EGFRvIII-Specific Chimeric Antigen Receptor T Cells Migrate to and Kill Tumor Deposits Infiltrating the Brain Parenchyma in an Invasive Xenograft Model of Glioblastoma

Hongsheng Miao¹, Bryan D. Choi¹,², Carter M. Suryadevara¹, Luis Sanchez-Perez¹, Shicheng Yang¹, Gabriel De Leon¹,³, Elias J. Sayour¹,², Roger McLendon²,⁴, James E. Herndon II⁵, Patrick Healy⁵, Gary E. Archer¹,²,⁎, Darell D. Bigner¹,²,⁴, Laura A. Johnson¹,⁴¤, John H. Sampson¹,²,⁴

¹ Duke Brain Tumor Immunotherapy Program, Division of Neurosurgery, Department of Surgery, Duke University Medical Center, Durham, North Carolina, United States of America, ² Department of Pathology, Duke University Medical Center, Durham, North Carolina, United States of America, ³ Department of Molecular Cancer Biology, Duke University Medical Center, Durham, North Carolina, United States of America, ⁴ The Preston Robert Tisch Brain Tumor Center, Duke University Medical Center, Durham, North Carolina, United States of America, ⁵ Department of Biostatistics and Bioinformatics, Duke University Medical Center, Durham, North Carolina, United States of America

Abstract

Glioblastoma (GBM) is the most common primary malignant brain tumor in adults and is uniformly lethal. T-cell-based immunotherapy offers a promising platform for treatment given its potential to specifically target tumor tissue while sparing the normal brain. However, the diffuse and infiltrative nature of these tumors in the brain parenchyma may pose an exceptional hurdle to successful immunotherapy in patients. Areas of invasive tumor are thought to reside behind an intact blood brain barrier, isolating them from effective immunosurveillance and thereby predisposing the development of "immunologically silent" tumor peninsulas. Therefore, it remains unclear if adoptively transferred T cells can migrate to and mediate regression in areas of invasive GBM. One barrier has been the lack of a preclinical mouse model that accurately recapitulates the growth patterns of human GBM in vivo. Here, we demonstrate that D-270 MG xenografts exhibit the classical features of GBM and produce the diffuse and invasive tumors seen in patients. Using this model, we designed experiments to assess whether T cells expressing third-generation chimeric antigen receptors (CARs) targeting the tumor-specific mutation of the epidermal growth factor receptor, EGFRvIII, would localize to and treat invasive intracerebral GBM. EGFRvIII-targeted CAR (EGFRvIII+CAR) T cells demonstrated in vitro EGFRvIII antigen-specific recognition and reactivity to the D-270 MG cell line, which naturally expresses EGFRvIII. Moreover, when administered systemically, EGFRvIII+CAR T cells localized to areas of invasive tumor, suppressed tumor growth, and enhanced survival of mice with established intracranial D-270 MG tumors. Together, these data demonstrate that systemically administered T cells are capable of migrating to the invasive edges of GBM to mediate antitumor efficacy and tumor regression.

Introduction

Glioblastoma (GBM) is the most common form of primary malignant brain tumor in adults and remains one of the most deadly neoplasms. Despite multimodal therapy including maximal surgical resection, radiation, and temozolomide (TMZ), the median overall survival is less than 15 months [1]. Moreover, these therapies are non-specific and are ultimately limited by factors which allow tumor-escape from treatment with ex vivo CAR T cells regardless of major histocompatibility complex (MHC) presentation of target antigen or MHC downregulation in tumors, suggesting that T cells can eradicate large, well-established tumors in mice and humans [3–7].

Chimeric antigen receptors (CARs) represent an emerging technology that combines the variable region of an antibody with T-cell signaling moieties, and can be genetically expressed in T cells to mediate potent, antigen-specific activation. CAR T cells carry the potential to eradicate neoplasms by recognizing tumor cells regardless of major histocompatibility complex (MHC) presentation of target antigen or MHC downregulation in tumors, factors which allow tumor-escape from treatment with ex vivo CAR T cells. Therefore, it remains unclear if adoptively transferred T cells can migrate to and mediate regression in areas of invasive GBM.
EGFRvIII-Specific CAR T Cells Kill Invasive GBM

Results

D-270MGFluc/GFP xenograft is highly invasive in NSG mice

In order to evaluate the antitumor efficacy of systemically delivered EGFRvIII+ CAR T cells against invasive intracerebral GBM tumors, we utilized the D-270MGFluc/GFP xenograft, which was isolated directly from a patient’s primary GBM tumor and has been previously validated to naturally express EGFRvIII [20,32]. We first sought to histologically evaluate and compare the characteristic growth patterns of D-270MGFluc/GFP tumor with U87MG.AEGRF, a xenograft derived from a previously described human glioma cell subline ([33,34]) that is among the most frequently used models in preclinical studies of GBM. U87MG.AEGRF is an EGFRvIII+ stably transfected subline of the parental human malignant astrocytoma cell line U87MG, which does not naturally express EGFRvIII. NSG mice received intracerebral tumor implants and were sacrificed after seven days. Brains were formalin-fixed, paraaffin-embedded, and 5 μm sections were stained with hematoxalin and cosin (H&E). NSG mice with no tumors were used as a control (Fig. 1a). Here, we show that U87MG.AEGRF xenografts produce tumors with well-defined boundaries that can be clearly delineated from normal brain (Fig. 1b, c). In stark contrast, D-270MGFluc/GFP tumors exhibit expanding borders with small perivascular streams of cells radiating from central tumor masses, and even subarachnoid infiltration of tumor cells (Fig. 1d, e, f). We found that D-270MGFluc/GFP xenografts reveal an invasive nodular proliferation of malignant cells with reduced eosinophilic cytoplasm and large nuclei with prominent nucleoli. We also observed focal areas of necrosis and frequent mitotic activity in tumors. Altogether, these data demonstrate that the in vivo phenotype of D-270MGFluc/GFP xenografts is consistent with the classic features of human GBM. Moreover, the in vivo growth patterns of this model recapitulate the diffuse and infiltrative nature of tumors found in patients, making D-270MGFluc/GFP an exemplary model for evaluating this CAR-based platform.

T cells expressing EGFRvIII+ CARs recognize D-270MGFluc/GFP tumors that naturally express EGFRvIII

Effective T-cell recognition and antitumor activity requires antigen-specific receptor expression and engagement of tumorigenic antigens. Therefore, we sought to determine if EGFRvIII+
CAR T cells would recognize D-270MGFLuc/GFP tumor cells, given their natural expression of EGFRvIII. Towards this end, we obtained human peripheral blood lymphocytes (PBLs) from patients and transduced them with a previously-described retrovirus encoding a third-generation EGFRvIII + CAR containing the humanized 139 anti-human EGFRvIII single-chain variable fragment in tandem with the hCD28-41BB-CD3ζ chain signaling domain [35]. Following transduction, we determined surface expression by flow cytometry, and T cells were found to efficiently express the EGFRvIII + CAR construct on their cell surface (Fig. 2a). In order to assess antigen-specificity of EGFRvIII + CAR T cells, we first quantitatively assessed levels of EGFRvIII expression in D-270MG FLuc/GFP, U87MG.ΔEGFR (EGFRvIII +), and U87MG (EGFRvIII−) control tumor cells. We found similar levels of EGFRvIII expression between D-270MGFLuc/GFP and U87MG.ΔEGFR (EGFRvIII +) tumor cells (Fig. 2b). Next, antigen-specific reactivity against EGFRvIII was determined in co-culture assays; while EGFRvIII + CAR T cells did not secrete observable levels of the type 1 cytokine interferon-gamma (IFN-γ) in response to the U87MG (EGFRvIII−) tumor cell line, they did produce IFN-γ in the presence of the U87MG.ΔEGFR (EGFRvIII−) cell line as measured by intracellular staining (ICS) (Fig. 2c; P = 0.0004; two-way ANOVA). Untransduced T cells alone showed no IFN-γ production versus either target. Importantly, recognition of the D-270MG FLuc/GFP cell line was observed only by EGFRvIII + CAR T cells and not by untransduced T cells (Fig. 2d; P = 0.002; one-way ANOVA). These data corroborate the specificity of EGFRvIII + CAR T cells and demonstrate their ability to elicit antitumor activity upon interaction with D-270MG FLuc/GFP tumors.

EFGRvIII + CAR T cells effectively migrate to invasive GBM tumors

Effective therapy in a clinical setting requires that systemically administered T cells effectively migrate to and encounter tumor cells in vivo. Unlike cancers of the periphery, GBM tumors rarely metastasize outside of the brain, instead shedding neoplastic cells that migrate away from main tumor cores and develop into highly invasive peninsulas residing within the normal brain, hiding within regions of intact BBB [24–26]. Although T cells are able to access bulky GBM lesions through dysfunction of the local BBB, it is unknown if they can effectively migrate into the invading tumor deposits that may reside behind an intact BBB. Therefore, we sought to evaluate the capacity of EGFRvIII + CAR T cells to localize to D-270MG FLuc/GFP intracerebral tumors, which closely mirror the invasive architecture of human GBM (Fig. 1d–f). To examine this, human donor PBLs were transduced with the external Gaussian luciferase (extGLuc) retrovirus or underwent a dual transduction with both the extGLuc and EGFRvIII + CAR retroviruses for use in bioluminescence imaging (BLI) analysis [36]. Following transduction, T cells were cultured in vitro prior to systemic infusion into NSG mice bearing established orthotopic D-270MG FLuc/GFP tumors. Imaging analysis two (Fig. 3a) and nine (Fig. 3b) days after T-cell injection revealed extGLuc signals in the brain area, demonstrating localization of EGFRvIII + CAR T cells to the tumor site. We also sought to evaluate whether EGFRvIII-specificity was necessary for efficient T-cell localization to tumor, and to do this, we compared the trafficking patterns of extGluc + EGFRvIII + T cells (Fig. 3a,b) with extGluc + T cells (Fig. 3c,d) in NSG mice bearing D-270MG FLuc/GFP tumors. We found that extGluc-only T cells (EGFRvIII−) failed to efficiently...
migrate across the BBB, whereas extGlu+ EGFRvIII+ CAR T cells rapidly localized in the brain. This suggests a necessity of EGFRvIII+ CAR expression for brain-trafficking or accumulation in NSG mice bearing D-270MG FLuc/GFP tumors. A separate group of mice were also sacrificed nine days after T-cell injection and tumor tissue was submitted for immunohistochemical analysis. Figure 3E shows that systemically administered EGFRvIII+ CAR T cells successfully migrated to the invasive edges of intracerebral tumor, particularly in areas of peninsula formation at the leading edge of tumor invasion. NSG mice receiving saline were used as a control (Fig. 3F). These results demonstrate that, in our model of invasive GBM, adoptively transferred EGFRvIII+ CAR T cells have the capacity to traffic to invasive areas of tumor thought to reside beyond the BBB.

**In vivo systemic delivery of EGFRvIII+ CAR T cells delays tumor growth and prolongs survival**

We sought to determine the therapeutic effect of systemically administered EGFRvIII+ CAR T cells against invasive intracerebral gliomas *in vivo*. Utilizing the NSG mouse model, D-270MG FLuc/GFP xenografts were implanted intracerebrally and allowed to engraft for three days prior to intravenous infusion with EGFRvIII+ CAR T cells. D-270MG FLuc/GFP cells were monitored using BLI every three days. No significant difference was observed between groups of mice that were either left untreated or infused with non-specific control CAR T cells. However, there was a significant delay in tumor growth in EGFRvIII+ CAR T-cell treatment groups compared to untreated and control CAR T cells, as detected by serial BLI recordings of tumor-cell photon emissions (Fig. 4a; P<0.0001; mixed model). Tumors were not visible in any group until day 11, at which point tumors began growing in
control and untreated mice. Complete suppression of tumor growth was evident in mice treated with EGFRvIII CAR T cells until day 17, but grew to achieve BLI values similar to untreated mice by day 26 (Fig. 4a). These growth kinetics translated into an 8–9 day survival advantage in mice treated with EGFRvIII CAR T cells when compared to both untreated mice and those receiving control CAR T cells (Fig. 4b; P<0.0001; generalized Wilcoxon test). Together, these preclinical results suggest that systemically administered third-generation EGFRvIII CAR T cells can inhibit invasive brain tumor growth and prolong survival.

**Discussion**

The therapeutic benefits of CAR-based adoptive cell therapy have been widely demonstrated in patients suffering with cancer [7,13–15]. Since its introduction, CAR design has evolved significantly to mediate a potent and robust T-cell immune response when directed against tumors in the periphery [6,37]. Importantly, we demonstrate here that similar immune responses can be achieved against established tumors in the immunologically privileged brain, even when directed against highly invasive cancers that are considered prone to immune evasion. The molecular properties and phenotype of GBM make it an extraordinarily difficult malignancy to treat from an immunologic perspective. Its invasive nature behind the BBB could confer a relatively high degree of isolation from immune activity, allowing tumors to grow silently with limited immune surveillance. Unlike previous studies, we have sought to examine the efficacy of adoptive T-cell therapy using the D-270MG^Luc^/GFP xenograft, which we demonstrate possesses diffuse intraparenchymal and perivascular invasion, consistent with the histopathological hallmarks of human GBM. Furthermore, unlike cell lines engineered...
to express EGFRvIII, such as U87MG.AEGFR. D-270 MG was isolated from a primary tumor that naturally expressed EGFRvIII and has maintained expression ex vivo and in vivo. This lends greater credence to its more accurate recapitulation of the clinical scenario.

We demonstrate here that EGFRvIII+ CAR T cells recognize tumors naturally expressing EGFRvIII, such as D-270MGFluc/GFP. We show that these EGFRvIII+ CAR T cells recognize tumor cells in an antigen-specific manner in vitro as measured by ICS (Fig. 2c, d). It is important to note that we measured a 10–15% frequency of IFN-γ+ cells in our in vitro ICS assays, which is less than our recorded frequency of CD3+ CAR+ T cells. This has been a consistent and expected result under the culture, transduction, and assay protocols described here. One explanation may be the varied differentiation states of CD3+ CAR+ T cells, since different stages of activation can alter the incubation time required for T-cell secretion of IFN-γ. We have found that longer incubation times (greater than the 18 h described here) and altered tumor: T cell ratios yield frequencies >50%, and we suspect that this is due to the inclusion of more cells occupying a greater spectrum of activation. However, we chose the assay conditions described here since incubation times >18 h decrease cell viability, and 18 h incubations have yielded consistent results to date [38].

Our data show that systemically delivered EGFRvIII+ CAR T cells have the capacity to migrate to invasive tumor deposits within the CNS. T-cell migration across endothelium requires molecular cues provided by chemokine–chemokine receptor interaction and engagement of adhesion molecules, which are thought to be independent of TCR engagement [39,40]. The cross-reactivity of murine adhesion molecules and chemokines with human T cells and their chemokine receptors is known to be limited [41], and although this could have negatively impacted T-cell localization, we instead observed a substantial influx of T cells into the invasive tumor. We are currently evaluating the contribution of tumor-derived human chemokines and EGFRvIII-specificity on CAR T cells to further elucidate the steps required for effective T-cell migration across the BBB in the invasive areas of GBM.

Importantly, we show here that systemically administered EGFRvIII+ CAR T cells have the capacity to inhibit tumor growth and prolong survival of mice with established D-270MGFluc/GFP tumors (Fig. 4a, b). Despite the evidence of an effective, antigen-specific immune response, it is important to note that brain tumors ultimately continued to grow and caused death even in mice receiving EGFRvIII+ CAR T cells (Fig. 4b).

Normalized BLI values from these treated mice eventually reached comparable values to untreated and CAR control groups, indicating tumor growth after a period of dormancy likely mediated by antitumor T-cell activity (Fig. 4a). One possible explanation is the loss of functional loss of EGFRvIII+ CAR T cells in this model. To test the antitumor efficacy of EGFRvIII+ CAR T cells in vivo, we chose an NSG mouse model, which has the advantage of evaluating promising preclinical therapies in an animal system using human tumor tissue. However, one major drawback of this approach is the fact that human T cells often have a limited lifespan and functional half-life in the murine background. For this reason, we sought to monitor T-cell persistence over time in vivo by BLI analysis. However, we unexpectedly found the administration of coelenterazine to be toxic at the manufacturer’s recommended dosage, and as such, terminated BLI measurements after day 9 (Fig. 3a, b). Our studies demonstrate that, despite this potential for CAR T cell loss, EGFRvIII+ CAR T cells were able to persist long enough to migrate to and mediate antitumor activity against invasive intracranial tumors. We are currently evaluating host conditioning regimens to support enhanced and long-term human T-cell survival and function in NSG mice, since these factors could, in theory, potentiate antitumor efficacy.

A second possible explanation for the eventual recurrence of tumor in our model is the concept of antigen-loss, wherein therapeutic pressure selects for tumor cells that do not express the target antigen. This explanation would be consistent with two recent clinical studies where recurrence in patients treated with CARs [42] or a vaccine targeting a single antigen [43] was characterized by outgrowth of antigen-loss variants. As such, given the theoretical limitations of targeting single tumor antigens, future efforts will likely focus on the identification of additional GBM-specific targets and multimodal therapies designed to target several antigens simultaneously through CAR-mediated or alternative

Figure 4. In vivo systemic delivery of EGFRvIII+ CAR T cells delays tumor growth and prolongs survival. NSG mice were implanted with 1×10⁶ D-270MGFluc/GFP tumor cells intracranially, randomized into three groups (n = 6-8), and monitored for tumor growth and survival. 5.0 - 10×10⁶ T cells were administered intravenously 3 days after tumor implantation. (a) Normalized BLI values associated with longitudinal monitoring of tumor growth for untreated, control CAR T-cell, and EGFRvIII+ CAR T-cell groups are shown (value = mean ± SD). The pattern of change in log BLI values significantly differs between the three treatment groups (P<0.0001; mixed model). (b) The survival of animals treated with EGFRvIII+ CAR T cells was significantly prolonged (P<0.0001; generalized Wilcoxon test) when compared to other treatment groups. Data represent one of two (n = 2) experiments with similar results. doi:10.1371/journal.pone.0094281.g004
T-cell-based approaches. Additional areas of further investigation may include determining factors involved in eliciting broader endogenous immune responses through mechanisms such as epitope spreading, which has emerged as a critical factor during clinical trials of immunotherapy for melanoma [44].

In the current study, we have demonstrated that systemically administered and tumor-specific EGFRvIII CAR T cells effectively migrate to areas of tumor invasion and mediate efficacy in a murine model of invasive human glioma. This work contributes to the rapidly growing literature supporting the utility of adoptively transferred CAR T cells targeting tumor-specific antigens as a potent treatment modality for invasive brain tumors.

Materials and Methods

Human GBM cell lines and xenografts

We utilized the previously described human glioma cell line, U87MG [29–31], which does not express EGFRvIII, and subline U87MG.AEGRF [33,34], which was stably transduced to express EGFRvIII. We also utilized the D-270 MG cell line, which was propagated directly as a xenograft from a primary human GBM harvested from a patient and has previously been shown to naturally express EGFRvIII [20,32]. Briefly, mechanically minced tumor tissue was enzymatically dissociated into single cells using the Papain Dissociation System. After wash, tissue was homogenized and passed through a 75 μm cell strainer, re-suspended with freezing medium containing 90% fetal bovine serum (FBS) and 10% dimethyl sulfoxide and frozen in individual vials using standard procedures. For further experimentation, cells were thawed at 37°C, washed, and counted with trypsin blue per standard practice.

Animals

NSG mice were obtained (Charles River Laboratories, Wilmington, MA) and bred under standard conditions at Duke University Medical Center (DUMC). Mice were kept and utilized under the accordance of protocols approved by the Duke University Institutional Animal Care and Use Committee (IACUC). All mice used in this study were healthy females between 6–8 weeks of age weighing 0.020–0.025 kg and were randomized to experimental or control groups. Mice were routinely monitored for health (every 2 days) and qualified for euthanasia if they demonstrated an inability to ambulate to food and water (i.e., in lateral recumbency and unable to right itself), or if they were unable to move forward two steps when prompted gently by touching a finger to the hind area. Moribund mice were humanely euthanized when they met these endpoints using CO2 asphyxiation followed by decapitation as approved by our Duke University IACUC protocol. In accordance with this protocol, mice did not receive any analgesics or anesthetics.

Human PBLs

Human PBLs used in this study were obtained from normal volunteers at Duke University Medical Center. The use of PBLs was approved under protocol 0009043 by the Duke University School of Medicine Institutional Review Board (irm.mc.duke.edu). Approved protocols conform to the Declaration of Helsinki protocols. All patients signed a written informed consent. PBLs were cultured in AIM-V medium (Life Technologies, Grand Island, NY) supplemented with 10% human AB serum (Valley Biomedical Inc., Winchester, VA), 50 units/mL penicillin, 50 μg/mL streptomycin (Life Technologies, Grand Island, CA) and 300 IU/mL interleukin-2 (IL-2) and maintained at 37°C with 5% CO2.

EGFRvIII+ CARs and extGLuc retroviral vector transduction

The extGLuc retroviral vectors were supplied by Renier Brentjens of Memorial Sloan-Kettering Cancer Center, New York, NY. The EGFRvIII+ CAR and extGLuc retroviral vectors were utilized to generate EGFRvIII+ CAR T cells and extGluc+ EGFRvIII+ CAR T cells. The transduction procedures have previously been described [36,43,46]. Briefly, peripheral blood mononuclear cells (PBMCs) from healthy donors and GBM patients (post-resection, prior to treatment) were thawed and cultured in AIM-V medium supplemented with 5% human AB serum, plus antibiotics, 300 IU/mL IL-2, and 50 ng/mL OKT-3. After 48 hours, T cells (0.25x10^6/mL) were transduced with a retroviral supernatant containing either the extGLuc, EGFRvIII+ CAR, or both vectors spun onto RetroNectin (Takara Bio Inc, Japan) coated non-tissue culture treated 6-well plates twice on two consecutive days as described by the manufacturer. Transduced cells were allowed to expand in AIM-V medium as above, without OKT-3.

Rapid Expansion Protocol

Transduced PBLs (or untransduced control PBLs from same donor) were expanded in vitro using rapid expansion protocol (REP) [47]. Briefly, T cells were cultured in complete AIM-V medium plus 10% human AB serum, 300 IU/mL IL-2, and 50 ng/mL OKT-3 in the presence of 100x excess 5000 rads irradiated allogeneic PBMC feeder cells, and allowed to expand 10–14 days.

Lentiviral transduction of D-270 MG with firefly-luciferase-GFP gene

The D-270 MG tumor cell line was transduced with a lentiviral vector encoding the firefly luciferase (Fluc) and EGFP genes linked by 2A peptide driven by an internal murine stem cell virus (MSCV) promoter. Briefly, lentiviral vectors were generated by transient transfection of HEK 293T cells with a four-plasmid system [48]. Six hours post-transfection, plates were washed twice with phosphate-buffered saline (PBS) and 20 mL fresh medium was added. The supernatant was collected 30–48 hours post-transfection and cell debris was removed by centrifugation at 6000 g for 10 minutes, followed by filtration on 0.45 μm polyvinylidene fluoride filters. The lentiviral supernatant was kept at ~8°C. D-270 MG cells were then quickly thawed at 37°C, washed, counted with trypan blue, and re-suspended in the lentiviral supernatant and zinc medium with 10% FBS and incubated at 37°C with 5% CO2 overnight. D-270MGFluc/GFP tumor cells were cell sorted based on GFP expression. Expression of Fluc was confirmed by data acquisition using the IVIS 100 in vivo BLI system (Caliper Life Sciences, Hopkinton, MA) coupled with Living Image software (PerkinElmer, Waltham, MA). EGFRvIII expression levels by D-270MGFluc/GFP and U87MG.AEGRF tumor cells were measured by qRT PCR as previously described [49].

Cell surface CAR expression and ICS of transduced T cells

Cells were stained for EGFRvIII+ CARs to detect cell-surface expression using an EGFRvIII multimer-PE as previously described [50]. Negative staining controls were conducted by staining untransduced cells from the same donor. ICS was performed by co-culturing T cells with tumors 1:1 over 18 hours with the BD GolgiPlug protein transport inhibitor containing brefeldin A (BD Sciences, San Jose, CA) in RPMI-1640 medium plus 10% FBS. Following co-culture, cells were submitted to...
surface staining for CD3, CD8 and intracellular IFN-γ stain using the BD Cytofix/Cytoperm method (BD Biosciences, San Jose, CA).

Monitoring tumor growth and T-cell trafficking using BLI
To monitor tumor growth, 24 NSG mice underwent intracranial implantation of 1 × 10^5 D-270MGFluc/GFP tumor cells in 5 μl PBS and methocell mixer using stereotactic coordinates 2 mm lateral and 4 mm intraparenchymal from the bregma on experimental day 0. Tumor growth was analyzed every three to five days for 26 days by BLI as previously described [31] until the study was terminated. Briefly, BLI was performed by injecting mice intraperitoneally with 150 mg D-luciferin/kg (Xenogen, Hopkinton, MA) 10 minutes prior to imaging and photon emission (photons s^{-1} cm^{-2} sr^{-1}) was recorded. To monitor T-cell trafficking, T cells were transduced with the extGLuc or co-transduced with the EGFRvIII CAR and extLuc retroviral vectors as described above (see EGFRvIII CARs and extLuc retroviral vector transduction). Mice receiving T cells were injected with 5.0 - 10 × 10^6 cells intravenously via the tail vein and imaged on days 2 and 9 after infusion. Briefly, 250 μg coelenterazine (NanoLight Technology, Pinetop, AZ) was injected IV in mice receiving either saline or EGFRvIII extGLuc CAR T cells and imaged within 90 seconds by measuring BLI. We obtained image data sets and measurement of signal intensity by using the IVIS 100 in vivo BLI system and through region of interest analysis using Living Image Software with normalized images displayed on each data set according to color intensity. Mean BLI values for control and treatment groups were calculated and plotted according to the corresponding day of imaging.

H&E Staining and Immunohistochemistry
In order to evaluate tumor architecture and growth patterns, NSG mice received intracerebral tumor implants (D-270MGFluc/GFP) 1 × 10^5 cells or U87MG.ΔEGFR/1 × 10^5 cells and were sacrificed after seven days. Brains were harvested, formalin-fixed and paraffin-embedded. 5 μm sections were stained with H&E. To evaluate T-cell migration to the invasive edge of tumor, we utilized two experimental groups, which included four tumor-bearing mice treated with saline or extGLuc EGFRvIII CAR 1 × 10^7 T cells. Mice were euthanized on day 9. Brains were harvested, formalin-fixed and paraffin-embedded. 5 μm coronal sections were immunostained with rabbit anti-human CD3 antibody (Thermo Lab Vision, Clone RM9107S) as recommended by the manufacturer at a 1:100 dilution and counterstained with hematoxylin.

Statistical methods
Statistical differences in group percentage cytotoxicity and bioluminescence were evaluated by either a one-way analysis of variance (ANOVA) or a two-way ANOVA model with interaction. The Kaplan-Meier estimator was used to generate survival curves, and differences between survival curves were calculated using a generalized Wilcoxon test. Patterns of change in normalized BLI values on the log scale over time were evaluated using a mixed model that included main effects for time and treatment group along with a time interaction with treatment group. This model accounted for within animal correlation of measurements by using a 1st degree autoregressive covariance structure.

Acknowledgments
We extend our gratitude to Steven A. Rosenberg and Richard A. Morgan of the Surgery Branch at the National Cancer Institute for providing us with the EGFRvIII CAR construct and Renier J. Brentjens of Memorial Sloan-Kettering Cancer Center for providing us with the extLuc retrovirus used in this study. We also thank Alina Boesteanu for providing histology images of U87MG.ΔEGFR xenograft implants and David Snyder for his contribution to animal care.

Author Contributions
Conceived and designed the experiments: HM BDC LSP CMS LAJ. Performed the experiments: HM BDC LSP SY GD EJS. Analyzed the data: RM JEH PH. Contributed reagents/materials/analysis tools: LSP CMS LAJ JHS. Wrote the paper: HM BDC LSP CMS LAJ JHS. The paper: HM BDC LSP CMS LAJ.

References
1. Stupp R, Mason WP, van den Bent MJ, Weller M, Fisher B, et al. (2005) Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. N Engl J Med 352: 987–996.
2. Imperato JP, Paleologos NA, Vick NA (1990) Effects of treatment on long-term survivors with malignant astrocytoma. Ann Neurol 28: 818–822.
3. Johnson LA, Morgan RA, Dudley ME, Cassard L, Yang JC, et al. (2009) Gene therapy with human and mouse T-cell receptors mediates cancer regression and targets normal tissues expressing cognate antigen. Blood 114: 355–346.
4. Hong JJ, Rosenberg SA, Dudley ME, Yang JC, White DE, et al. (2010) Successful treatment of melanoma brain metastases with adoptive cell therapy. Clin Cancer Res 16: 4892–4898.
5. Robbins PF, Morgan RA, Feldman SA, Yang JC, Sherry RM, et al. (2011) Tumor regression in patients with metastatic syringobulbia cell sarcoma and melanoma using genetically engineered lymphocytes reactive with NY-ESO-1. J Clin Oncol 29: 917–924.
6. Rosenberg SA (2011) Cell transfer immunotherapy for metastatic solid cancer—what clinicians need to know. Nat Rev Clin Oncol 8: 577–583.
7. Kochenderfer JN, Dudley ME, Feldman SA, Wilson WH, Spaner DE, et al. (2012) B-cell depletion and remissions of malignancy along with cytokine-associated toxicity in a clinical trial of anti-CD19 chimeric antigen-receptor-transduced T cells. Blood 119: 2709–2720.
8. Rosenberg SA, Yang JC, Robbins PF, Wunderlich JR, Hwu P, et al. (2012) CD20-specific adoptive immunotherapy for lymphoma using a chimeric antigen receptor with both CD20 and 4-1BB domains: pilot clinical trial results. Blood 119: 3940–3950.
9. Pol AE, Sarolvo B, Myers GD, Rossig C, Russell HV, et al. (2008) Virus-specific T cells engineered to coexpress tumor-specific receptors: persistence and antitumor activity in individuals with neuroblastoma. Nat Med 14: 1264–1270.
10. Brentjens RJ, Davila ML, Riviere I, Park J, Wang X, et al. (2013) CD19-targeted T cells rapidly induce molecular remissions in adults with chemotherapy-refractory acute lymphoblastic leukemia. Sci Transl Med 5: 177ra138.
11. Porter DL, Levine BL, Kalos M, Bagg A, June CH (2011) Chimeric antigen receptor-modified T cells in chronic lymphoid leukemia. N Engl J Med 365: 725–733.
12. Tilt BG, Jensen MC, Wang J, Qian X, Gopal AK, et al. (2012) CD20-specific adoptive immunotherapy for lymphoma using a chimeric antigen receptor with both CD20 and 4-1BB domains: pilot clinical trial results. Blood 119: 3940–3950.
13. Pule MA, Savoldo B, Myers GD, Rossig C, Russell HV, et al. (2008) Virus-specific T cells engineered to coexpress tumor-specific receptors: persistence and antitumor activity in individuals with neuroblastoma. Nat Med 14: 1264–1270.
14. Brentjens R, Yeh R, Bernal Y, Riviere I, Sadelain M (2010) Treatment of chronic lymphocytic leukemia with genetically targeted autologous T cells: case report of an unforeseen adverse event in a phase I clinical trial. Mol Ther 18: 666–668.
15. Morgan RA, Yang JC, Kitano M, Dudley ME, Laurencot CM, et al. (2010) Case report of a serious adverse event following the administration of T cells transduced with a chimeric antigen receptor recognizing ERBB2. Mol Ther 18: 654–657.
16. Wikstrander CJ, Hale LP, Batra SK, Hill ML, Humphrey PA, et al. (1995) Prognostic value of the blood-brain barrier. Journal of Neural Transmission 113: 477–485.
37. Sadelain M, Brentjens R, Riviere I (2013) The basic principles of chimeric antigen receptor design. Cancer Discov 3: 388–398.

38. Choi BD, Suryadevara CM, Gedeon PC, Herndon JF, E., Sanchez-Perez L, et al. (2014) Intracerebral delivery of a third generation EGFRvIII-specific chimeric antigen receptor is efficacious against human glioma. J Clin Neurosci 21: 109–190.

39. Engelhardt B, Ramohoff RM (2012) Capture, cradle, cross: the T cell code to breach the blood-brain barriers. Trends Immunol 33: 579–589.

40. Ramohoff RM (2009) Chemokines and chemokine receptors: standing at the crossroads of immunobiology and neurobiology. Immunity 31: 711–721.

41. Mestas J, Hughes CC (2004) Of mice and not men: differences between mouse and human immunology. J Immunol 172: 2731–2738.

42. Grupp SA, Kals M, Barrett D, Aplenc R, Porter DL, et al. (2013) Chimeric antigen receptor-modified T cells for acute lymphoid leukemia. N Engl J Med 365: 1509–1518.

43. Sampson JH, Heimberger AB, Archer GE, Aldape KD, Friedman AH, et al. (2010) Immunologic escape after prolonged progression-free survival with epidermal growth factor receptor variant III peptide vaccination in patients with newly diagnosed glioblastoma. J Clin Oncol 28: 4722–4729.

44. Butterfield LH, Ribas A, Dissette VB, Amarnani SN, Yu HT, et al. (2003) Determinant spreading associated with clinical response in dendritic cell-based immunotherapy for malignant melanoma. Clin Cancer Res 9: 998–1008.

45. Hughes MS, Yu YY, Dudley ME, Zheng Z, Robbins PF, et al. (2005) Transfer of a TCR gene derived from a patient with a marked antitumor response conveys highly active T-cell effector functions. Hum Gene Ther 16: 457–472.

46. Morgan RA, Dudley ME, Yu YY, Zheng Z, Robbins PF, et al. (2003) High efficiency TCR gene transfer into primary human lymphocytes affords avid recognition of melanoma tumor antigen glycoprotein 100 and does not alter the recognition of autologous melanoma antigens. J Immunol 171: 3267–3295.

47. Riddell SR, Greenberg PD (1990) The use of anti-CD3 and anti-CD28 monoclonal antibodies to clone and expand human antigen-specific T cells. J Immunol Methods 128: 189–201.

48. Yang X, Cohen CJ, Peng PD, Zhao Y, Cassard L, et al. (2008) Development of optimal bicistronic lentiviral vectors facilitates high-level TCR gene expression and robust tumor cell recognition. Gene Ther 15: 1411–1423.

49. Yoshimoto K, Deng J, Zhu S, Nathanson D, Huang T, et al. (2008) Development of a real-time RT-PCR assay for detecting EGFRvIII in glioblastoma samples. Clin Cancer Res 14: 488–493.

50. Sampson JH, Choi BD, Sanchez-Perez L, Suryadevara CM, Snyder DJ, et al. (2013) EGFRvIII-mCAR-modified T cell therapy cures mice with established intracerebral glioma and generates host immunity against tumor-antigen loss. Clin Cancer Res.

51. Szentirmai O, Baker CH, Lin N, Szucs S, Takahashi M, et al. (2006) Noninvasive bioluminescence imaging of Luciferase expressing intracranial U87 xenografts: correlation with magnetic resonance imaging determined tumor volume and longitudinal use in assessing tumor growth and antiangiogenic treatment effect. Neurosurgery 50: 365–372; discussion 365–372.