed then resuspended in PBS and acquired using a BD LSRFortessa with FACSDiva software (BD Biosciences). Post-acquisition analysis was performed using FlowJo software (TreeStar).

Cytotoxicity assay

Long-term tumor cell killing was determined by xCELLigence, an impedance-based real-time cytotoxicity assay (RTCA). Background impedance of RTCA E-plates and cell culture media was first measured. Tumor cells (U251 or U87) were seeded on untreated RTCA E-plates (20,000 cells per well). For the glioblastoma primary lines, wells were coated with 1 µg/cm^2 laminin and 40,000 cells were seeded in each well for 14–18 hours for cells to adhere. EphA3 CAR T cells were added at the same ratio as targets. Changes in impedance were reported every 30 min as Cell Index. Normalized CI values were used to calculate the percentage of cytolysis using the following: Percentage of cytolysis = ((Cell Index $_{\text{no effectors}}$ – Cell Index $_{\text{effector}}$)/Cell Index $_{\text{no effectors}}$) × 100. EC 50 values were calculated from the sigmoidal dose-response curve calculated with the GraphPad Prism V.9.4.0 software (GraphPad software).

Gene expression analysis

T cells were incubated with U251 cells at a 1:1 ratio. After overnight incubation, EphA3 CAR T cells were enriched using FACS sorting based on Live/Dead NIR and goat anti-mouse IgG AF546 (Thermo Fisher). RNA was extracted from purified CAR T cells using the RNeasy Mini kit (QIAGEN). Gene expression analysis was conducted using the NanoString nCounter gene expression platform (NanoString Technologies) according to the manufacturer's instructions. A custom code set consisting of genes involved in T-cell biology, immune regulation, and immune cellular markers was used as previously described. The samples were scanned at maximum scan resolution on the nCounter Digital Analyser and gene expression data normalized by housekeeping gene expression to identify differentially expressed genes. Expression clusters were identified using unsupervised hierarchical clustering.

GBO co-culturing with EphA3 CAR T cells

Tumor tissues were collected from patients with glioblastoma undergoing surgery at the Royal Brisbane and Women's Hospital, under Human Ethics approved project HREC/17/QRBW/577: P3420 Novel Therapies for Brain Cancer. Specimens were collected immediately following resection and processed for analysis at QIMR Berghofer laboratories. GBC418 and 420 were generated from patients with primary glioblastoma whereas GBC417 was generated from a recurrent tumor. GBOs were cultured and maintained as previously described.[45] Briefly, GBO were cultured for 2–3 weeks and organoids with 500–1,000 µm diameter were selected for the in vitro T-cell assays. Each GBO was placed in a separate well of an ultra-low attachment 24-well plates (Corning) in 500 µL GBO medium. EphA3 CAR T cells and non-transduced control T cells were resuspended in GBO medium supplemented with 5% FBS and IL-2 (120 units/mL). Approximately 30,000 T cells were added to each well equating to approximately 10 GBO cells per T cell. The GBO-T cell co-culture plates were placed on an orbital shaker placed in a sterile incubator, rotating at 120 rpm at 37°C, 5% CO_2, 90% humidity. Co-culture experiments were performed for three GBO samples with replicates for each condition (n=3 for QBC417 and 418 and n=5 for QBC420). Bright-field images of individual GBOs were taken using the EVOS FL Auto imaging system (Thermo Fisher). The CAR T treated GBO was embedded into Epredia Cryomatrix (Fisher Scientific) for immunohistology analysis at defined time points. Serial tissue sections 7–15 µm were generated using a cryostat and melted onto charged microscopy slides. Slides were dried at room temperature before storing at −80°C. For immunofluorescence staining, the tissue was fixed for 5 min in 75% acetone 25% ethanol and washed with PBS. Tissue sections were permeabilized and non-specific binding blocked using 1% bovine serum albumin (BSA) (w/v), 0.3% Triton X-100 (v/v) in Background Sniper (Biocare Medical) for EphA3, CD31 stains) or 10% donkey serum (v/v), 0.3% Triton X-100 (v/v), 1% BSA (w/v) in Sniper (for cleaved caspase 3, CD3 stains) for 30 min at room temperature. After washing in PBS with 0.05% Tween 20, tissue sections were incubated with the following antibodies: anti-EphA3 (clone IIIA4 at 40 µg/mL overnight incubation at 4°C), anti-CD31 (Dako, Clone JC70A, cat#M0823, 1:200, overnight incubation at 4°C), CD3 (Dako, polyclonal cat# A0452, 1:80, 1 hour incubation at room temperature), and cleaved caspase 3 Asp175 (Cell Signaling Technology, Clone D175, cat# 9664, 1:80, 1 hour incubation at room temperature). After washing in PBS with 0.05% Tween 20, the tissue sections were incubated with Alexa Fluor secondary antibodies (Thermo Fisher), diluted 1:300 PBS plus 0.05% Triton-X for 30 min in the dark at room temperature. After washing with PBS plus 0.05T Tween 20, slides were mounted in mounting solution (ProLong Diamond Antifade Mountant with DAPI, Thermo Fisher), coverslipped, and sealed with nail polish.

Imaging was performed on the Zeiss 780-NLO confocal microscope using ×10 and ×20 objectives. T-cell infiltration was quantified by segmenting organoids into cells based on the DAPI nuclear stain and quantifying the number cells that were CD3-positive, as a fraction of the total number of cells present in each organoid using QuPath StarDist V.0.4.3 software. Three organoids per condition were analyzed. Parameters: nucleus area=10–400 µm^2 (DAPI channel), cell expansion=2 µm including cell nucleus; intensity parameters: threshold=80, maximum background intensity=2 (CD3 channel).

Statistics

Data analysis was performed using GraphPad Prism V.9.4.0 software (GraphPad software) and presented as specified in individual figure legends. Comparisons were determined using Student's t-test (two groups), one-way analysis of variance (ANOVA) (three or more groups), or a two-way ANOVA when two independent variables were analyzed. For comparison between three or more groups, Bonferroni's multiple comparison tests were used to compare all or selected pair of data (95% CIs). Statistical significance was defined as *p<0.05 (two-sided).

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Contributors PM contributed to the conception and design, acquisition, analysis and interpretation of data, and wrote the manuscript. RCJD and NS contributed to the conception and design, acquisition, analysis, and interpretation of data involving mouse models and organoids. LL, SH, MR, and XL were involved with the acquisition and analysis of data involving CAR T cells. TL and FS were involved in the acquisition of data for the mouse studies. CS and JB contributed to the conception and design and interpretation of data. BWD provided the organoids and contributed to the conception and design and interpretation of data. RK made substantial contributions to conception, design and interpretation of data and is responsible for the overall content as the guarantor. All authors have read and approve the final manuscript.

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Competing interests RK and PM are listed as inventors on the patent application describing EphA3 CAR T-cell therapy.

Patient consent for publication Consent obtained directly from patient(s).

Ethics approval This study involving use of peripheral blood mononuclear cells from human participants was approved by QIMR Berghofer Medical Research Institute Human Research Ethics Committee (HREC:P2282 The role of the immune system in infection and cancer). Studies using glioblastoma organoids was approved by Metro North Health Human Research Ethics Committee A (Project HREC/17/QRBW/577 – P3420 Novel Therapies for Brain Cancer). Participants gave informed consent to participate in the study before taking part. All animal studies have been approved and comply with QIMR Berghofer Animal Ethics Committee (#A1805-604M, P2324).

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