

T Cells Redirected to EphA2 for the Immunotherapy of Glioblastoma

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Outcomes for patients with glioblastoma (GBM) remain poor despite aggressive multimodal therapy. Immunotherapy with genetically modified T cells expressing chimeric antigen receptors (CARs) targeting interleukin (IL)-13R α 2, epidermal growth factor receptor variant III (EGFRvIII), or human epidermal growth factor receptor 2 (HER2) has shown promise for the treatment of gliomas in preclinical models and in a clinical study (IL-13R α 2). However, targeting IL-13R α 2 and EGFRvIII is associated with the development of antigen loss variants, and there are safety concerns with targeting HER2. Erythropoietin-producing hepatocellular carcinoma A2 (EphA2) has emerged as an attractive target for the immunotherapy of GBM as it is overexpressed in glioma and promotes its malignant phenotype. To generate EphA2-specific T cells, we constructed an EphA2-specific CAR with a CD28- ζ endodomain. EphA2-specific T cells recognized EphA2-positive glioma cells as judged by interferon- γ (IFN- γ) and IL-2 production and tumor cell killing. In addition, EphA2-specific T cells had potent activity against human glioma-initiating cells preventing neurosphere formation and destroying intact neurospheres in coculture assays. Adoptive transfer of EphA2-specific T cells resulted in the regression of glioma xenografts in severe combined immunodeficiency (SCID) mice and a significant survival advantage in comparison to untreated mice and mice treated with nontransduced T cells. Thus, EphA2-specific T-cell immunotherapy may be a promising approach for the treatment of EphA2-positive GBM.

Received 22 November 2011; accepted 12 September 2012; advance online publication 16 October 2012. doi:10.1038/mt.2012.210

INTRODUCTION

Glioblastoma (GBM) is the most aggressive primary brain tumor in adults.¹ The current standard of care consists of surgical

resection, radiation, and chemotherapy with temozolomide but results in 5-year overall survival rates of <10%.^{2,3} Immunotherapy is an attractive strategy to improve outcomes for patients with GBM as it does not rely on the cytotoxic mechanisms employed by chemotherapy or radiation. Indeed, dendritic cell vaccines have shown encouraging results, producing clinical responses and increased progression-free survival in patients with recurrent and newly diagnosed GBM.⁴⁻⁶ Although these results await confirmation in randomized clinical trials, published studies have also shown that it is difficult to reliably induce GBM-specific T cells *in vivo*.

One strategy to overcome this limitation is to generate tumor-specific T cells *in vitro* by genetically modifying T cells to express chimeric antigen receptors (CARs), which consist of a single chain variable fragment, a transmembrane domain, and signaling domains derived from the T-cell receptor complex and costimulatory molecules.⁷ The clinical experience with CAR T cells in patients with GBM is limited, but given the recent encouraging clinical results using CAR T cells to treat GD2-positive neuroblastoma and CD19-positive leukemia, further exploration is warranted.^{8,9}

The success of CAR T-cell immunotherapies for GBM will require preventing immune escape by targeting antigens that are important for sustaining the malignant GBM phenotype. The erythropoietin-producing hepatocellular carcinoma A2 (EphA2) receptor, a member of the Eph family of receptor tyrosine kinases, has emerged as a target antigen as such. EphA2 is overexpressed in GBM^{10,11} and is associated with poor outcomes.^{12,13} EphA2 overexpression induces pro-oncogenic effects including enhanced tumorigenesis,¹⁴ tumor cell migration and invasion,¹⁵ angiogenesis, and metastasis.¹⁶⁻¹⁹

Here, we report the development of an EphA2-specific CAR to redirect T cells to EphA2-positive GBMs. We show that these T cells are able to recognize and kill EphA2-positive glioma cells and glioma-initiating cells *in vitro* and induce tumor regression in an orthotopic xenograft severe combined immunodeficiency (SCID) mouse model of GBM.

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RESULTS

EphA2 is expressed in glioma cell lines and primary GBM

We confirmed the expression of EphA2 in GBMs by western blot analysis. EphA2 was expressed in the glioma cell lines U87 and U373 but not in normal whole brain or frontal lobe tissue, T cells, or the leukemia cell line K562 (Figure 1a). To determine the expression of EphA2 in primary GBM, protein was extracted from cell lines established after short-term culture of five different GBM tumor biopsies.²⁰ EphA2 was detected in 5/5 primary GBM cell lines, although the level of expression varied between patients (Figure 1b). These results confirm that EphA2 is expressed in GBM in contrast to normal brain.

Generation of EphA2-specific CAR-modified T cells

To redirect T cells to the EphA2 receptor, a second-generation EphA2-specific CAR was designed based on the humanized EphA2 monoclonal antibody (MAb) 4H5.^{21,22} A codon-optimized synthetic gene encoding 4H5 in single chain variable fragment format was cloned into a SFG retroviral vector upstream of an IgG1-CH2CH3 domain, a CD28 transmembrane domain, and costimulatory domains derived from CD28 and CD3- ζ (Figure 2a). Gibbon ape leukemia virus-pseudotyped retroviral particles encoding the EphA2-specific CAR were used to transduce CD3/CD28-activated T cells from normal healthy donors. Following T-cell transduction, fluorescence activated cell sorting (FACS) analysis was used to determine the cell surface expression of the EphA2-specific CAR. The percentage of CAR-expressing T cells ranged from 49.9 to 95.0% with a median of 73.2% ($n = 7$; Figure 2b). The resultant T-cell lines consisted of a mixed population of CD4- and CD8-positive cells, with both subsets expressing EphA2-specific CARs (Figure 2b).

EphA2-specific T cells recognize and kill EphA2-positive gliomas

We used ELISAs and cytotoxicity assays to test the functionality of EphA2-specific T cells *in vitro*. EphA2-specific T cells were cocultured with EphA2-positive and negative targets, and after 24 hours, we determined the concentration of interferon- γ (IFN- γ) and interleukin (IL)-2 in the cell culture supernatants by ELISA. EphA2-specific T cells recognized EphA2-positive glioma cell lines U373 and U87 as evidenced by the production of significantly higher levels of IFN- γ and IL-2 in comparison to the EphA2-negative T cells and K562 (Figure 3a). Nontransduced-T cells produced little to no IFN- γ or IL-2 in response to all targets (Figure 3a). In standard 4-hour ⁵¹Cr release assays, EphA2-specific T cells had significant cytotoxic activity against U373 and U87 whereas nontransduced T cells did not ($P < 0.0004$ and $P < 0.002$; Figure 3b). Although low levels of killing of the EphA2-negative targets was observed in the presence of EphA2-specific T cells and nontransduced T cells, there was no difference between both effector T-cell populations ($P > 0.15$ for T cells and $P > 0.27$ for K562; Figure 3b). Recognition of EphA2-positive tumor cells by EphA2-specific T cells was confirmed for three other EphA2-positive tumor cell lines (Supplementary Figure S1). In addition, all five primary EphA2-positive GBM cell lines were killed in cytotoxicity assays

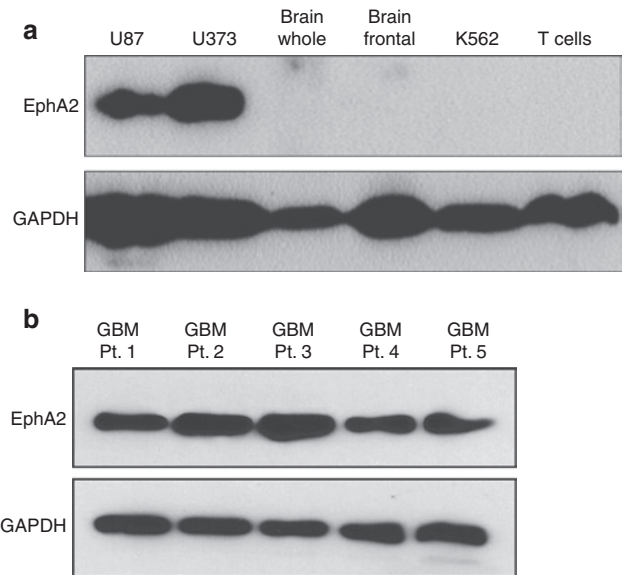


Figure 1 Erythropoietin-producing hepatocellular carcinoma A2 (EphA2) is expressed in glioma but not in normal brain. (a) Western blot showed high expression of EphA2 in the glioma cell lines U87 and U373. EphA2 was not detectable in normal brain tissue (whole brain or frontal lobe), K562, or normal T cells. (b) EphA2 was detected in 5/5 primary cell lines established from tumors of patients with GBM. GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GBM, glioblastoma.

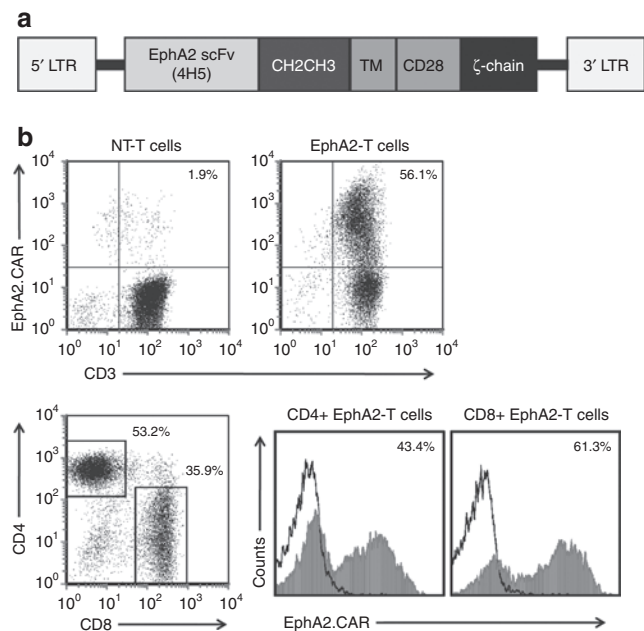


Figure 2 Generation of erythropoietin-producing hepatocellular carcinoma A2 (EphA2)-specific T cells. (a) The EphA2-specific chimeric antigen receptors (CAR) was generated by cloning a single chain variable fragment derived from the EphA2 monoclonal antibody 4H5 upstream of an IgG1-CH2CH3 domain, a CD28 TM domain, and costimulatory domains derived from CD28 and CD3- ζ into an SFG retroviral vector. (b) EphA2-CAR expression was detected by staining T cells with a CH2CH3 antibody. Fluorescence activated cell sorting analysis revealed expression of EphA2-specific CARs on the cell surface of transduced T cells as compared with controls (median = 73.2%, $n = 7$, range = 49.9–95.0%, representative plot shown). Transduced T cells consisted of CD4- and CD8-positive cells with both subsets expressing EphA2-specific CARs. LTR, long terminal repeats; NT, nontransduced; TM, transmembrane.

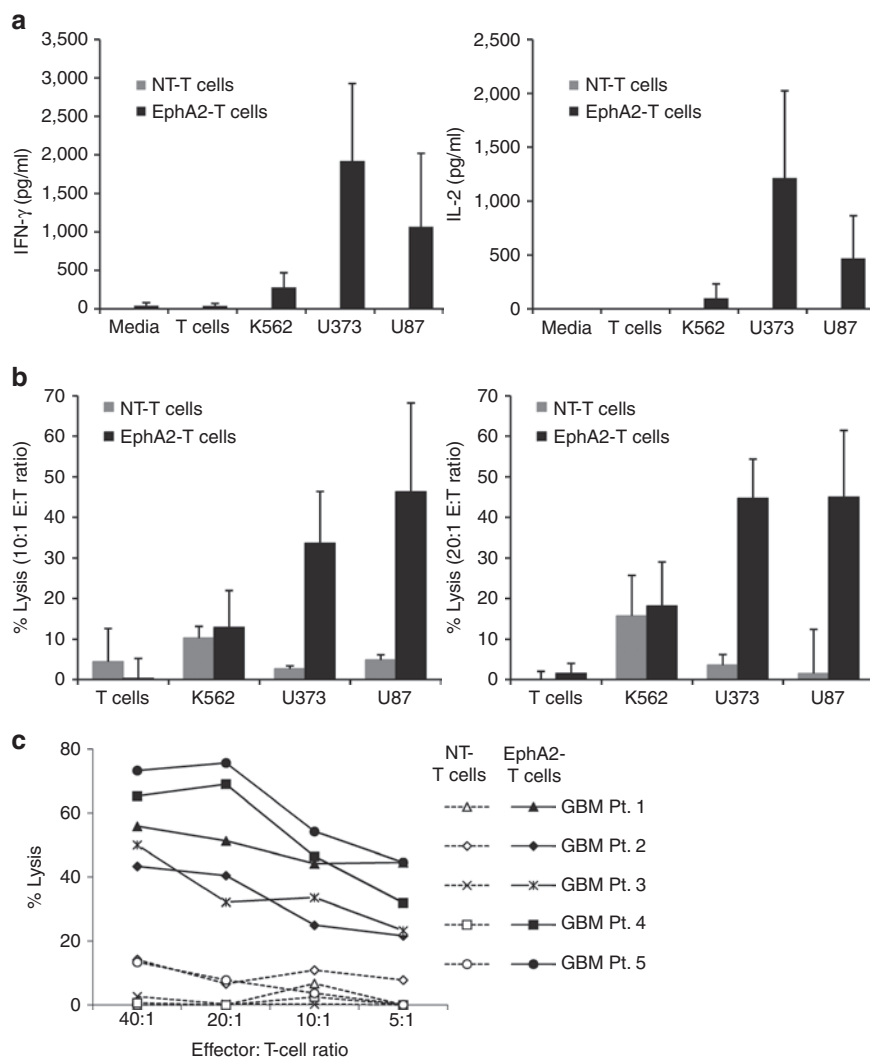


Figure 3 Erythropoietin-producing hepatocellular carcinoma A2 (EphA2)-specific T cells recognize and kill EphA2-positive gliomas. **(a)** Nontransduced (NT) or EphA2-specific T cells were cocultured with target cells at a 1:5 ratio, and after 24 hours, the production of interferon- γ (IFN- γ) and interleukin-2 (IL-2) by T cells was determined by ELISA. EphA2-specific T cells produced significantly higher levels of IFN- γ (U373 versus K562 $P = 0.006$; U373 versus T cells $P = 0.007$; U87 versus K562 $P = 0.049$; U87 versus T cells $P = 0.034$); and IL-2 (U373 versus K562 $P = 0.011$; U373 versus T cells $P = 0.014$; U87 versus K562 $P = 0.018$; U87 versus T cells $P = 0.029$) in response to EphA2-positive targets U373 and U87 than to EphA2-negative targets K562 and normal T cells (mean + SD; $n = 5$). **(b)** EphA2-specific T cells were tested in 4-hour chromium release assays. EphA2-specific T cells had significant cytotoxic activity against U373 and U87 whereas NT-T cells did not at effector to target (E:T) ratios of 10:1 (left panel) or 20:1 (right panel; U373: $P < 0.0004$; U87 $P < 0.002$ at both E:T ratios). There was no difference between both effector T-cell populations for EphA2-negative targets (T cells: $P > 0.15$; K562: $P > 0.27$ at both E:T ratios; mean + SD; $n = 5$). **(c)** Cytotoxicity assays with EphA2-specific T cells and NT-T cells as effectors and five primary EphA2-positive glioblastoma cell lines as targets.

whereas nontransduced T cells produced only background levels of killing (Figure 3c). To provide further evidence that the recognition of EphA2-specific T cells depends on the expression of EphA2, we genetically modified K562 cells to express EphA2 (K562-EphA2). EphA2-specific T cells killed K562-EphA2 as efficiently as U87 cells, but had no effect on parental K562 cells. By contrast, nontransduced T cells had no cytotoxic activity on any of these target cells. (Supplementary Figure S2a–c). In addition, U87 cells, genetically modified to express an EphA2-specific shRNA, expressed lower levels of EphA2 and were less sensitive to EphA2-specific T-cell killing (Supplementary Figure S2d,e). These results indicate that T-cell activation and

killing depend on the expression of EphA2-specific CARs and the presence of EphA2 on target cells.

Neurospheres express EphA2 and are killed by EphA2-specific T cells

Glioma-initiating cells are resistant to traditional chemotherapy and radiation therapy.^{23,24} To determine whether EphA2-specific T cells have activity against this cell population, we generated neurospheres from a U87 cell line that was genetically modified with a retrovirus encoding an eGFP.FFLuc fusion gene. Secondary neurospheres expressed EphA2 at similar levels to U87 cells cultured in monolayer (Figure 4a). EphA2-specific T cells killed U87 cells

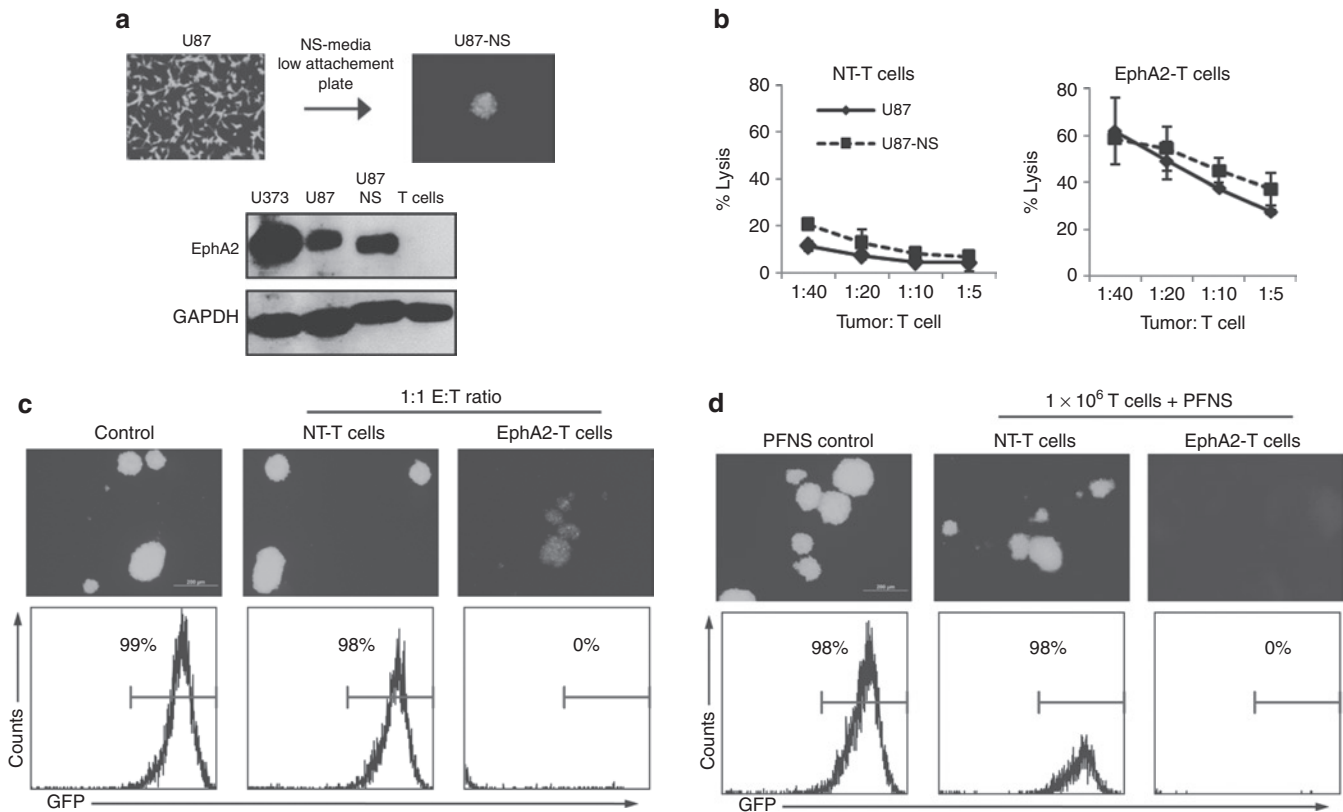


Figure 4 Erythropoietin-producing hepatocellular carcinoma A2 (EphA2)-specific T cells inhibit neurosphere formation and destroy established neurospheres. **(a)** Neurospheres were formed by culturing U87.eGFP.FFLuc in neurosphere (NS) media on low-attachment plates. Western blot showed that these U87-neurospheres expressed an equivalent amount of EphA2 as compared with U87 cells cultured in monolayer. **(b)** EphA2-specific T cells killed U87-NS in a 4-hour chromium release assay (mean + SD; $n = 2$). **(c)** Nontransduced (NT) or EphA2-specific T cells were plated with U87-NS cells at a 1:1 ratio in neurosphere media on low-attachment plates. Although NT-T cells had no effect on the ability of U87-NS cells to form neurospheres, EphA2-specific T cells prevented neurosphere formation as shown by fluorescence microscopy and fluorescence activated cell sorting (FACS) analysis for GFP-positive U87-NS. **(d)** 1×10^6 NT or EphA2-specific T cells were plated with PFNS. Only EphA2-specific T cells were able to destroy preformed neurospheres as judged by fluorescence microscopy and FACS analysis for GFP-positive U87-NS (data shown are representative of two independent experiments). GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GFP, green fluorescent protein; PFNS, preformed U87-NS.

isolated from secondary neurospheres as equally well as U87 cells cultured in monolayer in standard cytotoxicity assays (**Figure 4b**).

To provide further evidence that EphA2-specific T cells have activity against glioma-initiating cells, we determined if EphA2-specific T cells prevent the formation of neurospheres. U87 cells derived from neurospheres were plated at a ratio of 1:1 with nontransduced or EphA2-specific T cells under neurosphere culture conditions. EphA2-specific T cells inhibited the formation of neurospheres as shown by fluorescence microscopy and FACS analysis whereas nontransduced T cells did not (**Figure 4c**). In addition, EphA2-specific T cells, but not nontransduced T cells, were able to destroy intact neurospheres (**Figure 4d**). These results indicate that EphA2-specific T cells have potent antineurosphere activity.

EphA2-specific T cells induce GBM regression *in vivo*

To evaluate the anti-GBM activity of EphA2-specific T cells *in vivo*, we used an orthotopic xenograft mouse model described earlier.²⁰ U373 glioma cells were modified to express an eGFP.FFLuc fusion protein (U373.eGFP.FFLuc) allowing us to track tumor growth using serial noninvasive *in vivo* bioluminescence imaging. 1×10^5 U373.

eGFP.FFLuc cells were injected into the brains of SCID mice on day 0. Tumors engrafted and grew exponentially in the week post-tumor cell injection. On day 7, the mice were injected intracranially with 2×10^6 EphA2-specific T cells or nontransduced T cells into the previous stereotactic tumor coordinates. Untreated mice served as controls. Although control mice and mice treated with nontransduced T cells showed continuous tumor growth, mice treated with EphA2-specific T cells did not (**Figure 5a,b**). Comparison of bioluminescence imaging results revealed no significant difference between all three groups on the day of T-cell injection; however, mice treated with EphA2-specific T cells had significantly lower tumor signals as early as one day post-treatment in comparison to untreated mice or mice treated with nontransduced T cells ($P = 0.002$ and $P = 0.007$; **Table 1**). Treatment with EphA2-specific T cells significantly prolonged survival of mice as compared with no treatment ($P = 0.009$) or treatment with nontransduced T cells ($P = 0.010$). By contrast, nontransduced T cells did not prolong survival in the untreated mice ($P = 0.852$; **Figure 5c**).

To evaluate the potency of EphA2-specific T cells, we modified the studies and injected fourfold fewer T cells into day 7 tumors or the same number of T cells into day 14 tumors (**Figure 6a,b**).

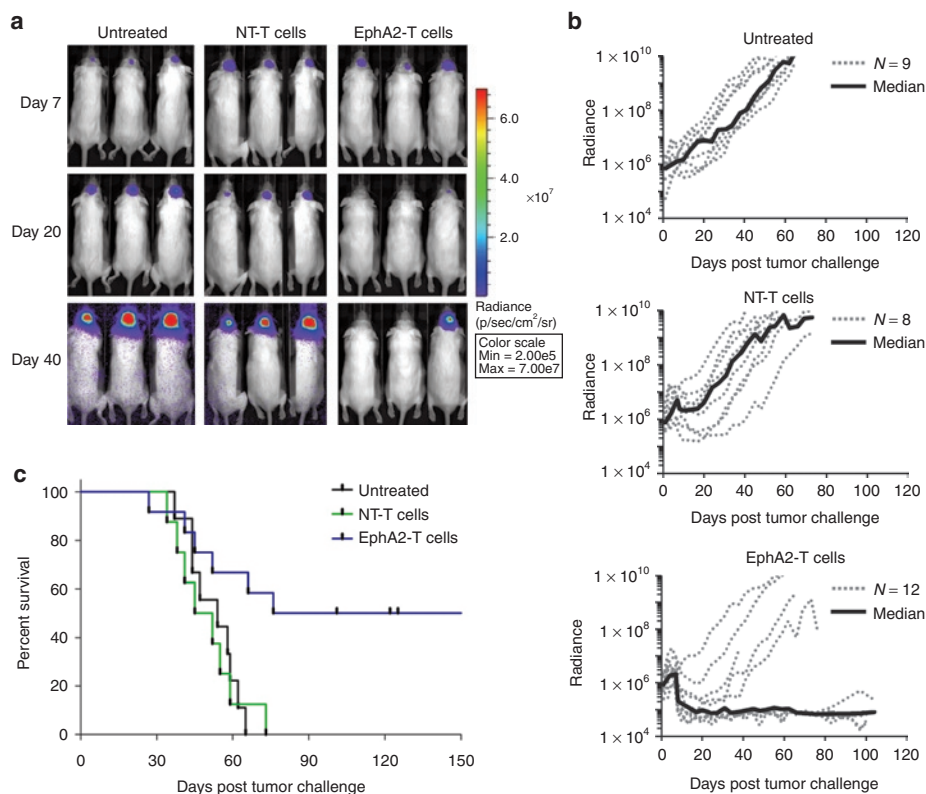


Figure 5 Erythropoietin-producing hepatocellular carcinoma A2 (EphA2)-specific T cells induce regression of established glioblastoma *in vivo*. 1×10^5 U373.eGFP.FLuc were injected intracranially into severe combined immunodeficiency mice on day 0 and treated with a single injection of 2×10^6 EphA2-specific T cells into the same tumor coordinates on day 7. Untreated mice and mice treated with nontransduced (NT) T cells served as controls. **(a)** Bioluminescence imaging was used to follow tumor progression. All mice had detectable tumors just prior to treatment (day 7). By day 40, all mice in the control groups had progressive tumors (9/9 for untreated, 8/8 for NT-T cells). The tumor signal decreased in all mice treated with EphA2-specific T cells, and 50% (6/12) were long-term survivors. **(b)** The bioluminescence signal from the tumors (radiance = photons/sec/cm²/sr) over time. Dotted lines represent each individual mouse while the solid black line represents the median radiance for the group at the given time. **(c)** Mice treated with EphA2-specific T cells had a significantly prolonged survival compared with untreated ($P = 0.009$) and NT-T cell ($P = 0.010$) treated mice. There was no significant difference between the survival of untreated versus NT-T cell treated mice ($P = 0.852$).

Table 1 Tumor signal comparison

Tumor signal comparison	P ^a
Day 7	
EphA2-T cells ($n = 12$) versus NT-T cells ($n = 8$)	0.622
EphA2-T cells ($n = 12$) versus untreated ($n = 9$)	0.916
NT-T cells ($n = 8$) versus untreated ($n = 9$)	0.531
Day 8	
EphA2-T cells ($n = 12$) versus NT-T cells ($n = 8$)	0.007
EphA2-T cells ($n = 12$) versus untreated ($n = 9$)	0.002
NT-T cells ($n = 8$) versus untreated ($n = 9$)	0.744
Day 20	
EphA2-T cells ($n = 12$) versus NT-T cells ($n = 8$)	0.007
EphA2-T cells ($n = 12$) versus untreated ($n = 9$)	<0.0001
NT-T cells ($n = 8$) versus untreated ($n = 9$)	0.130

EphA2, erythropoietin-producing hepatocellular carcinoma A2; NT, nontransduced.

^at-test.

All mice ($n = 4$) injected on day 7 with a lower number of T cells had regression of their tumors with one mouse having a complete response (CR), and two of four mice with day 14 tumors had a response, including one CR (Figure 6d). To discover if the systemic administration of human EphA2-specific T cells have antitumor

activity in our xenograft model, mice with day 7 tumors received 1×10^7 EphA2-specific T cells by tail vein injection (Figure 6c,d). No antitumor effects were observed.

DISCUSSION

In this study, we describe the development and characterization of a novel CAR specific for the EphA2 receptor and show that T cells expressing this CAR can effectively target and kill EphA2-positive GBM. We show that EphA2-specific T cells produce the immunostimulatory cytokines IFN- γ and IL-2 when cocultured with EphA2-positive targets and cause tumor cell lysis in cytotoxicity assays *in vitro*. EphA2-specific T cells were also able to target glioma-initiating cells as shown in neurosphere inhibition and killing assays. Finally, we demonstrated that EphA2-specific T cells have potent antitumor activity *in vivo*.

Over the last decade, several tumor-associated antigens have been identified in GBM, including the membrane-bound tumor antigens, epidermal growth factor receptor variant III (EGFRvIII), IL-13R α 2, human epidermal growth factor receptor 2 (HER2), transmembrane glycoprotein NMB, and EphA2.^{10,11,25-29} T cells expressing EGFRvIII-, IL-13R α 2-, and HER2-specific CARs have been evaluated in preclinical animal models with encouraging results,^{20,30,31} the clinical experience with these CAR T cells,

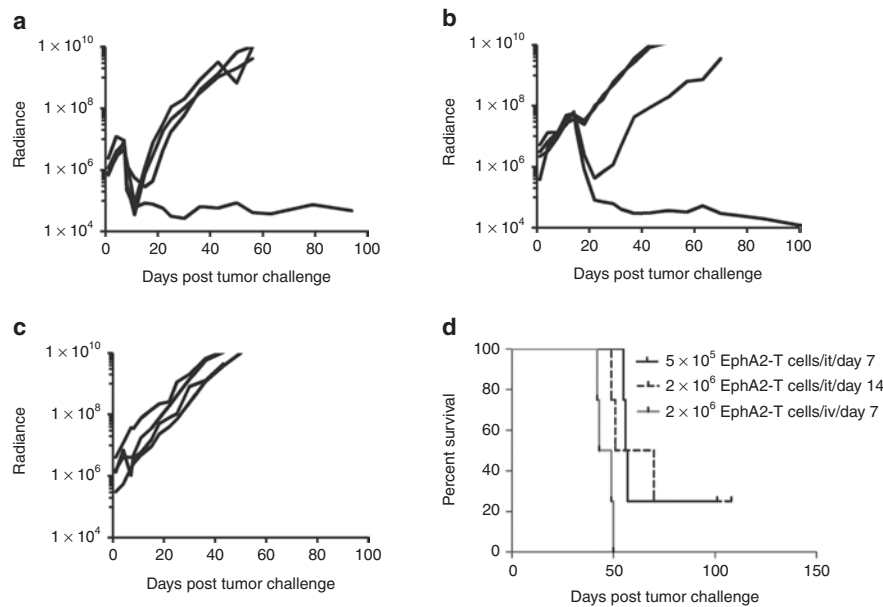


Figure 6 Antitumor activity of erythropoietin-producing hepatocellular carcinoma A2 (EphA2)-specific T cells *in vivo* depends on T-cell number and tumor size. **(a)** 5×10^5 EphA2-specific T cells were injected intratumorally on day 7 or **(b)** 2×10^6 EphA2-specific T cells on day 14. All mice ($n = 4$) injected on day 7 had a regression of their tumors with one mouse having a complete response (CR). Two of four mice treated on day 14 had a response, including one CR. **(c)** Mice with day 7 tumors received 1×10^7 EphA2-specific T cells intravenously through their tail vein ($n = 4$). No antitumor effects were observed. **(d)** Kaplan–Meier survival curve of all treated groups of mice.

however, is limited. Nevertheless, limitations of individual CARs are starting to emerge. Three patients with GBM have received intratumoral injection of IL-13R α 2-specific CAR T cells. T-cell administration was safe, and tumor responses were observed; however, tumors recurred, and these malignant cells were negative for IL-13R α 2.³² The risk of immune escape is also high for EGFRvIII targeted T-cell therapy given the emergence of GBM antigen loss variants in patients who received EGFRvIII peptide vaccines.⁵ In addition, there are safety concerns with regards to HER2-targeted T-cell therapies due to the death of a patient who received a high dose of HER2-CAR T cells.³³

Given these limitations of currently available CARs for GBM, new targets need to be explored. Moreover, a panel of GBM-specific CARs may facilitate clinical protocols in which patients are infused with T-cell products that target multiple GBM antigens. EphA2 has emerged as an attractive target for GBM immunotherapy owing to recent findings that EphA2 signaling is involved in glioma cell proliferation, migration, and invasion.^{12,15}

We detected the expression of EphA2 by western blot analysis in all GBM samples tested. Western blot was used, as commercially available EphA2 antibodies failed to reliably detect EphA2 by FACS analysis. Coculture of EphA2-specific T cells with EphA2-positive target cells resulted in secretion of IFN- γ and IL-2 in an antigen-dependent manner. EphA2-specific T cells also prevented neurosphere formation and destroyed intact neurospheres. Although CAR T cells as well as conventional antigen-specific T cells are able to kill CD133-positive glioma-initiating cells,^{20,34,35} recent studies showed that a subset of CD133-negative glioma cells also have glioma-initiating cell characteristics.³⁶ Thus, our finding that EphA2-specific T cells destroy neurospheres and prevent their formation provides further evidence that glioma-initiating cells are sensitive to immune-mediated killing.

In vivo, EphA2-specific T cells induced the regression of GBMs grown in the brains of SCID mice. Mice were injected with 1×10^5 tumor cells and at the time of T-cell injection (day 7) the tumor signal had increased 4.8-fold (median). All mice had a decrease in their tumor signal, and 6 of 12 mice treated with 2×10^6 and one of four mice treated with 5×10^5 EphA2-specific T cells had a CR. There was no significant difference in tumor size at the time of EphA2-specific T-cell injection between long-term survivors and mice which died. These data imply that this model requires the tumor cell:T-cell ratio to be approximately equivalent to induce complete tumor regression. In support of this approximate assessment is our observation that of four mice treated on day 14, when tumor had a 16.8-fold (median) increase in signal over baseline, 2×10^6 EphA2-specific T cells induced a response in two animals in one of which there was a CR. Hence, we suggest that the overall CR rate of 40% is likely a reflection of the limited persistence and expansion of human T cells in SCID mice *in vivo*. Although this limitation may be overcome by modifying the tumor cells to secrete T-cell growth factors such as IL-2,³¹ such manipulations remain highly artificial and may not provide any better prediction of future clinical potency.

Is targeting EphA2 with EphA2-specific T cells safe? The EphA2 epitope recognized by the CAR is preferentially accessible in dividing malignant cells and not in quiescent normal epithelial cells, though these may also express EphA2 at low levels.^{22,37} As shown in **Figure 1a** and as reported by others, normal adult brain does not express EphA2.¹⁰ In addition, we observed no acute toxicities following local and systemic injection of EphA2-specific T cells. Histological examination of the brains of two tumor-free mice sacrificed at 118 days post T-cell injection showed normal brain morphology (**Supplementary Figure S3**). These results are particularly reassuring since our EphA2-specific CAR recognizes

both human and murine EphA2 as judged by the ability of EphA2-specific T cells to secrete IFN- γ in response to recombinant murine EphA2 protein, and to kill murine target cells in an EphA2-specific manner (**Supplementary Figure S4**). This favorable safety profile is supported by findings of other investigators who found that intravenous administration of a bispecific EphA2-specific antibody targeting the same EphA2 epitope and human CD3 (bscEphA2xCD3) in combination with human CD3-positive T cells resulted in no systemic side effects.³⁷ In addition, patients with GBM receiving a dendritic cell vaccine loaded with a cocktail of HLA-A2 restricted peptides derived from IL-13R α 2, EphA2, YKL-40, and gp100 had no adverse immune events.⁴

In conclusion, T cells redirected to EphA2 by an EphA2-specific CAR have potent antitumor activity against glioma and glioma-initiating cells *in vitro*, and induce the regression of established GBM xenografts *in vivo*, indicating that they may be of value in the treatment of EphA2-positive GBM.

MATERIALS AND METHODS

Blood donors, primary tumor cells, and cell lines. Blood samples from healthy donors and primary tumor cells from patients with GBM were obtained in accordance to protocols approved by the Institutional Review Board of Baylor College of Medicine. The GBM cell lines U373 and U87 and the leukemia cell line K562 were purchased from the American Type Culture Collection (ATCC; Manassas, VA). U373 and U87 cells expressing enhanced green fluorescent protein and firefly luciferase (U373.eGFP.FFLuc and U87.eGFP.FFLuc) were generated by retroviral transduction. T cells and cell lines were grown in Roswell Park Memorial Institute or Dulbecco's Modified Eagle's Medium (Thermo Scientific HyClone, Waltham, MA; Lonza, Basel, Switzerland) with 10% fetal calf serum (HyClone, Logan, UT) and 2 mmol/l GlutaMAX-I (Invitrogen, Carlsbad, CA). The "Characterized Cell Line Core Facility" at MD Anderson Cancer Center, Houston, TX, performed cell line validation. Primary GBM cell lines were established as previously described²⁰ and were grown in Dulbecco's Modified Eagle's Medium with 10% fetal calf serum, 2 mmol/l GlutaMAX-I, 1.5 g/l sodium bicarbonate, 0.1 mmol/l nonessential amino acids, and 1.0 mmol/l sodium pyruvate (all media supplements from Invitrogen).

Western blot. Cells were dissociated with phosphate buffered saline + 3 mmol/l EDTA and lysed in a buffer containing 50 mmol/l Tris, 150 mmol/l NaCl, 5 mmol/l EDTA, 1% Triton X-100 (all from Sigma, St. Louis, MO), and protease inhibitors (Thermo Scientific, Waltham, MA). Protein concentrations were determined using a Bio-Rad protein assay (Bio-Rad, Hercules, CA) with bovine serum albumin as the standard. Samples were denatured in Laemmli buffer (Bio-Rad) at 95°C for 5 minutes. Electrophoresis-ready protein lysates from normal human brain tissue (whole brain and frontal lobes) were obtained from Clontech (Mountain View, CA). Protein of 10 μ g were loaded per well and run on an 8% polyacrylamide gel. Proteins were transferred to Immobilon-P polyvinylidene difluoride membranes (Millipore, Billerica, MA). Using the SNAP i.d. protein detection system (Millipore), membranes were blocked with 0.25% milk powder in tris-buffered saline + 0.1% Tween-20 (Sigma) and then probed with EphA2 (clone D7; Sigma) or glyceraldehyde 3-phosphate dehydrogenase (sc-47724; Santa Cruz Biotechnology, Santa Cruz, CA) mouse monoclonal antibodies followed by a horseradish peroxidase conjugated goat mouse IgG antibody (sc-2005; Santa Cruz Biotechnology). Blots were developed using SuperSignal West Dura Extended Duration Substrate (Thermo Scientific) and exposed to GeneMate Blue Basic Autoradiography Film (BioExpress, Kaysville, UT).

Generation of an EphA2-specific CAR. The EphA2-specific single chain variable fragment was derived from the EphA2 MAb 4H5, a humanized

version of the EphA2 MAb EA2.^{21,22} A codon-optimized gene was synthesized by GeneArt (Invitrogen) containing the immunoglobulin heavy-chain leader peptide,³⁸ the 4H5 heavy-chain, a glycine (G) serine (S) linker [(G₃S)₃], and the 4H5 light chain flanked by 5' NcoI and 3' BamHI sites. This mini gene was subcloned into an SFG retroviral vector containing the human IgG1-CH2CH3 domain, a CD28 transmembrane domain, and costimulatory domains derived from CD28 and the CD3 ζ -chain.^{39,40} The cloning of the EphA2-specific CAR was verified by sequencing (Seqwright, Houston, TX).

Retrovirus production and transduction of T cells. Retroviral particles were generated by transient transfection of 293T cells with the EphA2-specific CAR encoding SFG retroviral vector, Peg-Pam-e plasmid containing the sequence for MoMLV gag-pol, and pMEVSVg plasmid containing the sequence for VSV-G, using GeneJuice transfection reagent (EMD Biosciences, San Diego, CA). Supernatants containing the retrovirus were collected after 48 and 72 hours. The VSV-G pseudotyped viral particles were used to transduce the PG-13 producer cell line for the production of retroviral particles for T-cell transduction. To generate EphA2-specific CAR T cells, peripheral blood mononuclear cells were isolated by Lymphoprep (Greiner Bio-One, Monroe, NC) gradient centrifugation and then stimulated on nontissue culture treated 24-well plates, which were precoated with OKT3 (Ortho Biotech, Bridgewater, NJ) and CD28 (Becton Dickinson, Mountain View, CA) antibodies. Recombinant human interleukin-2 (IL-2; Proleukin; Chiron, Emeryville, CA) of 100 U/ml was added to cultures on day 2. On day 3, OKT3/CD28-stimulated T cells (2.5×10^5 cells/well) were transduced on RetroNectin (Clontech, Mountainview, CA) coated plates in the presence of IL-2. On day 5 or 6, T cells were transferred into new wells and subsequently expanded with 50–100 U/ml IL-2. Nontransduced T cells, used as controls, were activated with OKT3/CD28 and expanded in parallel with 50–100 U/ml IL-2. EphA2-specific CAR expression was determined 3 to 4 days post-transduction. In all experiments, the functionality of matched (from the same donor) transduced and nontransduced T cells was compared.

Flow cytometry. A FACSCalibur instrument (BD, Becton Dickinson, Mountain View, CA) was used to acquire immunofluorescence data which were analyzed with CellQuest (BD) or FCS Express software (De Novo Software, Los Angeles, CA). Isotype controls were immunoglobulin G1-fluorescein isothiocyanate (IgG1-FITC; BD), IgG1-phycoerythrin (IgG1-PE; BD), IgG1-peridinin chlorophyll protein (IgG1-PerCP; BD), and isotype Cy5 (Jackson ImmunoResearch Laboratories, West Grove, PA). T cells were analyzed for EphA2-CAR expression using a CH2CH3 Cy5 antibody (Jackson ImmunoResearch Laboratories) along with anti-CD8 FITC, -CD4 PE, and -CD3 PerCP. U87 cells were analyzed for CD133 expression using a CD133 PE antibody (Miltenyi Biotec, Bergisch Gladbach, Germany). Forward and side scatter gating were used to discriminate live cells from dead cells. Cells were collected and washed once with phosphate buffered saline (Sigma) containing 1% fetal bovine serum (Hyclone; FACS buffer) prior to the addition of antibodies. Cell were incubated for 30 minutes on ice in the dark, washed once, and fixed in 0.5% paraformaldehyde/FACS buffer prior to analysis.

Analysis of cytokine production. EphA2-specific or nontransduced T cells from healthy donors were cocultured with normal T cells, K562, U373, or U87 cells at a 1:5 effector to target (E:T) ratio in a 48-well plate. After 24 hours of incubation, culture supernatants were harvested, and the presence of IFN- γ and IL-2 was determined by ELISA as per the manufacturer's instructions (R&D Systems, Minneapolis, MN).

Cytotoxicity assay. Standard chromium (⁵¹Cr) release assays were performed as previously described.⁴¹ Briefly, 1×10^6 target cells were labeled with 0.1 mCi (3.7 MBq) ⁵¹Cr and mixed with decreasing numbers of effector cells to give effector to target ratios of 40:1, 20:1, 10:1, and 5:1. Target cells incubated in complete medium alone or in 1% Triton X-100 were used to determine spontaneous and maximum ⁵¹Cr release, respectively.

After 4 hours, supernatants were collected and radioactivity was measured in a gamma counter (Cobra Quantum; PerkinElmer; Wellesley, MA). The mean percentage of specific lysis of triplicate wells was calculated according to the following formula: $[\text{test release} - \text{spontaneous release}] / [\text{maximal release} - \text{spontaneous release}] \times 100$.

Neurosphere assay. Primary U87-neurospheres were formed by seeding 1×10^5 U87 or U87.eGFP.FFLuc glioma cells in serum-free neurosphere media (1:1 Dulbecco's Modified Eagle's Medium:F-12 supplemented with 2mmol/l GlutaMAX-I, 1x B27, 50ng/ml epidermal growth factor, and 20ng/ml fibroblast growth factor) on ultra-low attachment 6-well plates (Corning, Lowell, MA). These primary neurospheres were then dissociated with trypsin and reseeded to form secondary neurospheres. To determine if EphA2-specific T cells could inhibit neurosphere formation, secondary neurospheres were dissociated and cocultured with T cells in a 1:1 ratio in neurosphere media on ultra-low attachment 24-well plates (Corning). To determine if the T cells could destroy established neurospheres, secondary spheres were collected without dissociating and cocultured with 1×10^6 T cells. Cultures were imaged by fluorescent microscopy on day 4, and analyzed by FACS analysis on day 6.

Orthotopic xenograft SCID mouse model. All animal experiments followed a protocol approved by the Baylor College of Medicine Institutional Animal Care and Use Committee. Experiments were performed as described previously²⁰ with a few modifications. ICR-SCID mice were purchased from Taconic (IcrTac:ICR-Prkdc^{scid}; Fox Chase C.B-17 SCID ICR; Taconic, Hudson, NY). Male 8- to 12-week-old mice were anesthetized with rapid sequence inhalation isoflurane (Abbot Laboratories, England, UK) followed by an intraperitoneal injection of 225–240mg/kg Avertin solution and then maintained on isoflurane by inhalation throughout the procedure. The head was shaved and the mice were immobilized in a CunninghamMouse/Neonatal Rat Adaptor (Stoelting, Wood Dale, IL) stereotaxic apparatus fitted into an E15600 Lab Standard Stereotaxic Instrument (Stoelting), then scrubbed with 1% povidone-iodine. A 10 mm skin incision was made along the midline. The tip of a 30G ½ inch needle mounted on a Hamilton syringe (Hamilton, Reno, NV) served as the reference point. A 1 mm burr-hole was drilled into the skull 1 mm anterior and 2 mm to the right of the bregma. Firefly-luciferase expressing U373 cells (U373.eGFP.FFLuc; 1×10^5 in 2.0 µl) were injected 3 mm deep to the bregma, corresponding to the center of the right caudate nucleus over 5 minutes. The needle was left in place for 3 minutes, to avoid tumor cell extrusion, and then withdrawn over 5 minutes. Seven days after tumor cell injection, animals were treated with 2×10^6 nontransduced or EphA2-specific T cells from the same donor in 2 µl to the same tumor coordinates. The incision was closed with 2–3 interrupted 7.0 Ethilon sutures (Ethicon, Somerville, NJ). A subcutaneous injection of 0.03–0.1 mg/kg buprenorphine (Buprenex RBH, Hull, England) was given for pain control.

Bioluminescence imaging. Isoflurane anesthetized animals were imaged using the IVIS system (IVIS, Xenogen, Alameda, CA) 10–15 minutes after 150 mg/kg D-luciferin (Xenogen) was injected per mouse intraperitoneally. The photons emitted from the luciferase-expressing tumor cells were quantified using Living Image software (Caliper Life Sciences, Hopkinton, MA). A pseudo-color image representing light intensity (blue least intense and red most intense) was generated and superimposed over the grayscale reference image. Animals were imaged every other day for one week after injections, then twice weekly thereafter. Mice were euthanized when the tumor radiance was $>1 \times 10^9$ on two occasions or when they met euthanasia criteria (neurological deficits, weight loss, signs of distress) in accordance with the Center for Comparative Medicine at Baylor College of Medicine.

Statistical analysis. For the mouse experiments, tumor radiance data were log-transformed and summarized using mean \pm SD at baseline and multiple subsequent time points for each group of mice. Changes in tumor radiance from baseline at each time point were calculated and compared between

groups using t-test. Survival determined from the time of tumor cell injection was analyzed by the Kaplan–Meier method and by the log-rank test.

SUPPLEMENTARY MATERIAL

Figure S1. EphA2-specific T cells recognize EphA2-positive lung cancer cell lines.

Figure S2. EphA2-specific target cell killing depends on the presence of EphA2.

Figure S3. Normal brain morphology in mice treated with EphA2-specific CAR T cells.

Figure S4. EphA2-specific T cells recognize murine EphA2.

ACKNOWLEDGMENTS

We thank Cliona M. Rooney and Malcolm K. Brenner (Center for Cell and Gene Therapy) for helpful discussion and advice. The authors declare no conflict of interest. K.K.H.C. is a Melnick scholar and was supported by NIH grants 5T32HL092332 and 5T32GM007330, S.N. by CPRIT grant RP101499, S.G. by the Clayton Foundation for Research, the James McDonald Foundation and CPRIT grant RP110553, and S.G. and N.A. by the Dana Foundation. The Characterized Cell Line Core Facility at MD Anderson Cancer Center is funded by NCI no. CA16672.

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