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Transgenic Expression of IL15 Improves Antiglioma Activity of IL13R α 2-CAR T Cells but Results in Antigen Loss Variants

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Abstract

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G. Dotti reports receiving other commercial research support from Bluebird Bio and is a consultant/advisory board member for Cell Medica. I.V. Balyasnikova has ownership interest in a submitted patent application. S. Gottschalk has ownership interest in a patent and is a consultant/advisory board member for Merrimack. No potential conflicts of interest were disclosed by the other authors.

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Glioblastoma (GBM) is the most aggressive primary brain tumor in adults and is virtually incurable with conventional therapies. Immunotherapy with T cells expressing GBM-specific chimeric antigen receptors (CAR) is an attractive approach to improve outcomes. Although CAR T cells targeting GBM antigens, such as IL13 receptor subunit $\alpha 2$ (IL13R $\alpha 2$), HER2, and EGFR variant III (EGFRvIII), have had antitumor activity in preclinical models, early-phase clinical testing has demonstrated limited antiglioma activity. Transgenic expression of IL15 is an appealing strategy to enhance CAR T-cell effector function. We tested this approach in our IL13R $\alpha 2$ -positive glioma model in which limited IL13R $\alpha 2$ -CAR T-cell persistence results in recurrence of antigen-positive gliomas. T cells were genetically modified with retroviral vectors encoding IL13R $\alpha 2$ -CARs or IL15 (IL13R $\alpha 2$ -CAR.IL15 T cells). IL13R $\alpha 2$ -CAR.IL15 T cells recognized glioma cells in an antigen-dependent fashion, had greater proliferative capacity, and produced more cytokines after repeated stimulations in comparison with IL13R $\alpha 2$ -CAR T cells. No autonomous IL13R $\alpha 2$ -CAR. IL15 T-cell proliferation was observed; however, IL15 expression increased IL13R $\alpha 2$ -CAR T-cell viability in the absence of exogenous cytokines or antigen. *In vivo*, IL13R $\alpha 2$ -CAR.IL15 T cells persisted longer and had greater antiglioma activity than IL13R $\alpha 2$ -CAR T cells, resulting in a survival advantage. Gliomas recurring after 40 days after T-cell injection had downregulated IL13R $\alpha 2$ expression, indicating that antigen loss variants occur in the setting of improved T-cell persistence. Thus, CAR T cells for GBM should not only be genetically modified to improve their proliferation and persistence, but also to target multiple antigens.

Introduction

Glioblastoma (GBM) represents the most common and aggressive primary brain tumor in humans (1). Despite a multimodal therapeutic approach including surgery, chemotherapy, and radiation, outcomes for pediatric and adult GBM patients remain poor (2–4). Novel therapeutics could improve outcomes. Immunotherapy promises to meet this need as it does not rely on the cytotoxic pathways employed by the aforementioned standard therapies.

T cells, genetically engineered to be GBM specific, can be a foundation for adoptive immunotherapy of GBMs (5, 6). Several groups, including ours, have developed GBM-specific chimeric antigen receptors (CAR) targeting tumor-associated antigens (TAA), including IL13R $\alpha 2$, HER2, EphA2, and EGFRvIII (7–11). On the basis of preclinical studies that demonstrated GBM activity of CAR T cells in xenograft models, phase I testing of IL13R $\alpha 2$ -, HER2-, and EGFRvIII-specific CAR T cells in humans is currently in progress (12–15). These clinical studies demonstrated safety, but the anti-GBM activity of CAR T cells was limited (12–15). CAR T-cell therapy studies showed similar results for solid tumors (16, 17). In contrast, CD19-specific CAR T cells showed potent antitumor activity for B-cell-derived hematologic malignancies (18–20).

Lack of efficacy of CAR T cells for brain and solid tumors is likely multifactorial, including limited proliferation and expansion of T cells at tumor sites (21, 22). Genetic modification strategies being pursued to overcome this obstacle include expression of cytokines (e.g., IL12, IL15), or manipulation of cytokine receptors (e.g., TGF β , IL4, or PD-1) to block

inhibitory signals in the tumor microenvironment or convert inhibitory into stimulatory signals (23–30).

The effectiveness of secondary genetic modifications to enhance the effector function of CAR T cells for GBM has not been explored. T cells expressing an IL13R α 2-CAR with a CD28. ζ domain (IL13R α 2-CAR T cells) have potent antitumor activity in a glioma xenograft model (8). However, antigen-positive tumors in mice treated with IL13R α 2-CAR T cells recurred due to limited T-cell persistence (8). Here, we demonstrate that transgenic expression of IL15 enhances the effector function and antiglioma activity of IL13R α 2-CAR T cells *in vitro* and *in vivo*. Gliomas in mice treated with IL13R α 2-CAR.IL15 T cells still recurred albeit at later time points in comparison with mice treated with IL13R α 2-CAR T cells. Mechanistic studies revealed that “late recurring tumors” had downregulated the expression of IL13R α 2, highlighting the need not only to optimize T-cell persistence and expansion, but also to target multiple TAAs expressed in GBM.

Materials and Methods

Cell lines

U373 (GBM), 293T (human embryonic kidney), and Raji (Burkitt lymphoma) cell lines were purchased from the ATCC). GBM6 is a primary adult GBM cell line (kindly provided by Dr. C. David James, Northwestern University, Chicago, IL; ref. 31). The generation of U373 cells expressing enhanced GFP and firefly luciferase (U373.eGFP.ffLuc), 293T cells expressing GFP (293T. GFP) or IL13R α 2 and GFP (293T.IL13R α 2.GFP) was reported previously (11). Cell lines were grown in RPMI or DMEM (GE Healthcare Life Sciences HyClone Laboratories) with 10% FBS (GE Healthcare Life Sciences HyClone) and 2 mmol/L GlutaMAX-I (Invitrogen). Cell lines were purchased between 2008 and 2011. The “Characterized Cell Line Core Facility” at MD Anderson Cancer Center (Houston, TX) performed cell line validation (2011). Once thawed, cell lines were kept in culture for a maximum of 3 months before new reference vials were thawed. All cell lines were tested on a regular basis for mycoplasma and were negative.

Generation of retroviral vectors encoding IL13R α 2-specific CAR and IL15

The generation of the IL13R α 2-specific CAR with a short spacer region, a CD28 transmembrane domain, and a CD28. ζ endodomain was described previously (8). The IL15-encoding retroviral vector was generated by replacing the CD34 gene in pSFG.iC9-2A-CD34-2A-IL15 (28) with a cytoplasmic domain-truncated nerve growth factor receptor gene (NGFR; pSFG.iC9-2A-NGFR-2A-IL15). RD114-pseudotyped retroviral particles were generated by transient transfection of 293T cells as described previously (11) using GeneJuice transfection reagent (EMD Biosciences). Super-natants containing retroviral particles were collected 48 hours after transfection for T-cell transduction.

Generation of CAR T cells

Human peripheral blood mononuclear cells (PBMC) from healthy donors were obtained under a Baylor College of Medicine Institutional Review Board–approved protocol, after informed consent was obtained in accordance with the Declaration of Helsinki. To generate

IL13R α 2-CAR T cells expressing IL15, PBMCs were isolated by Lymphoprep (Greiner Bio-One) gradient centrifugation and then stimulated on nontissue culture-treated 24-well plates, which were precoated with OKT3 (CRL-8001, ATCC) and CD28 (BD Biosciences) antibodies. Recombinant human IL7 and IL15 (IL7, 10 ng/mL; IL15, 5 ng/mL; PeproTech) were added to cultures on day 2 (32). On day 3, OKT3/CD28 stimulated T cells (2.5×10^5 cells/well) were doubly transduced on RetroNectin (Clontech)-coated plates in the presence of IL7 and IL15. On day 3 or 4, T cells were transferred into new wells and subsequently expanded with IL7 and IL15. Nontransduced T cells were activated with OKT3/CD28 and expanded in parallel with IL7 and IL15. IL13R α 2-CAR and IL15 expression was determined 4 to 5 days posttransduction and at later time points (day 12 and day 20).

Flow cytometry

FACSCanto II (BD Biosciences) or BC Gallios (Beckman Coulter, Inc.) instruments were used to acquire immunofluorescence data, which were analyzed with FACSDiva (BD Biosciences) or BC Gallios (Beckman Coulter, Inc.), respectively. FlowJo v.9 (FlowJo, LLC) or Kaluza v1.2 (Beckman Coulter, Inc.) was used for final data analysis and graphic representation. Isotype controls were immunoglobulin G1-FITC (IgG1-FITC; BD Biosciences) and IgG1-PE (IgG1-PE; BD Biosciences). IL13R α 2-CAR expression was detected by staining T cells with human IL13R α 2 chimera (R&D Systems, Inc.) followed by Fc-FITC (Millipore) or Fc-PE (SouthernBiotech). U373 cells and recurrent tumors were analyzed for IL13R α 2, HER2, and EphA2 expression using anti-IL13R α 2, HER2-APC (BD Biosciences), and anti-EphA2 antibodies (R&D Systems, Inc.), respectively. Secondary antibodies anti-goat IgG-AF647 and anti-mouse IgG-AF647 were used for IL13R α 2 and EphA2, respectively (both from Invitrogen). Cell phenotype was evaluated by staining T cells with CD4-Pacific Blue, CD8-PerCP, CCR7-FITC, and CD45RA-AF750 (BD Biosciences). Forward and side scatter gating were used to distinguish normal lymphocytes. Cells were collected and washed once with PBS (Sigma) containing 1% FBS (GE Healthcare Life Sciences HyClone Laboratories; FACS buffer) prior to the addition of antibodies. Cells were incubated for 30 minutes on ice in the dark, washed once, and fixed in FACS buffer with 0.5% paraformaldehyde (BD Biosciences) prior to analysis.

Western blot analysis

Cells were dissociated with PBS + 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), and protease inhibitors (Thermo Fisher Scientific). Protein concentrations were determined using a Bio-Rad protein assay (Bio-Rad) with BSA as the standard. Samples were denatured in Laemmli buffer (Bio-Rad) at 95°C for 5 minutes. Cell lysates (5–10 μ g/lane) were run on a 10% SDS polyacrylamide gel and transferred to nitrocellulose membranes (Bio-Rad). Membranes were blocked with 5% milk powder in TBS + 0.1% Tween-20 (all from Sigma) and then probed with anti-CD3 ζ (sc-1239, Santa Cruz Biotechnology, Inc.) or GAPDH (sc-47724, Santa Cruz Biotechnology, Inc.) mouse mAbs or anti-caspase-9 rabbit antibody (9502, Cell Signaling Technology), followed by a horseradish peroxidase-conjugated goat anti-mouse IgG antibody (Santa Cruz Biotechnology, Inc.) or goat anti-rabbit IgG antibody (Jackson ImmunoResearch, Inc.). Blots were developed using SuperSignal West Dura

Extended Duration Substrate (Thermo Fisher Scientific) and exposed to GeneMate Blue Basic Autoradiography Film (BioExpress).

Coculture assays

Recombinant protein coculture assay—Nontissue culture-treated 24-well plates were precoated with recombinant human IL13R α 1, IL13R α 2 (R&D Systems, Inc.), or OKT3 (CRL-8001, ATCC) proteins at a final concentration of 500 ng/well. Plates were washed once using RPMI, and CAR, CAR.15 or NT T cells were plated. After 24 hours, supernatants were harvested, and IL15 release was measured by ELISA as per the manufacturer's instructions (R&D Systems, Inc.).

Cell coculture assay—CAR T cells were cocultured with target cells at a 2:1 effector-to-target (E:T) ratio in a 24-well plate. Non-transduced T cells served as controls. After 24 hours, culture supernatants were harvested, and the presence of cytokines was determined by Multiplex assay (HSTCMAG28SPMX13, EMD Millipore).

Repeated stimulation assay—CAR T cells were cocultured with target cells at a 2:1 E:T ratio in a 24-well plate. CAR T cells were restimulated with fresh target cells every 7 days. Culture supernatants were harvested 24 hours after each stimulation, and the presence of cytokines was determined by Multiplex assay. CAR T cells were counted every 7 days, before the addition of fresh U373 cells.

Cytotoxicity assay

Standard chromium (^{51}Cr) release assays were performed as described previously (11). Briefly, 1×10^6 target cells were labeled with 0.1 mCi (3.7 MBq) ^{51}Cr and mixed with decreasing numbers of effector cells to give E:T 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 ^{51}Cr release, respectively. After 4 hours, supernatants were collected, and radioactivity was measured in a gamma counter (Cobra Quantum; PerkinElmer). The mean percentage of specific lysis of triplicate wells was calculated according to the following formula:

$$\frac{[\text{test release} - \text{spontaneous release}]}{[\text{maximal release} - \text{spontaneous release}]} \times 100$$

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 previously (8). Briefly, 7- to 9-week-old ICR-SCID mice were anesthetized. The head was shaved and the mice were immobilized in a Cunningham Mouse/Neonatal Rat Adaptor (Stoelting) stereotactic apparatus and then scrubbed with 1% povidoneiodine. A 10-mm skin incision was made along the midline. A 1-mm burr hole was drilled into the skull 1 mm anterior and 2 mm to the right of the bregma. U373.eGFP.ffLuc cells (1×10^5) in 2.0 μL were injected 3 mm deep to the bregma 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 effector cells in 2 μ L to the same tumor coordinates. The incision was closed with 2 to 3 interrupted 7.0 Ethilon sutures (Ethicon, Inc.). A subcutaneous injection of 0.03 to 0.1 mg/kg buprenorphine (Buprenex RBH) was given for pain control.

Recurrent tumor cell isolation—Recurrent tumors were microdissected under sterile conditions from CAR or CAR.IL15-treated xenograft mouse models. The isolates were manually dissociated and filtered through a 40- μ m mesh filter. The cell suspension was washed with PBS and cultured in RPMI + 10% FBS.

Bioluminescence imaging

Isoflurane-anesthetized animals were imaged using the IVIS system (IVIS, Xenogen Corp.) 10 to 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). A pseudo-color image representing light intensity (blue least intense and red most intense) was generated and superimposed over the grayscale reference image. Mice were euthanized when the tumor radiance was greater than 1×10^9 on two occasions or when they met euthanasia criteria (neurologic deficits, weight loss, signs of distress) in accordance with the Center for Comparative Medicine at Baylor College of Medicine (Houston, TX).

Statistical analysis

All *in vitro* experiments were performed at least in triplicate. Data were summarized using descriptive statistics. Comparisons were made between groups using Wilcoxon rank sum test or *t* test, whichever is appropriate, for continuous variables. Changes from baseline to follow-up measures were compared using paired *t* test. Linear regression analysis was performed to evaluate the trend in cytokine secretion relationship between CAR and CAR.IL15. Survival time from the time of tumor cell injection was estimated by the Kaplan–Meier method, and differences in survival between groups were compared by the Wilcoxon test. GraphPad Prism 5 software (GraphPad software, Inc.), SAS 9.4, and R 3.3.2 were used for statistical analysis. *P* values <0.05 were considered statistically significant.

Results

Generation of IL13R α 2-specific CAR T cells releasing transgenic IL15

To generate IL13R α 2-specific CAR T cells that secrete transgenic IL15 (IL13R α 2-CAR.IL15 T cells), we genetically modified T cells with a retroviral vector encoding an IL13R α 2-specific scFv (scFv47) with a CD28. ζ endodomain (IL13R α 2-CAR; ref. 8), and a retroviral vector encoding inducible caspase-9 (iC9), NGFR with a truncated cytoplasmic domain (NGFR), and IL15 separated by 2A sequences (iC9-2A-NGFR-2AIL15; Fig. 1A). CD3/CD28-activated T cells from healthy donors were transduced with RD114-pseudotyped retroviral particles, and 4 to 5 days posttransduction, CAR expression was determined by FACS analysis. As controls, we generated T cells that only expressed IL13R α 2-CARs, IL15, or IL13R α 2-CARs in which the endodomain was deleted (IL13R α 2-

CAR.). Transduction efficiency was determined by FACS analysis for CAR and NGFR expression (Fig. 1B and C). Single transduction with retroviral vectors encoding CAR, CAR. , or iC9-2A- NGFR-2A-IL15 yielded mean transduction efficiencies of 66.5% (SD \pm 12.1%), 66.1% (SD \pm 13.6%), and 56.3% (SD \pm 14.5%), respectively. In IL13R α 2-CAR.IL15 T-cell lines, on average, 35.4% (SD \pm 7.4%) of T cells were genetically modified with both vectors and in IL13R α 2-CAR. .IL15 T-cell lines 39.5% (SD \pm 9.6%). CAR expression was confirmed by Western blot for CD3. ζ (Fig. 1D). Phenotypic analysis of transduced cells revealed a mixture of CD4- and CD8-positive T cells and the presence of naïve (CD45RA⁺/CCR7⁺), central memory (CD45RA⁻/CCR7⁺), effector memory (CD45RA⁺/CCR7⁻), and terminally differentiated effector memory (CD45RA⁻/CCR7⁻) T cells (Supplementary Fig. S1). Genetic modification with CAR, CAR. , and/or iC9-2A- NGFR-2A-IL15 did not change the CD4:CD8 ratio or T-cell subset composition in comparison with nontransduced T cells. Cytotoxicity assays were performed with all T-cell populations using IL13R α 2-negative (239T-GFP) and IL13R α 2-positive (239T-GFP. IL13R α 2, U373, GBM6) target cells (Supplementary Fig. S2). Only IL13R α 2-CAR T cells and IL13R α 2.IL15-CAR T cells killed IL13R α 2-positive target cells in contrast to IL13R α 2-CAR. .IL15, IL15, and nontransduced T cells, demonstrating that genetic modification of T cells with iC9-2A- NGFR-2A-IL15 did not induce unspecific T-cell killing or influence the lytic activity of IL13R α 2-CAR T cells (Fig. 1E).

IL13R α 2-CAR.IL15 T cells displayed activation-dependent IL15 production

To determine IL15 production by IL15, IL13R α 2-CAR. .IL15, IL13R α 2-CAR.IL15, or nontransduced T cells at baseline and after activation, we cultured T cells on tissue culture plates that were coated with recombinant IL13R α 1 or IL13R α 2 proteins. Non-coated plates or plates coated with OKT3 served as controls. After 24 hours, IL15 concentration in culture media was measured by ELISA. At baseline IL15, IL13R α 2-CAR. .IL15, and IL13R α 2-CAR. IL15 T cells produced similar amounts of IL15 as did nontransduced T cells. However, in the absence of exogenous cytokines, viability of IL15-expressing T cells was improved in comparison with their unmodified counterpart after 14 days of culture (Supplementary Fig. S3). After OKT3 activation, IL15 (mean, 159.5 pg/mL \pm 70.8), IL13R α 2-CAR. .IL15 (mean, 137.9 pg/mL \pm 73.5), and IL13R α 2-CAR.IL15 (mean, 152.2 pg/mL \pm 72.4) T cells produced significantly ($P < 0.001$) more IL15 in comparison with nontransduced T cells (mean, 16.3 pg/mL \pm 2.6; Fig. 2A). IL13R α 2-CAR.IL15 T cells produced significantly ($P < 0.001$) more IL15 when stimulated with recombinant IL13R α 2 protein (mean, 176.7 pg/mL; \pm 55.4), whereas no increase in IL15 production was observed for all T-cell populations in the presence of recombinant IL13R α 1 protein. Thus, IL15 production by IL15, IL13R α 2-CAR. .IL15, and IL13R α 2-CAR.IL15 T cells was activation dependent. This was not specific for IL15, because we observed an activation-dependent increase in expression also for a second transgene (Supplementary Fig. S4).

We next determined whether IL13R α 2 expression on glioma cells is sufficient to induce IL15 production of IL13R α 2-CAR.IL15 T cells. IL15, IL13R α 2-CAR. .IL15, IL13R α 2-CAR.IL15, or non-transduced T cells were cultured with Raji (IL13R α 1-/IL13R α 2-), 293T (IL13R α 1⁺/IL13R α 2⁻), or U373 (IL13R α 1⁺/IL13R α 2⁺) cells, or media alone. After 24 hours, IL15 concentration in culture media was determined by ELISA. Only

IL13R α 2-CAR.IL15 T cells produced significant amounts of IL15 in the presence of IL13R α 2-positive cells (U373) compared with IL15, IL13R α 2-CAR, IL15, or nontransduced T cells (Fig. 2B). These data were confirmed using 293T cells genetically modified to express IL13R α 2 (293T-GFP.IL13R α 2; Supplementary Fig. S5). Target cells that were only positive for IL13R α 1 (293T cells) or negative for IL13R α 1 and IL13R α 2 (Raji) did not induce significant IL15 production in any of the tested T-cell populations. Thus, as for the experiments with recombinant protein, antigen-specific CAR activation induces IL15 production by IL13R α 2-CAR.IL15 T cells. As the IL15 vector also encodes iC9, we wanted to determine its presence and functionality. Western blot analysis for C9 at baseline revealed the presence of iC9 in IL15 and IL13R α 2-CAR.IL15 T-cell lines. However, 24 hours postexposure to 10 nmol/L CID (Chemical Inducible Dimerizer; A/C Heterodimerizer, Clontech), only native C9 was present, indicating the killing of iC9-positive T cells (Supplementary Fig. S6A). Cell killing was confirmed by FACS analysis for Annexin V and 7-AAD staining (Supplementary Fig. S6B).

IL13R α 2-CAR.IL15 T cells proliferate more than IL13R α 2-CAR T cells

To determine the benefits of transgenic IL15 expression on the effector function of CAR T cells, IL13R α 2-CAR and IL13R α 2-CAR.IL15 T cells were stimulated every 7 days with U373 or 293T cells without exogenous cytokines. Prior to each stimulation T cells were counted and CAR and NGFR expression was determined by FACS analysis. IL15 and nontransduced T cells served as controls. Repeat stimulations with U373 cells resulted in a significant ($P < 0.05$) expansion of IL13R α 2-CAR and IL13R α 2-CAR.IL15 T cells in comparison with IL15 and NT T cells (Fig. 3A). There was no significant difference between IL13R α 2-CAR and IL13R α 2-CAR.IL15 T-cell expansion after the first and second simulation. With subsequent stimulations, only IL13R α 2-CAR.IL15 T cells continued to expand. Addition of exogenous IL15 rescued IL13R α 2-CAR T cells, indicating that the observed effect was specific to IL15 (Supplementary Fig. S7). 293T cells did not induce T-cell expansion, confirming antigen specificity. Successive stimulations resulted in enrichment of IL13R α 2-CAR.IL15 T cells as judged by FACS analysis for each single transgene (before the first stimulation compared with after the sixth stimulation: for CAR: $P < 0.05$; for IL15: $P < 0.01$) in contrast to controls (nontransduced, IL15, IL13R α 2-CAR T cells; Fig. 3B).

Transgenic IL15 expression maintains cytokine production

Having established that IL13R α 2-CAR.IL15 T cells expand better than IL13R α 2-CAR T cells after repeated antigen-specific stimulations, we next determined the benefits of transgenic expression of IL15 on cytokine production. NT, IL15, IL13R α 2-CAR, or IL13R α 2-CAR.IL15 T cells were stimulated weekly with U373 cells (up to 5 stimulations), and 24 hours after each stimulation, an aliquot of media was removed to determine the concentrations of cytokines by Multiplex analysis. After the first stimulation, only IL13R α 2-CAR and IL13R α 2-CAR.IL15 T cells produced TH1 (IFN γ , TNF α , GM-CSF, IL2), TH1/2 cytokine (IL6), and TH2 (IL4, IL5, IL10) cytokines (Fig. 4). There was no difference in cytokine production between both T-cell populations. IL15 and nontransduced T cells produced negligible amounts of cytokines, confirming antigen specificity. With subsequent stimulations, there was a decline in cytokine production by IL13R α 2-CAR T and IL13R α 2-

CAR.IL15 T cells. However, the decline in IFN γ , TNF α , and IL5 production by IL13R α 2-CAR T cells was significantly ($P=0.0008$, $P=0.0253$, $P=0.0291$, respectively) greater in comparison with IL13R α 2-CAR.IL15 T cells as evaluated by the trend in cytokine secretion relationship between CAR and CAR.IL15 T cells. In addition, we measured IL15 secretion in long-term cultures and confirmed continuous production of IL15 as judged by IL15 ELISA (Fig 4; bottom right).

IL13R α 2-CAR.IL15 T cells have improved antitumor activity

Finally, we evaluated the impact of IL15 on the antitumor activity of IL13R α 2-CAR T-cell in the U373 human glioblastoma orthotopic xenograft mouse model. On day 0, U373.eGFP.ffLuc cells were injected into the brains of SCID mice followed by the intratumoral injection of IL13R α 2-CAR and IL13R α 2-CAR.IL15 T cells on day 7. Mice injected with IL13R α 2-CAR.IL15 T cells served as controls. Mice treated with IL13R α 2-CAR.IL15 T cells showed continuous tumor growth within 4 days of T-cell injection, whereas mice treated with IL13R α 2-CAR \pm IL15 T cells did not (Fig. 5A and B). Comparison of bioluminescence imaging results revealed no difference between IL13R α 2-CAR T cells and the IL13R α 2-CAR.IL15 T-cell groups up to 28 days after T-cell injection. However, mice treated with IL13R α 2-CAR.IL15 had lower tumor signals starting 35 day posttreatment (Supplementary Table 1). This resulted in greater progression-free survival (PFS) and overall survival (OS) of mice treated with IL13R α 2-CAR.IL15 T cells than mice treated with IL13R α 2-CAR T cells (CAR vs. CAR.IL15: PFS, $P=0.0021$; OS, $P=0.0019$; Fig. 5C) without inducing GVHD from nonspecific T-cell activation. To investigate the mechanism of improved PFS and OS of IL13R α 2-CAR.IL15 T cells, we compared the *in vivo* persistence of IL13R α 2-CAR and IL13R α 2-CAR.IL15 T cells. U373 glioma-bearing mice were injected on day 7 with IL13R α 2-CAR, IL13R α 2-CAR.IL15, or CAR.IL15 T cells that were also genetically modified to express eGFP.ffLuc. T cells modified only with eGFP.ffLuc served as controls. IL13R α 2-CAR.IL15 persisted longer than nontransduced ($P<0.05$) and IL13R α 2-CAR T cells ($P<0.01$). However, there was no difference between IL13R α 2-CAR.IL15 and CAR.IL15 T cells (Fig. 5D). No T cells were detected outside the brain as judged by bioluminescence imaging.

Therapy failure is due to antigen loss variants

Although IL13R α 2-CAR.IL15 T cells persisted longer than IL13 α 2-CAR T cells *in vivo*, IL13R α 2-CAR.IL15 T cells only persisted for 10 days after T-cell injection as judged by bioluminescence imaging. To differentiate between lack of long-term T-cell persistence and antigen escape as an etiology of therapy failure, recurrent gliomas from IL13R α 2-CAR and IL13R α 2-CAR.IL15 T-cell-treated mice were harvested, and FACS analysis was performed for IL13R α 2 and two other glioma-associated antigens (HER2, EphA2) after short-term culture. Tumors that recurred within 20 days after T-cell injection were IL13R α 2 positive, whereas more than 80% of cells in tumors that recurred after day 40 were IL13R α 2 negative (Fig. 6A and B). Tumors recurring between day 20 and 40 exhibited variable levels of IL13R α 2 expression. In contrast, HER2 and EphA2 expression was preserved in all tumors except in one late-recurring tumor (day 63) that was HER2 negative (Fig. 6B, middle). Cytotoxicity assays with CAR T cells were used to confirm loss of IL13R α 2 and HER2

expression (Supplementary Fig. S8). Thus, antigen negative immune escape is the most likely explanation for late (>day 40 after T-cell injection) tumor recurrence.

Discussion

Here, we described the generation and characterization of T cells expressing an IL13R α 2-CAR with a CD28. ζ signaling domain and IL15. We showed that transgenic expression of IL15 in IL13R α 2-CAR T cells enhanced their effector function as judged by cytokine production and T-cell expansion after repeated stimulations, resulting in selection of T cells that expressed CAR and IL15. *In vivo*, IL13R α 2-CAR.IL15 T cells had greater antitumor activity in comparison with IL13R α 2-CAR T cells. Recurring tumors presented as antigen loss variants, highlighting the need to target multiple TAAs once the effector function of monospecific T cells is optimized.

We genetically modified T cells with our SFG retroviral vector encoding iC9-2A-NGFR-2A-IL15. Modification was confirmed by FACS analysis for NGFR and Western blot for iC9. At baseline, there was no increase in IL15 production in comparison with unmodified T cells. Upon T-cell activation, IL15 production increased in comparison with IL15 gene-unmodified T cells, which was confirmed for a second transgene. The expression of all transgenes in SFG retroviral vectors, including IL15 and GFP. fLuc, is driven by the Molony Murine Leukemia Virus (MoMuLV) long terminal repeat (LTR) promoter/enhancer. The MoMuLV LTR enhancer is located within the U3 region of the LTR and contains binding sites for at least six distinct transcription factors (33). T-cell activation results in a broad upregulation of multiple transcription factors, including NFAT and NF- κ B (34), which likely enhance the expression of LTR-driven genes including IL15 and GFP.fLuc. Our findings are consistent with previous findings that demonstrated activation-dependent transgenic expression of IL15 in Epstein–Barr virus-specific or CD19-CAR T cells that were genetically modified with a SFG retroviral vector encoding IL15 (27, 28).

We did not observe autonomous growth of IL13R α 2-CAR.IL15, IL13R α 2-CAR. IL15, or IL15 T cells, which is in agreement with previous studies (27, 28); however, baseline IL15 expression enhanced T-cell survival in the absence of antigen. This should be a favorable attribute for T-cell therapy of brain and solid tumors in which TAAs are not readily available to activate infused T cells, as in the setting of leukemia. Repeat stimulation resulted in an enrichment of T cells expressing IL15 and IL13R α 2-CARs with approximately 100% of T cells expressing both transgenes after six stimulations. Selection of IL13R α 2-CAR.IL15 T cells after repeat stimulations argues for an autocrine loop in which IL15 is only secreted in sufficient amounts to enhance the function of IL15 gene-modified T cells. As we observed enrichment of IL13R α 2-CAR.IL15 T cells after repeat stimulations, we did not sort double-positive cells for the *in vivo* experiments.

Transgenic expression of IL15 did not change the T-cell memory subsets as defined by CD45RA and CCR7 expression in comparison with nontransduced, CAR, and CAR. T cells. Transgenic expression of membrane-bound or “tethered” IL15, and the generation of CAR T cells in IL7 and IL15 have been reported to increase the percentage of T cells with memory stem cell-like (CD45RA⁺, CCR7⁺) phenotype (32, 35, 36). We expected no

phenotypic differences between *IL15* gene–modified and unmodified T cells, as we routinely generated T cells in the presence of IL7 and IL15.

Transgenic expression of IL15 did not improve IL13R α 2-CAR T-cell proliferation or cytokine secretion after the first antigen-specific stimulation; however, starting with the third stimulations, there were significant (proliferation, $P < 0.05$; IFN γ , IL2, TNF α secretion, $P < 0.05$) differences between IL13R α 2-CAR and IL13R α 2-CAR.IL15 T cells. After the third stimulation, no viable IL13R α 2-CAR T cells remained, whereas IL13R α 2-CAR.IL15 T cells continued to expand and produce cytokines for at least two additional stimulations. Cytokine production with subsequent stimulations declined in IL13R α 2-CAR.IL15 T cells. This finding is consistent with an exhausted T-cell phenotype (37), which is expected after T-cell culture for more than 40 days. Studies are in progress to evaluate this in detail for IL13R α 2-CAR.IL15 T cells.

As in our previous study, IL13R α 2-CAR T cells did not expand *in vivo* after local injection (8). IL13R α 2-CAR.IL15 T cells also did not expand, but persisted longer than IL13R α 2-CAR T cells. These findings are in contrast to previous studies in which T cells, expressing only a CAR with a CD28 ζ signaling domain, initially expand in an antigen-dependent manner after intravenous or intraperitoneal administration (38, 39).

The benefits of transgenic expression of cytokines, such as IL12 and IL15, have been explored by several groups of investigators for solid tumors (23, 24, 40, 41). For example, transgenic expression of IL12 enhanced effector function and antitumor activity of MUC-16-CAR T cells, as judged by increased T-cell persistence and prolonged survival of tumor-bearing mice (40). In addition, transgenic expression of IL12 in VEGF-CAR T cells enhanced their antitumor activity by reversing the immunosuppressive tumor microenvironment (41). Finally, transgenic expression of IL15 in GD2-CAR NKT cells rendered NKT resistant to hypoxia and tumor-associated macrophages resulting in improved activity against neuroblastoma (24). Our study extends these findings to glioma and highlights that improving T-cell persistence by transgenic expression of IL15 leaves T cells vulnerable to other mechanisms of immune escape.

Antigen-negative immune escape was the major mode of CAR T-cell therapy failure in tumors recurring more than 40 days after T-cell injection. Antigen escape was specific for the targeted antigens, as all tested recurring tumors continued to express the glioma-associated antigen EphA2, and all but one expressed HER2. Finding an HER2-negative, recurring, tumor highlights the need to profile TAA expression in recurring tumors to determine the best second antigen to target. Investigators have targeted two TAAs either by mixing two CAR T-cell populations, expressing two CARs within the same T-cell, or developing CARs that contain two antigen-binding domains (42–44). On the basis of our findings, we favor targeting IL13R α 2 and EphA2 with CAR T cells to prevent immune escape. How to best target both antigens needs to be determined experimentally, given the growing literature that there is an intricate structural/functional relationship between the targeted antigen and the CAR (8, 45). In addition, CARs can multimerize, resulting in baseline or tonic signaling (45–47), which can have detrimental effects on T-cell function (46, 47). Expressing single-specificity CARs in T cells that recognize TAAs through their

endogenous $\alpha\beta$ TCR, or bispecific T-cell engagers (BiTEs) in CAR T cells present alternative strategies to generate bi- or multispecific T-cell products for glioma, which should mitigate the risk of heterodimerization of multiple CARs expressed in a single T cell.

A limitation of our study and the majority of published CAR T-cell therapy studies is the use of xenograft models, which do not recapitulate the immunosuppressive tumor microenvironment created by tumors, including glioma (48–50). Others have reported the development of an immunocompetent glioma model to evaluate CAR T-cell therapies; however, this model does not recapitulate physiologic TAA expression, as glioma cells were genetically modified to express the targeted TAA, EGFRvIII (48). Our xenograft model allowed for the study of human tumor and human T cells *in vivo*, which enabled us to delineate attributes of effective CAR T-cell products for glioma. We are planning to establish an immunocompetent glioma model to evaluate the effects of transgenic expression of IL15 on resident immune cell.

In conclusion, we showed that transgenic expression of IL15 in IL13R α 2-CAR T cells enhanced their effector function, resulting in improved antiglioma activity *in vitro* and *in vivo*. Recurring gliomas had downregulated the expression of IL13R α 2, highlighting the need to target multiple TAAs once the effector function of monospecific T cells is optimized. Hence, optimal CAR T-cell products for glioma should be engineered to increase their effector function and to target multiple TAAs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Summary

Glioblastoma responds imperfectly to immunotherapy. Transgenic expression of IL15 in T cells expressing CARs improved their proliferative capacity, persistence, and cytokine production. The emergence of antigen loss variants highlights the need to target multiple tumor antigens.

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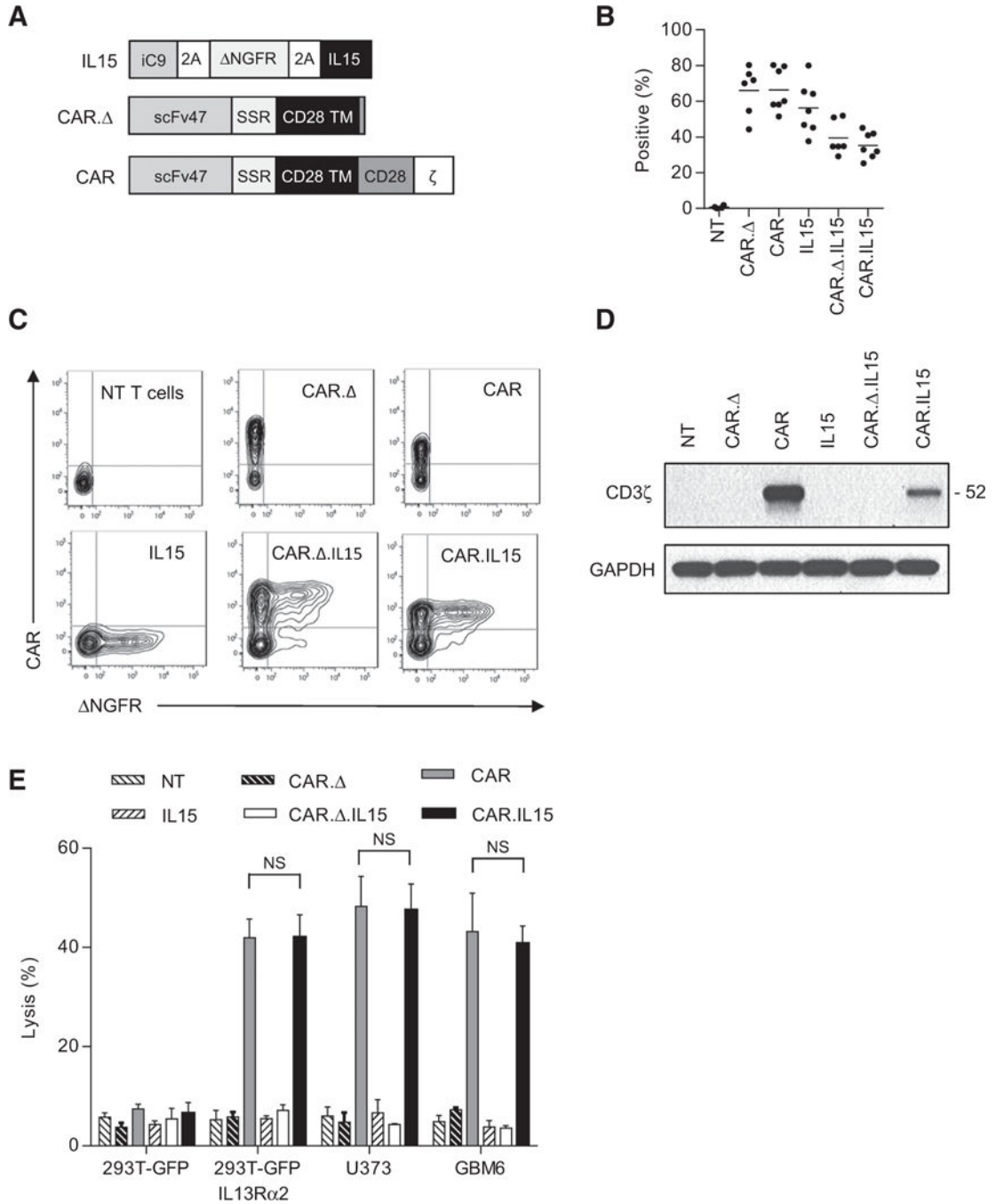


Figure 1. Generation of IL13Rα2-specific CAR T cells expressing transgenic IL15. **A**, Scheme of IL13Rα2-specific CAR and IL15 retroviral vectors. scFv47 is the name of the scFv that specifically recognize IL13Rα2. **B** and **C**, CAR and IL15 expression was confirmed using FACS analysis. Summary data [**B**; range, 37.7%–80.4% for single transduction, 30%–51.1% for double transduction (CAR. ΔIL15 and CAR.IL15), $n = 6-7$ per construct; 4–5 independent experiments] and representative plots (**C**) are shown. **D**, Expression of full-length CAR by Western blot analysis using a CD3-ζ antibody. **E**, Four-hour cytotoxicity

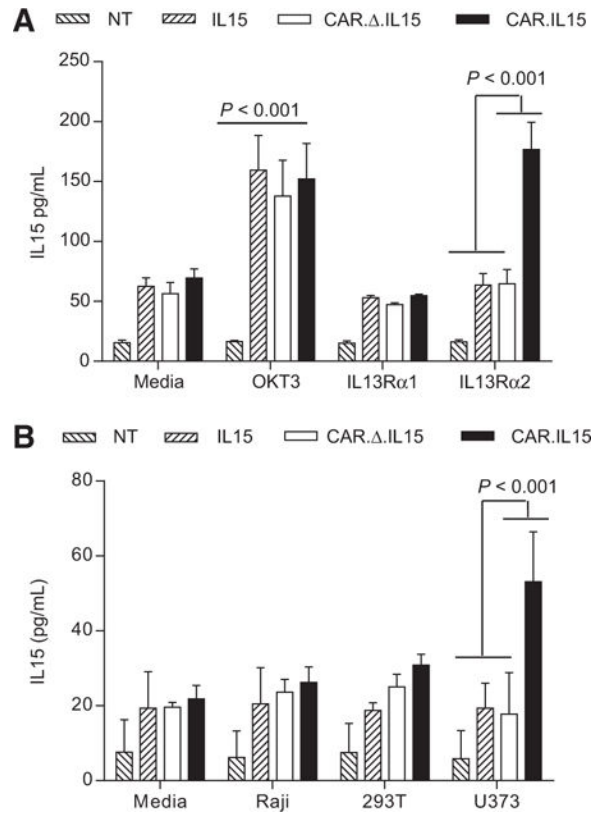
assay at an E:T ratio of 10:1 ($n = 4$; two independent experiments); CAR versus CAR.IL15: NS; two-way ANOVA; error bars, SEM). Targets: 293T-GFP, 293T-GFP-IL13R α 2, U373, and GBM6 cells (all positive for IL13R α 2 except 293T-GFP).

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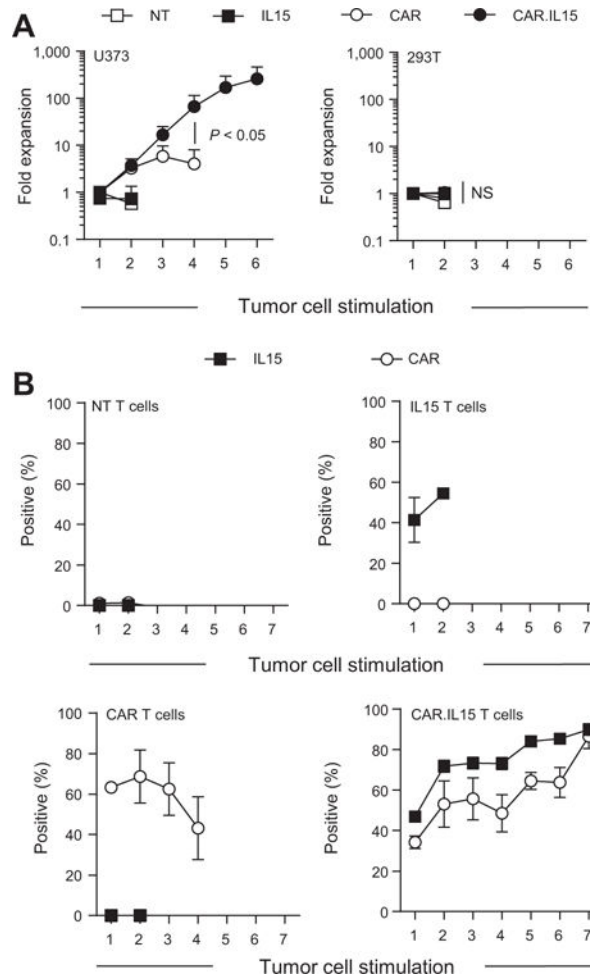
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**Figure 2.**

IL13Rα2-CAR.IL15 T cells display activation-dependent IL15 production. **A**, IL13Rα2-CAR or nontransduced (NT) T cells were stimulated with recombinant IL13Rα1 or IL13Rα2 protein, or OKT3. After 24 hours, IL15 was measured by ELISA. T cells expressing IL15 and CAR.IL15 constructs, but not controls, expressed significant concentrations of IL15 when stimulated with recombinant IL13Rα2 protein and OKT3 in comparison with IL13Rα1 stimulated T cells ($n = 6$; three independent experiments); poststimulation with OKT3: NT versus IL15/CAR/CAR.IL15 $P < 0.001$; poststimulation with IL13Rα2: NT/IL15/CAR. IL15 versus CAR.IL15 $P < 0.001$; two-way ANOVA; error bars, SEM). **B**, IL13Rα2-CAR T cells were cocultured with Raji, 293T, and U373 cells at a 2:1 E:T ratio. NT, IL15, and CAR.Δ.IL15 T cells served as controls. After 24 hours IL15 was measured by ELISA ($n = 4$; two independent experiments); poststimulation with U373: NT/IL15/CAR versus CAR.IL15 $P < 0.001$; two-way ANOVA; error bars, SEM).

**Figure 3.**

IL13R α 2-CAR.IL15 T cells have greater proliferative capacity compared with IL13R α 2-CAR T cells. CAR and CAR.IL15 T cells were cocultured with U373 cells at a 2:1 E:T ratio. T cells were stimulated weekly with fresh U373 cells, and T cells were counted before addition of fresh target cells. **A**, Cumulative data of CAR T-cell expansion ($n = 4$; two independent experiments); post third stimulation with U373: CAR versus CAR.IL15 $P < 0.05$; t test; error bars, SEM). **B**, Transgene expression after each co-culture with U373 tumor cells as determined before each stimulation by FACS analysis. Bottom right, percent of CAR or IL15-positive cells in CAR.IL15 T-cell lines ($n = 4$; two independent experiments); pre first versus post sixth stimulation: for CAR: $P < 0.05$; for IL15: $P < 0.01$; paired t test; error bars, SEM).

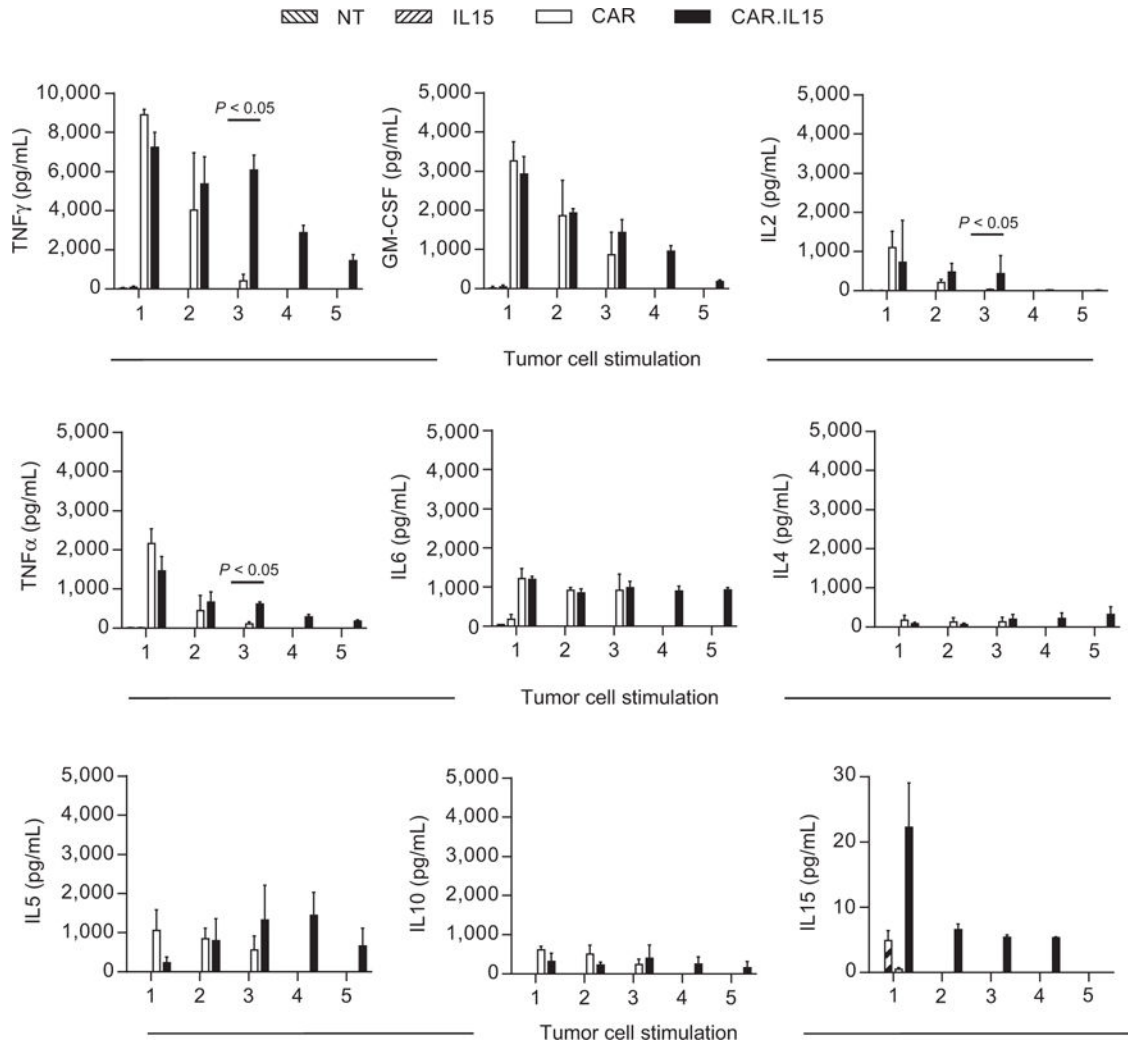


Figure 4. Transgenic IL15 expression improves cytokine production after repeated stimulations of IL13R α 2-CAR.IL15 T cells with glioma cells. CAR and CAR.IL15 T cells were cocultured with U373 cells at a 2:1 E:T ratio. T cells were stimulated weekly with U373 cells, and 24 hours after each stimulation, a small aliquot of media was removed to determine the concentrations of cytokines by Multiplex assay (HSTCMAG28SPMX13, EMD Millipore) and IL15 ELISA Kit (R&D Systems, Inc.). Nontransduced and IL15 T cells served as controls ($n = 4$; two independent experiments); post third stimulation CAR versus CAR.IL15: for IFN γ , IL2, and TNF α : $P < 0.05$; Wilcoxon rank sum test; error bars, SEM).

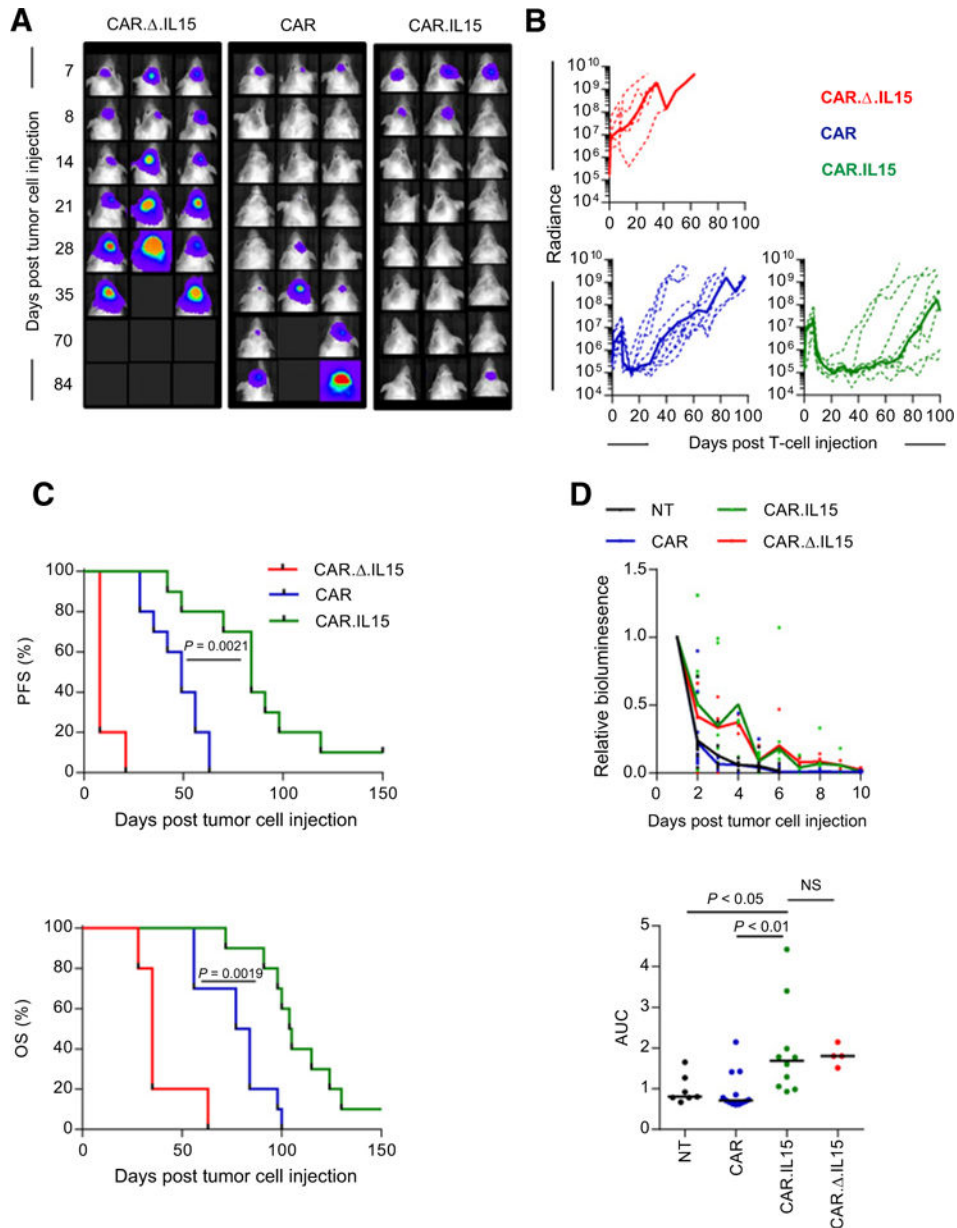


Figure 5. Treatment of glioma xenografts with IL13R α 2-CAR.IL15 T cells resulted in tumor regression and improved OS compared with IL13R α 2-CAR T cells. U373 glioma-bearing mice were treated on day 7 with CAR ($n = 10$), CAR.IL15 ($n = 10$; two independent experiments) T cells. CAR.Δ.IL15 T cells ($n = 5$; one independent experiment) served as a control. **A** and **B**, Representative images for each group (**A**) and quantitative bioluminescence (**B**; radiance = photons/sec/cm²/sr) imaging data for all mice are shown (dotted lines: individual mice; solid lines: median). **C**, Kaplan–Meier survival analysis (CAR versus CAR.IL15, $P = 0.0019$) and PFS (CAR vs. CAR.IL15, $P = 0.0021$). No GVHD from nonspecific activation was observed. **D**, Unmodified U373 cells (1×10^5) were injected intracranially into mice. On day 7, mice received 2×10^6 CAR. eGFP.ffLuc or

CAR.IL15.eGFP.ffLuc CAR T cells intracranially using the same tumor coordinates. Top, bioluminescence measurement was used to monitor T-cell persistence; bottom, AUC of bioluminescence data [nontransduced (NT; $n = 7$; two independent experiments) vs. CAR.IL15 ($n = 10$; two independent experiments), $P < 0.05$; CAR ($n = 13$; three independent experiments) vs. CAR.IL15 ($n = 10$; two independent experiments), $P < 0.01$; CAR.IL15 vs. CAR.IL15 ($n = 4$; one independent experiment), ns; t test].

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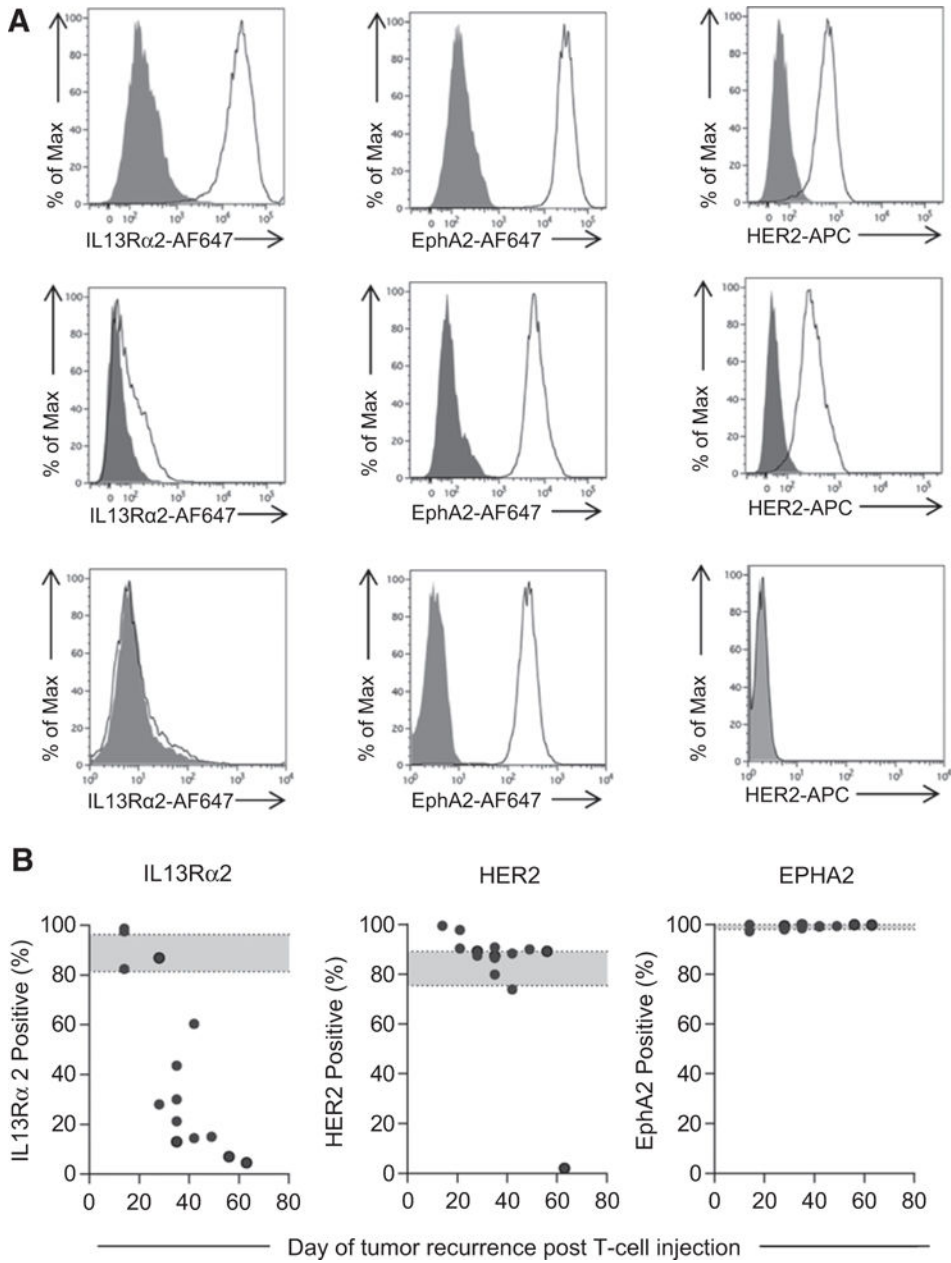


Figure 6. Late-recurring U373 gliomas did not express IL13R α 2. Cells isolated from recurrent tumors were analyzed for IL13R α 2, EphA2, and HER2 expression using primary goat anti-IL13R α 2 (AF146, R&D Systems), mouse anti-EphA2 (MAB3035, R&D Systems), and HER2-APC (340554, BD Biosciences) followed by secondary (except for HER2) rabbit anti-goat or goat anti-mouse IgG Alexa647 (Life Technologies). **A**, Representative FACS plots. Top row, IL13R α 2-positive recurrent tumors; middle row, IL13R α 2 low-expressing recurrent tumors; bottom row, IL13R α 2-negative recurrent tumors; gray, isotype control; and white, antigen-specific antibody. **B**, Summary data of GBM-associated antigen

(IL13R α 2, HER2, EphA2) expression; each dot represents a recurring tumor ($n = 13$; one independent experiment per one recurrence, total 13).

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