Cancer Therapy: Preclinical

EGFRvIII mCAR-Modified T-Cell Therapy Cures Mice with Established Intracerebral Glioma and Generates Host Immunity against Tumor-Antigen Loss

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Abstract

**Purpose:** Chimeric antigen receptor (CAR) transduced T cells represent a promising immune therapy that has been shown to successfully treat cancers in mice and humans. However, CARs targeting antigens expressed in both tumors and normal tissues have led to significant toxicity. Preclinical studies have been limited by the use of xenograft models that do not adequately recapitulate the immune system of a clinically relevant host. A constitutively activated mutant of the naturally occurring epidermal growth factor receptor (EGFRvIII) is antigenically identical in both human and mouse glioma, but is also completely absent from any normal tissues.

**Experimental Design:** We developed a third-generation, EGFRvIII-specific murine CAR (mCAR), and performed tests to determine its efficacy in a fully immunocompetent mouse model of malignant glioma.

**Results:** At elevated doses, infusion with EGFRvIII mCART cells led to cures in all mice with brain tumors. In addition, antitumor efficacy was found to be dependent on lymphodepletive host conditioning. Selective blockade with EGFRvIII soluble peptide significantly abrogated the activity of EGFRvIII mCAR T cells in vitro and in vivo, and may offer a novel strategy to enhance the safety profile for CAR-based therapy. Finally, mCAR-treated, cured mice were resistant to rechallenge with EGFRvIII^NEG^ tumors, suggesting generation of host immunity against additional tumor antigens.

**Conclusion:** All together, these data support that third-generation, EGFRvIII-specific mCARs are effective against gliomas in the brain and highlight the importance of syngeneic, immunocompetent models in the preclinical evaluation of tumor immunotherapies. Clin Cancer Res; 20(4); 972–84. ©2013 AACR.

Introduction

Glioblastoma is the most common brain tumor and also the most deadly. Despite aggressive treatment, including surgical resection, dose-intensive radiation, and multimodal chemotherapy, prognosis remains exceedingly grim with a median survival of less than 15 months from the time of diagnosis (1). Moreover, the effects of current therapies are often nonspecific, causing significant collateral damage to healthy cells and adjacent brain.

In contrast, immunotherapy offers an extremely precise approach with the potential to eliminate cancer specifically while leaving normal tissues intact. Substantial evidence suggests that T cells in particular have the ability to eradicate large, well-established tumors in mice and humans (2–7). As such, a number of attempts have been made to establish large populations of tumor-reactive T cells in vivo, mainly via adoptive transfer of expanded tumor-infiltrating lymphocytes (TIL) or autologous T cells that have been transduced to express specific T-cell receptors (TCR; refs. 2, 3, and 5). Although promising, these approaches have been limited by a number of technical and functional drawbacks. Although TILs are difficult to isolate in most cancers, TCR-transduced T cells recognize only specific MHC alleles, restricting them to a subset of patients and making them vulnerable to MHC downregulation by tumors (8).
To address these limitations, a versatile class of receptors known as chimeric antigen receptors (CAR) has been generated by combining the variable region of an antibody with a T-cell signaling molecule, usually CD3 (9). Because their capacity for antigen recognition is derived from antibody binding, CARs have the ability to mimic endogenous immune system contribution to immunity. This work describes a relevant in vivo model for translational cancer research, namely, using immune-intact mice for adoptive T-cell–based cancer therapy, and has direct clinical implications that have previously been largely overlooked.

An additional advantage of the CAR platform is the incorporation of costimulatory molecules such as CD28 and 4-1BB into the CD3 signaling domain to improve T-cell expansion, survival, cytokine secretion, and tumor lysis (12–14). Clinical trials utilizing these second- and third-generation CARs have now targeted a variety of antigens and malignancies and have demonstrated their remarkable potential (15–18). However, severe adverse events have occurred when these CARs have been directed against antigens shared by normal tissues, such as ERBB2/HER2 (19). As such, the lack of truly tumor-specific targets for CARs and a poor toxicity profile to date represent critical barriers to the safe and effective translation of this promising therapy.

EGFRvIII is a tumor-specific mutation of the epidermal growth factor receptor that is absent from normal tissues, but commonly expressed on the surface of glioblastomas and other neoplasms (20). Functionally, EGFRvIII is a constitutively active version of the wild-type receptor, conferring enhanced tumorigenicity (21, 22), invasiveness (23), and therapeutic resistance (24) to tumor cells. Because this mutation results in the translation of a unique extracellular epitope, it is readily recognized by a number of previously described monoclonal antibodies (20); EGFRvIII thus represents an ideal target for CAR-based therapeutic development.

With few exceptions, the great majority of preclinical studies for CARs have been performed in vitro or in vivo with xenogeneic models wherein human T cells are tested against human tumors implanted into immunocompromised mice (25–30). This strategy is often the only available option, due to the lack of immunocompetent rodent models possessing surface molecules of equivalent binding affinities and function to those found in humans. Unfortunately, preclinical reports of gene-modified T cells in xenograft systems have not been predictive of dramatic toxicities that occurred upon translation in early clinical trials (13, 19). In addition to inadequately assessing autoimmunity, these xenograft models also do not permit realistic analyses of parameters that may critically impact efficacy in humans, such as the influence of host-conditioning regimens, species-specific immunosuppressive factors, and the potential generation or priming of endogenous immunity (26).

In this study, we directly address the limitations of previous immunocompromised models by generating a murine-derived, third-generation, EGFRvIII-specific CAR [EGFRvIII murine CAR (mCAR)] for evaluation in a fully immunocompetent mouse model of malignant glioma (31). In addition, we target a murine homologue of EGFRvIII that demonstrates identical antibody-binding characteristics to the human EGFRvIII (32). Our results demonstrate that murine T cells transduced with the EGFRvIII mCAR (mCAR T cells) express IFN-γ specifically in the presence of target cells expressing the EGFRvIII mutation. Despite conventional notions of immune-privilege, treatment with mCAR T cells led to complete eradication of 3- to 5-day established, syngeneic, EGFRvIII-expressing gliomas located subcutaneously and in the brain. Therapeutic effects were shown to be dose dependent and required host lymphodepletion before adoptive transfer for efficacy. We also show the ability to block this mCAR T-cell function in vivo with systemic administration of EGFRvIII peptide. Finally, successfully cured mice did not develop tumors upon rechallenge with EGFRvIII mCAR (mCAR T cells) express IFN-γ specifically in the presence of target cells expressing the EGFRvIII mutation. Together, our findings support that third-generation CARs can be used to produce effective immune responses against tumors in the brain. In addition, these data highlight the requirement of lymphodepletive host conditioning for efficacy, and also suggest that epitope spreading may contribute to favorable outcomes in the setting of CAR-mediated therapies. Ultimately, these studies lend credence to the utilization of fully immunocompetent models in the setting of adoptive transfer, with the potential to inform critical aspects of clinical trial design.
Materials and Methods

Cell lines and media
The murine glioma parental cell line SMA560 (EGFRvIII\textsuperscript{NEG}), and its subline, SMA560vIII (EGFRvIII\textsuperscript{POS}) have been previously described (31). Murine T cells were stimulated in complete RPMI-1640 media (R10; HEPES, Pen/Step, NEAA, L-Glut, 2ME, RPMI-1640 plus 10% FBS) supplemented with 50 IU/mL rh interleukin (IL)-2 or 1 ng/mL rhIL-7 for 2 days, followed by expansion in 50 IU/mL IL-2.

Retrovirus design and T-cell transduction
The murine (m) 139 single-chain antibody variable fragment (scFv) mCAR retrovirus was generated similarly to the published human (h) 139 scFv hCAR (34). The 139 scFv was inserted in tandem with mCD8 trans-membrane, mCD28, m4-1BB, and mCD3\textsubscript{z} intracellular regions in the MSGV1 retroviral vector as previously described (13, 35). Retroviral supernatant was generated by transient cotransfection of HEK 293T cells by Lipofectamine 2000 Transfection Reagent (Invitrogen), along with pCL-Eco helper plasmid (Imgenex). The resulting retroviral supernatant was used to transduce murine splenocytes as previously described (36). Briefly, mouse splenocytes were collected from VM/Dk mice, disaggregated, and passed through a 70-µm mesh filter to generate a single-cell suspension. Cells were cultured in complete R10 mouse T-cell media supplemented with 1 ng/mL rmIL-7 and activated on day 0 with 2.5 µg/mL Concanavalin A (ConA). T cells were transduced on RetroNectin-coated plates (Takara Inc.) at a density of 1 × 10\textsuperscript{5}/mL on day 2 after stimulation. Cells were then maintained at 1 to 2 × 10\textsuperscript{6}/mL in mouse T-cell media with 50 IU/mL rhIL-2 for 4 to 7 days.

Flow cytometric analysis
Live cells were gated on the lymphocyte population by forward scatter/side scatter (FSC/SSC). Transduced T cells were stained for mCAR surface expression using goat–anti-human F(ab)\textsuperscript{2}-biotin primary and streptavidin–phycoerythrin (SA–PE) secondary antibodies as previously described (34), or using a PEPvIII-PE multimer generated in the lab. Transduction efficiency was confirmed by paired GFP-transduced controls. SMA560 and SMA560vIII tumors were stained for surface EGFRvIII expression using the L8A4 mAb (Duke University) with a goat–anti-mouse Ig-PE secondary antibody.

IFN-γ ELISpot assay
T-cell responses to EGFRvIII\textsuperscript{POS} tumor target cells were measured ex vivo by direct IFN-γ ELISpot assay. Single-cell suspensions of splenic lymphocytes from naive or mice treated with GFP or EGFRvIII mCAR T cells in R10 medium were prepared by physical disruption. After red blood cell lysis and 2 washes with R10 medium, lymphocytes were isolated by density gradient centrifugation on Lympholyte M (Cedarlane), washed 2 times, and resuspended in fresh R10 medium. Splenic lymphocytes (2.5 × 10\textsuperscript{5}/well) were added to duplicate wells of ELISpot assay plates coated with anti-mouse IFN-γ monoclonal antibody (AN18) and allowed to settle for 1 hour. SMA560 or SMA560vIII tumor targets were subsequently added after 1 hour at an effector-to-tumor cell ratio of 1:1 and incubated overnight at 37°C. Plates were washed with PBS Tween-20, incubated with biotinylated, anti-mouse IFN-γ mAb [R4-6A2 (2 hours), avidin–peroxidase complex (1 hour), and substrate (3-amino-9-ethylcarbazole) for 4 minutes], separated by PBS washes at room temperature. Plates were dried and shipped to Zellnet Consulting for spot enumeration by automated analysis with a Zeiss KS ELISpot system. This assay was MIATA compliant.

Syngeneic murine model for brain tumor immunotherapy
SMA560vIII cells were harvested with trypsin, washed one time in serum-containing medium, and washed 2 times in Dulbecco’s PBS (DPBS). Cell pellets were resuspended in DPBS at the appropriate concentration of viable cells as determined by trypan blue dye exclusion, mixed with an equal volume of 10% methylcellulose in Zinc Option Modified Eagle medium (MEM) and loaded into a 250-µL syringe with an attached 25-gauge needle. In VM/Dk mice, the tip of the needle was positioned at bregma and 2 mm to the right of the cranial midline suture and 4 mm below the surface of the cranium using a stereotactic frame (David Kopf Instruments). Each mouse received 5 × 10\textsuperscript{5} tumor cells implanted into the brain in a volume of 5 µL. Three to five days following implantation, mice were treated with 1 × 10\textsuperscript{5} to 1 × 10\textsuperscript{6} mCAR or GFP control VM/Dk T cells delivered systemically via tail–vein injection. Absolute numbers of T cells were used for calculations, rather than number of mCAR positive T cells (mCAR surface expression ranged from 50% to 75%). In experiments evaluating efficacy against subcutaneous tumors, 5 × 10\textsuperscript{5} SMA560vIII tumor cells were injected into the right flank, in a volume of 100 µL PBS, followed 3 days later by 5 × 10\textsuperscript{5} mCAR or GFP control T cells delivered systemically via tail–vein injection. Where indicated, mice were conditioned with 5 Gy total body irradiation (TBI) immediately before T-cell transfer. For each in vivo experiment, groups consisted of 8 mice, unless stated otherwise. All experiments were repeated a minimum of 2 times. According to Duke University and Institutional Animal Care and Use Committee (IACUC) protocols, animal survival was monitored, with mortality recorded or mice sacrificed upon reaching predetermined morbidity endpoints. For subcutaneous experiments, tumors were measured for width and length at widest diameters to obtain size in mm\textsuperscript{2}, animals were sacrificed upon any single diameter reaching 20 mm or upon ulceration.

Soluble PEPvIII blockade in vitro
The effect of peptide blocking on EGFRvIII mCAR T cells was measured utilizing the ELISpot assay described above. 24-well tissue-culture plate wells were coated with 50 µg/mL of soluble EGFRvIII peptide, PEPvIII (LEEKKG-NYVTIDHC), or control. EGFRvIII mCAR or GFP control T cells were added to the wells in presence or absence...
of 10 or 100 μg/ml of PEPvIII or irrelevant isocitrate dehydrogenase (IDH) peptide (GWVKPIIIGHHAYGD-QYR). Cells (5 × 10^5/well) were incubated for 18 hours and submitted for IFN-γ ELISpot analysis.

**Soluble PEPvIII blockade in vivo**

PEPvIII peptide was prepared as a stock solution in dimethyl sulfoxide, then diluted 10× in PBS before injecting 100 μg in 100 μL via tail vein into mice that had previously received mCAR or GFP control T cells. Injections were administered 2 to 4 hours after T-cell transfer, and repeated 10 days later.

**In vivo passaging of SMA560p tumor**

The SMA560 tumor line was cultured in Zinc Option MEM media for 1 week before implantation. At the time of implantation, cells were harvested with trypsin, washed 1 time in serum-containing medium, washed 2 times in DPBS, and adjusted to a concentration of 5 × 10^6 cells/mL. One hundred microliters of cells were subcutaneously injected into the right flank of VM/Dk mice and were allowed to grow and establish for 14 days. Tumors were then harvested and made into a single-cell suspension by passing them through a 70 μm cell strainer and submitted to EGFRvIII staining as described above.

**Analysis of CAR persistence in murine peripheral blood lymphocytes**

Whole blood obtained by retro-orbital bleeding was analyzed for EGFRvIII mCAR T cells as follows: 50 μL of blood was incubated with the antibody CD3-APC, and PEPvIII-PE multimer in 150 μL FACS buffer protected from light for 15 minutes at room temperature. Red blood cells were lysed and cells were fixed using 1 mL 1× BD FACS Lysing Solution (BD Bioscience, Cat. No. 349202) and incubated overnight at 4°C. Cells were then washed and resuspended in 2% paraformaldehyde and submitted to flow cytometry analysis. All samples were analyzed on a FACScalibur flow cytometer (BD Biosciences). Absolute numbers per microliter of blood were calculated using Flowcount beads from Beckman Coulter according to manufacturer instructions.

**Rechallenge with EGFRvIII^NEG^ tumor**

Either tumor naïve mice, tumor naïve mice receiving T cells, or mice that had been cured of previous subcutaneous SMA560vIII tumors following treatment with EGFRvIII mCAR T cells were rechallenged with 5 × 10^5 SMA560 (EGFRvIII^NEG^) tumors injected subcutaneously into the left (contralateral) flank.

**Results**

**Generation of a third-generation, EGFRvIII-specific mCAR**

To evaluate CAR T-cell function in a syngeneic, immunocompetent brain tumor model, we chose the murine glioma cell line, SMA560, derived from an astrocytoma that arose spontaneously in a VM/Dk mouse (37), in its parental form (SMA560) or stably transfected with EGFRvIII (SMA560vIII; Fig. 1A). This astrocytoma mirrors human gliomas phenotypically and morphologically, and secretes immunosuppressive cytokines, including TGF-βs secreted by human gliomas (38). To develop an appropriate mCAR, we combined murine signaling domains for CD28, 4-1BB, and CD3ε with a scFv isolated from a human antibody clone specific for EGFRvIII, mAb 139, inserted into the MSGV1 retroviral backbone (ref. 13; Fig. 1B). The selection of mAb 139 was based on observations that this clone, compared with 6 others, could be consistently expressed in retrovirally transduced cells and recognize EGFRvIII antigen with high avidity and specificity (34). Also, because 139 possesses known specificity for the naturally occurring EGFRvIII epitope in both mouse and human tumors, its incorporation into preclinical models offers translational potential as well as biologic principle.

Next, we sought to determine the expression of the EGFRvIII mCAR on the surface of retrovirally transduced splenocytes isolated from VM/Dk mice. The same retrovirus encoding GFP was used to transduce control T cells. Flow cytometric analysis following transduction revealed expression of GFP (data not shown), and surface expression of the EGFRvIII mCAR at 58% (MFI 328 vs. negative control of <1%, MFI 8; Fig. 1C). Importantly, when tested for reactivity against tumor cell lines, GFP control T cells did not display a detectable response to EGFRvIII^POS^ SMA560vIII by IFN-γ ELISpot, whereas T cells transduced with the EGFRvIII mCAR yielded a significant number of spot-forming units (P < 0.05; Fig. 1D), which was found to be dependent on EGFRvIII expression on target tumor cells.

To test receptor specificity in vitro, we leveraged a previously described EGFRvIII-derived soluble peptide, PEPvIII, that corresponds with the extracellular antigenic epitope of the EGFRvIII tumor mutation (33). We precoated tissue culture plates with PEPvIII or a negative-control peptide, and added EGFRvIII mCAR or vector control T cells. Only EGFRvIII mCAR T cells plated on PEPvIII showed reactivity by producing IFN-γ, and this reactivity was blocked up to 75% in a concentration-specific manner by adding increasing amounts of soluble PEPvIII (P < 0.01), but not by the nonspecific IDH control peptide (Fig. 1E).

**mCAR T cells show efficacy against intracerebral tumors that is dependent upon lymphodepleting preconditioning**

Recent clinical trials of TCR- and CAR-engineered T-cell therapy have used systemic delivery to treat widely disseminated disease (2, 3, 5, 15, 17, 39, 40). Previous studies have demonstrated that adoptively transferred T cells administered intravenously can even treat intracerebral tumor metastases (2, 15, 41); however, in contrast to tumors arising from central nervous system (CNS) parenchyma, metastatic lesions tend to be associated with vascular border zones at the gray and white matter junction (42) and may not accurately recapitulate challenges associated with...
tumors arising from tissues originating behind the blood-brain barrier. To overcome the criticisms of previously used xenograft models (26), we selected an immune-intact murine model with a synegetic, invasive glioma to evaluate the preclinical efficacy of EGFRvIII mCAR T cells in vivo.

Previous studies have shown that the survival and antitumor function of adoptively transferred T cells in vivo can be dependent upon lymphodepletive host conditioning (43–45). However, the impact of this effect has been under- appreciated for CAR T cells, given the few available immune-competent animal models for preclinical testing. Using our synegetic murine system, we sought to determine if EGFRvIII mCAR T cells with or without lymphodepletive host conditioning with TBI could demonstrate efficacy against tumors in the brain.

To investigate the ability of EGFRvIII mCAR T cells to localize and treat gliomas in the CNS, VM/Dk mice were implanted intracerebrally with SMA560vIII gliomas. Three to five days later, mice were treated with or without 5 Gy TBI to induce lymphodepletion, and were then injected intravenously with either control or EGFRvIII mCAR T cells and monitored for survival endpoints. In the absence of TBI host conditioning, mice treated with EGFRvIII mCAR T cells did not display any survival benefit over mice receiving negative control GFP T cells (Fig. 2A). However, significantly prolonged survival was observed using the same number of EGFRvIII mCAR T cells, compared with GFP T cells in the setting of 5 Gy TBI ($P<0.01$; Fig. 2B). Thus, in our synegetic, immunocompetent mouse model, lymphodepletion is shown to be a critical factor in promoting the efficacy of CAR-modified T cells against tumors in the CNS.

**EGFRvIII mCAR T-cell activity is abrogated by soluble peptide**

Perhaps the greatest drawback of CAR T-cell therapy has been the lethal toxicity resulting from interaction between T cells and cognate antigen coexpressed in both tumors and normal tissues (19). Thus, a great need exists for novel strategies to reduce the toxicity profile of CARs targeting shared antigens on nontumor cells. As proof-of-concept, we sought to determine whether a short peptide corresponding to the antigenic epitope expressed on target cells could be applied to competitively inhibit and reduce the functional activity of EGFRvIII mCAR T cells in vivo, similar to the effects we saw in vitro.

To perform antigen-specific blockade in vivo in our model system, VM/Dk mice bearing intracerebral SMA560vIII tumors were irradiated and treated with EGFRvIII mCAR of $P<0.05$. E, soluble EGFRvIII peptide (PEPvIII) at 50 μg/mL was used to coat tissue culture plates before addition of EGFRvIII mCAR or GFP control T cells. Cells were cultured 18 hours in the presence of increasing dosages of soluble PEPvIII or irrelevant peptide (IDH) and subjected to IFN-γ ELISpot assay. Statistical analysis was performed by unpaired $t$ test comparing groups defined by EGFRvIII target expression. Horizontal bar represents a statistical significance of $P<0.05$. Experiments are representative of 2 independent repeats with similar results.
EGFRvIII mCAR T cells treat 3- to 5-day established, syngeneic subcutaneous, and intracranial tumors in a dose-dependent manner

To evaluate if treatment effect was dose-dependant, we repeated the intracranial glioma treatment experiment, using increasing numbers of EGFRvIII mCAR T cells. T-cell treatment numbers were based upon total number of cells and were transduced 50% to 75%. At the lowest dose ($0.7 \times 10^5$), there was no apparent impact on survival over control T-cell–treated mice. However, there was a significant, dose-dependent improvement in survival in mice receiving $3.5 \times 10^5$ to $1.0 \times 10^7$ EGFRvIII mCAR T cells, and at the highest dose, all animals were cured of intracranial tumors without apparent toxicity ($P<0.0002$; Fig. 3A). Taken together, these data demonstrate that systemic administration of EGFRvIII mCAR T cells can potently eliminate EGFRvIII-expressing tumors, even in the immunologically privileged CNS.

To follow tumor growth characteristics in the context of CAR treatment, VM/Dk mice were implanted subcutaneously with SMA560vIII, and tumor growth was recorded to measure treatment effect. Tumors in mice lymphodepleted with 5 Gy TBI before receiving GFP control T cells did not exhibit significantly altered tumor growth compared with untreated mice. However, lymphodepleted mice receiving

T cells followed by PEPvIII via intravenous infusion. Using only 2 separate injections of $100 \mu g$ PEPvIII, given 10 days apart, we observed a significant reduction in EGFRvIII mCAR T-cell activity in vivo, demonstrated by a reduction in overall survival compared with animals receiving EGFRvIII-targeted T cells alone ($P<0.05$; Fig. 2C). These data support that soluble cognate peptide may be used to selectively inhibit mCAR T cells in vivo. In addition, this selective blockade also provides evidence supporting the antigen specificity of the 139 scFv EGFRvIII mCAR.
EGFRvIII mCAR T cells exhibited compelling treatment effects with subcutaneous tumor growth completely eliminated in 5 of 8 mice (P < 0.0001; Fig. 3B).

EGFRvIII mCAR T cells show long-term persistence in vivo

T-cell persistence was evaluated in peripheral blood lymphocytes (PBL) of 5-Gy–irradiated mCAR-treated mice more than 35 days, and mCAR T cells were detected at all collection timepoints. Over time, the percentage of circulating PBL that were EGFRvIII mCAR-positive declined from 4.5% after 7 days to 1.5% on day 35, with the steepest decline occurring between days 14 and 28 (Fig. 4A). As this is the same time period in which endogenous lymphocytes would be expected to begin recovering from TBI in irradiated mice, we also assessed the total absolute number of mCAR T cells in the periphery. The total numbers of circulating EGFRvIII mCAR T cells in mice did not change significantly between weeks 1 to 5 after infusion, maintaining approximately 15 to 25 per μL of blood volume (Fig. 4B). The same trend was observed in mice receiving GFP control T cells (data not shown). This suggested that the overall number of transferred EGFRvIII mCAR T cells were maintained in vivo over time, remaining relatively constant, whereas endogenous lymphocytes recovered and increased in numbers in the periphery, reducing the percentage of circulating EGFRvIII mCAR T cells, but not the total amount.

EGFRvIII mCAR treatment of mice with EGFRvIIINEG expressing tumors provides long-term protection against rechallenge and EGFRvIII tumor-antigen loss

Substantial evidence supports that immune-based therapies have the ability to eradicate tumors based on the expression of specific antigens (7). However, a limitation of current strategies is that they often result in tumor escape owing to heterogeneous antigen expression; indeed, although EGFRvIII peptide vaccines have been shown to successfully eliminate EGFRvIII-expressing tumor cells in mice and humans, outgrowth of EGFRvIII<sup>NEG</sup> cells ultimately leads to tumor recurrence (33).

One suggested mechanism by which T-cell–based therapies in particular may be able to protect against tumor immune escape is through the triggering of epitope spreading, initiated by a localized endogenous immune response by the release of inflammatory cytokines at the tumor. The resulting recruitment of the endogenous immune system in the face of tumor destruction may confer the ability to generate new responses to tumor cells that do not express the original target antigen. To date, the ability of third-generation CAR T cells to effectively initiate this activity remains unknown, again owing to the relative dearth of available preclinical models with fully competent immune systems capable of mounting such a response.

We sought to determine whether treatment with EGFRvIII mCAR T cells could elicit de novo priming against additional tumor antigens, which could then serve as protection against tumor-antigen loss. Specifically, we wanted to test whether mice that were previously treated with mCARs could acquire protective immunity against tumors that did not contain the original targeted antigen of interest, in this case, EGFRvIII. To do this, we challenged mice—either tumor naïve mice with no treatment, or those previously given EGFRvIII mCAR T cells and cured of SMA560vIII tumor. In the control group, tumors grew rapidly and all reached humane size endpoints within 40 days. In contrast, previously cured mice were completely protected against rechallenge with EGFRvIII<sup>NEG</sup> tumor (Fig. 5A).

The ability of EGFRvIII<sup>NEG</sup> glioma cell lines to express EGFRvIII upon transfer in vivo has been recently demonstrated (46). We sought to rule out the possibility that this was the reason for protection against EGFRvIII<sup>NEG</sup> tumor...
To evaluate whether SMA560 upregulates EGFR-vIII in vivo, we implanted SMA560 tumors subcutaneously in mice. Fourteen days later, we excised the tumors, stained them for EGFRvIII expression, and performed a flow cytometric analysis. Figure 5B shows that although the EGFRvIII expression did increase slightly, from 0.18% in vitro to 20%, the increase was not statistically significant. We also measured the tumor size and the percentage of EGFRvIII mCAR T cells in blood before and after tumor challenge. Figure 5C–E shows that the percentage of EGFRvIII mCAR T cells in blood increased significantly after tumor challenge, with a peak at 7 days after tumor challenge. The tumor size was also monitored, and Figure 5F shows that the tumor size decreased significantly after tumor challenge. We concluded that treatment with EGFRvIII mCAR T cells could elicit de novo priming against antigen-loss variants, as long-term survivors of subcutaneous SMA560vIII tumors were rechallenged with EGFRvIII mCAR therapy and their survival was monitored. Additionally, the vector control T cells were replaced with EGFRvIII mCAR T cells, and the percentage of EGFRvIII mCAR T cells in blood increased significantly after tumor challenge. We also observed that the tumor size decreased significantly after tumor challenge. The experiment was representative of 2 independent repeats with similar results. Statistical significance of P < 0.0001 was determined using 2-way ANOVA.
1.20% post passing in vivo, the majority (>98%) of tumor cells did not exhibit EGFRvIII expression, making it unlikely to be the cause of tumor rejection.

To further evaluate the possibility of EGFRvIII antigen expression in vivo on SMA560, we looked for mCAR T-cell proliferation in vivo in response to SMA560 challenge. We used groups of mice that had received EGFRvIII mCAR T cells in the absence of tumor, and mice that had received the same T cells in the presence of SMA560vIII, and had successfully cleared the tumor. Both T-cell–injected tumor naïve and cured mice were found to have circulating EGFRvIII mCAR T cells in their blood 5 weeks after injection, before SMA560 challenge (Fig. 5C and D). Both groups then received the EGFRvIII<sup>NEG</sup> SMA560 tumor implanted subcutaneously, and were re-evaluated for circulating mCAR T cells 7 days later. In the tumor naïve mice, there was no change in absolute numbers or percentages of EGFRvIII mCAR T cells before and after tumor challenge. However, unexpectedly, the cured mice showed a significant (P < 0.05) increase in both absolute numbers and percentages of circulating EGFRvIII mCAR T cells after the antigen-negative tumor rechallenge. This was surprising, as we found no significant increase in EGFRvIII expression on the tumor in vivo, and because EGFRvIII mCAR T cells alone were not sufficient to protect the mice against SMA560 rechallenge (Fig. 5E and Table 1). Importantly, these data suggest that the mechanism responsible for rejection of the rechallenge parental tumor in vivo may have included EGFRvIII mCAR T cells; however, additional elements were necessary for antitumor response. These results show that the mCAR T cells have some response to antigen-negative tumor rechallenge in vivo, whether by direct tumor interaction, or indirectly by another generalized immune stimulation in vivo. Together, these results suggest that third-generation, mCAR-modified T cells may contribute to the development of host immunity against tumor-antigen escape.

Discussion

Several groups have demonstrated that T cells can be successfully redirected by CARs to induce potent antitumor immune responses; however, unexpected toxicity has been observed because of shared target antigen expression on normal tissues (11, 19). Because of its exquisite tumor specificity, EGFRvIII has been previously shown to be a promising target for gene-modified T-cell therapy immune responses both in vitro and in xenogeneic, immunodeficient mouse models (25, 34, 47). Our findings in this study demonstrate that a third-generation, EGFRvIII-specific mCAR can be used to treat 3- to 5-day established intracerebral glioma in a fully immunocompetent, syngeneic murine model.

With 3 notable exceptions, CD19 (48, 49), vascular endothelial growth factor receptor (VEGFR)-2 (48), and fibroblast activation protein (FAP) (50), previous preclinical studies of CAR-redirected T cells have been performed in vitro or have been limited to in vivo studies against xenografts in immune-deficient mice (25, 28–30, 51). Xenograft models, while enabling important experiments using human tumors and reagents, suffer from numerous shortcomings that have been reviewed in detail elsewhere (26). Briefly, because these mice lack physiologic immune systems, a number of signaling ligands, effector molecules, and other circulating factors are absent that could otherwise impact T-cell function, trafficking, and persistence. Similarly, human tumor cells generally do not grow in mouse tissues as they do in patients, owing to several species-specific stromal interactions and autologous growth factors thought to promote highly aggressive and invasive characteristics of human tumors in situ. In this study, our utilization of an immunocompetent mouse model with tumors derived from a syngeneic, spontaneously arising glioma, addresses many of these limitations.

The utilization of our immunocompetent mouse model revealed a number of findings that had previously been impossible to evaluate in a xenograft setting. One finding was the need for lymphodeplettive host preconditioning to achieve antitumor efficacy using adoptively transferred mCAR T cells. We speculate this may be because of a number of factors, including making "space" for the engineered lymphocytes to expand in the periphery, lack of cytokine sinks for lymphoid survival factors, removal of circulating regulatory cells, and possible increased Toll-like receptor signaling in immune cells, induced by damaging radiation (52, 53).

Total mCAR T-cell numbers in PBL were maintained in mice for more than a 35-day period, although percentage of mCAR T cells diminished over time because of

### Table 1. Eradication of SMA560vIII by EGFRvIII 139 CAR T cells is required for immunity against SMA560 (EGFRvIII-negative) parental tumors

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of mice</th>
<th>SMA560vIII challenge</th>
<th>CAR T cells</th>
<th>SMA560vIII cures</th>
<th>SMA560 challenge</th>
<th>SMA560 cures</th>
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<td>–</td>
<td>–</td>
<td>N/A</td>
<td>+</td>
<td>0/8</td>
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<td>0/15</td>
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<tr>
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<td>–</td>
<td>+</td>
<td>N/A</td>
<td>+</td>
<td>0/4</td>
</tr>
<tr>
<td>mCAR T cells, SMA560vIII exposed</td>
<td>15</td>
<td>+</td>
<td>+</td>
<td>10/15</td>
<td>+</td>
<td>10/10</td>
</tr>
</tbody>
</table>

NOTE: Day 0, mice received SMA560vIII tumors subcutaneously; day 5, mice received 5 Gy TBI followed by T cells intravenously; day 28, mice were challenged with SMA560 parental tumors.
reconstitution of endogenous lymphocytes (Fig. 4). It is not known what proportion of T cells may have migrated into tumor, tissue, or lymphoid organs, however, so this may be an underestimate of total mCAR T-cell retention in the VM/Dk model. It remains unknown what happens to the cells in vivo following ACT. A number of factors may, and likely do contribute, including body size, metabolic processes, extent of tumor infiltration, and potential suppressive, or immune activation factors in the periphery or at the tumor site. Although total numbers of surviving mCAR T cells in our mouse model are unknown, it is possible that each CAR T-cell could function to kill many tumor cells, effectively generating "serial killers," as has been reported clinically for the CD19 CAR in patients with leukemia (17, 54), suggesting that clinically, the quality of the cell may be more important than the quantity.

One of the biggest clinical problems with glioblastoma is its infiltrative micrometastases. Although glioblastoma rarely metastasizes outside the brain, it does recur generally in the area around the initial tumor excision, indicating that tumor cells remain after gross total resection. One unexpected finding in our study is that mice treated with EGFRvIII mCARs developed long-lasting immunity against syngeneic EGFRvIII<sup>NEG</sup> tumors, suggesting that this approach may be superior to prior peptide vaccine approaches that have associated favorable clinical responses with the ability to either mutate or downregulate a targeted antigen of interest (33). A major mechanism by which tumors have been shown to evade the immune system is through the process of immune editing, whereby tumor cells are favoredly selected for the ability to either mutate or downregulate a targeted antigen of interest (55). These phenomena likely contributed to observations in our previous clinical studies of an EGFRvIII peptide vaccine, in which multiple patients achieved significant survival benefits but eventually had tumor recurrence owing to the outgrowth of EGFRvIII antigen-loss variant tumors (33). Unlike the EGFRvIII vaccine, which was found to elicit a predominantly humoral immune response, EGFRvIII CARs directly mediate a potent, highly avid T-cell response. Importantly, T-cell immunity in particular is known to play central roles in the promotion of endogenous priming associated with epitope spreading (56), and is also capable of protecting against antigen loss through direct bystander effects (57).

We speculate that the expanded immune response we see may be occurring because of mCAR T-cell interaction with cognate target-bearing tumors, causing T-cell stimulation, and resulting in proliferation and production of multiple cytokines, including the inflammatory IL-1, IL-6, and IL-8, plus TNF-α and IFN-γ. These inflammatory cytokines are known to have direct effects on tumors, including upregulation of antigen expression on tumor cells, and in addition can recruit endogenous innate immune cells, including professional antigen-presenting cells (APC) such as dendritic cells and macrophages to the site of tumor destruction. The combination of mCAR T cells causing tumor cell lysis, recruitment of APC, and an inflammatory milieu of pro-type 1 immune cytokines would create an optimal environment for the priming and activation of dendritic cells in the presence of tumor antigen. Previously referred to as a cytokine storm (58, 59), this "perfect storm" may be sufficient to cause activated APCs to engulf and present tumor-specific antigens to naïve T cells in situ, or at the draining lymph nodes, resulting in priming of endogenous T cells against novel tumor antigens. Although we cannot rule out a possible immune response against foreign antigens found on the tumors (i.e., fetal calf serum from in vitro culture), this would likely require the same immune priming process in vivo.

The development of endogenous antitumor (non-EGFRvIII) immunity in our mCAR-treated mice was somewhat unexpected, as our mice were lymphodepleted, although not myeloablated with radiation (5 Gy) at the time of their mCAR T-cell transfer. Even more surprising was the in vivo expansion of EGFRvIII mCAR T cells observed upon rechallenge with an EGFRvIII<sup>NEG</sup> tumor. It is unknown whether this increase in mCAR T cells was the result of proliferation induced by cognate antigen binding, or if another mechanism allowed for expansion of these cells in vivo, such as bystander proliferation as a result of IL-2 production by other activated T cells. We also postulate that the lymphodepleted periphery may have provided an ideal environment for adoptively-transferred mCAR T cells with endogenous tumor-reactive TCRs, or newly emergent naïve lymphocytes from the bone marrow with endogenous TCR recognition of tumor antigens, to selectively expand. Without competition for progrowth cytokines or suppression by T regulatory and other suppressive cells, activation of endogenous tumor-specific TCRs would provide a selective advantage to those cells to proliferate and expand into the lymphopenic periphery. Given that recent clinical studies have associated favorable clinical responses with the ability to produce broad immunity to previously untargeted antigenic determinants (60), further study about the ability for CARs to elicit these secondary responses should be an emerging priority.

The remarkable cytotoxic potential of CAR-based T-cell therapy is perhaps most unfortunately illustrated by the dramatic toxicities observed in clinical trials of CARs targeting antigens coexpressed in both tumors and normal tissues. Thus, the selection of a potential target antigen must be carefully considered to reduce the risk of life-threatening autoimmunity. To our knowledge, the EGFRvIII-specific CAR represents the first truly tumor-specific application of the CAR platform, and promises to circumvent toxicities that have been previously observed for genetically engineered T cells targeting shared antigens, including MART-1 (5), gp100 (2), CEA (40), CAIX (11), ERBB2 (19), and CD19 (18, 39). However, in situations where these antigens continue to be targets for CAR therapy, our data presented here support that strategies utilizing soluble peptide blockade may be further developed to provide a novel antidote for reducing undesired CAR-mediated T-cell activation in vivo.

Although not directly addressed in this study, our immunocompetent murine model could prove useful in the evaluation of endogenous suppressive factors that have
emerged as major impediments to successful antitumor immunotherapy. Many of these interactions are physiologically complex and as such are not adequately modeled by in vitro systems or immune-deficient animal models of human tumor xenograft. Molecular interactions such as those observed between PD-1, CTLA-4, and their respective ligands (61), as well as the impact of suppressive regulatory T cells and TGF-β in patients with glioma (62) have been cited as promising therapeutic targets for the reversal of tumor-associated immune suppression. Studies designed to address these and other questions in immunocompetent animal models may provide useful alternatives and will certainly gain relevance as CAR-mediated therapies progress through clinical trials. Here, we begin to provide evidence that immune-replete, preclinical models can reveal critical details for therapy that may inform clinical trial design.

Together, these data support that third-generation EGFRVIII CAR T cells have the ability to treat established syngeneic gliomas in the brain and may in addition confer host immunity against tumor-antigen loss variants through epitope spreading. As shown here, preclinical models with competent immune systems represent an opportunity to greatly advance our understanding of adoptive transfer therapies. Although it will be important to evaluate treatment at later timepoints following tumor implantation to better mirror the clinical situation, EGFRVIII-targeted CAR T cells may provide a highly specific, promising therapeutic candidate for patients with tumors in the CNS and are now in phase I clinical trials at the NCI Surgery Branch (NCT01454596, www.clinicaltrials.gov).

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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