

Characterization and Functional Analysis of scFv-based Chimeric Antigen Receptors to Redirect T Cells to IL13R α 2-positive Glioma

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Immunotherapy with T cells expressing chimeric antigen receptors (CARs) is an attractive approach to improve outcomes for patients with glioblastoma (GBM). IL13R α 2 is expressed at a high frequency in GBM but not in normal brain, making it a promising CAR T-cell therapy target. IL13R α 2-specific CARs generated up to date contain mutated forms of IL13 as an antigen-binding domain. While these CARs target IL13R α 2, they also recognize IL13R α 1, which is broadly expressed. To overcome this limitation, we constructed a panel of IL13R α 2-specific CARs that contain the IL13R α 2-specific single-chain variable fragment (scFv) 47 as an antigen binding domain, short or long spacer regions, a transmembrane domain, and endodomains derived from costimulatory molecules and CD3. ζ (IL13R α 2-CARs). IL13R α 2-CAR T cells recognized IL13R α 2-positive target cells in coculture and cytotoxicity assays with no cross-reactivity to IL13R α 1. However, only IL13R α 2-CAR T cells with a short spacer region produced IL2 in an antigen-dependent fashion. *In vivo*, T cells expressing IL13R α 2-CARs with short spacer regions and CD28. ζ , 41BB. ζ , and CD28.OX40. ζ endodomains had potent anti-glioma activity conferring a significant survival advantage in comparison to mice that received control T cells. Thus, IL13R α 2-CAR T cells hold the promise to improve current IL13R α 2-targeted immunotherapy approaches for GBM and other IL13R α 2-positive malignancies.

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INTRODUCTION

The outcome for glioblastoma (GBM) remains poor and immunotherapy with vaccines or GBM-specific T cells is one attractive strategy to improve survival, since it does not rely on the cytotoxic mechanisms employed by conventional therapies such as chemotherapy and radiation.^{1–3} Genetic modification with

chimeric antigen receptors (CARs) allows for the rapid generation of tumor-specific T cells. For GBM-targeted CAR T-cell therapies, several antigens are actively being pursued including interleukin 13 receptor α 2 (IL13R α 2), human epidermal growth factor receptor 2 (HER2), epidermal growth factor variant III (EGFRvIII), and erythropoietin-producing hepatocellular carcinoma A2 (EphA2).^{4–11}

IL13R α 2, a cancer testis antigen, is an ideal target for CAR T-cell therapy for GBM since it is expressed in 50–80% of GBM cells and glioma-initiating cells, which are resistant to conventional therapies, but not in normal brain.^{12–15} IL13R α 2 expression is associated with poor prognosis,¹⁵ and while initial studies indicated that IL13R α 2 is a “decoy receptor”, more recent studies have demonstrated that IL13R α 2 prevents apoptosis and induces TGF- β secretion.^{16–18}

CARs consist of an ectodomain, which is most commonly derived from a single-chain variable fragment (scFv), a spacer region, a transmembrane domain, and an endomain.^{19–21} Current IL13R α 2-specific CARs do not contain scFvs, but an IL13-mutein with one or two amino acid substitutions to preferentially redirect T cells to IL13R α 2.^{7–10} While one study showed that T cells expressing IL13-mutein CARs did not recognize IL13R α 1-positive cells,²² other studies including ours demonstrated that IL13-mutein CAR T cells readily recognize IL13R α 1-positive targets raising concerns of ‘on target/off cancer toxicity’.^{7,8}

To selectively target IL13R α 2, we previously had generated a monoclonal antibody (MAb) and scFv that specifically recognized IL13R α 2 (clone 47; scFv47) with a high affinity and no cross-reactivity to IL13R α 1.^{23,24} Here, we report the development of IL13R α 2-specific CARs with a scFv47-based antigen-binding domain (IL13R α 2-CARs). We show that IL13R α 2-CARs require a short spacer region for optimal functionality, and that IL13R α 2-CAR T cells are able to recognize and kill only IL13R α 2-positive and not IL13R α 1-positive target cells *in vitro*. In addition, IL13R α 2-CAR T cells induce tumor regression in an orthotopic xenograft mouse model of GBM, which was associated with a significant survival advantage.

RESULTS

Generation of IL13R α 2-CAR T cells

We initially generated two retroviral vectors encoding CARs based on the IL13R α 2-specific MAb clone 47 (Figure 1a).^{23,24} Both CARs contained a N-terminal leader sequence, a codon-optimized synthetic gene encoding scFv47, a spacer region, a CD28 transmembrane domain, and signaling domains derived from CD28 and CD3. ζ (Figure 1a). As the spacer region, we either used the IgG1 hinge (16 amino acids; short spacer region; IL13R α 2-CAR.SSR.CD28. ζ) or the IgG1-CH2CH3 domain (239 amino acids; long spacer region; IL13R α 2-CAR.LSR.CD28. ζ). As controls, LSR and SSR IL13R α 2-CARs without signaling domains were constructed (IL13R α 2-CAR.SSR. Δ , IL13R α 2-CAR.LSR. Δ ; Figure 1a). CD3/CD28-activated T cells from healthy donors were transduced with RD114-pseudotyped retroviral particles, and 4 to 5 days post-transduction T-cell phenotype and CAR expression was determined by fluorescence activated cell sorting (FACS) analysis. CARs were expressed on the cell surface with the transduction efficiency ranged from 74.1 to 93.3% and no significant differences between constructs (Figure 1b,c). Expression of full-length IL13R α 2-CAR.SSR.CD28. ζ and IL13R α 2-CAR.LSR.CD28. ζ was confirmed by western blot using a CD3. ζ antibody for detection (Figure 1d). Western blot analysis under nonreducing conditions revealed no significant differences in dimer/multimer formation between LSR AND SSR CARs (Supplementary

Figure S2). Phenotypic analysis revealed a mixture CD4- and CD8-positive T cells. While the ratio of CD8- to CD4-positive T cells was ~3:1 for IL13R α 2-CAR.SSR.CD28. ζ , IL13R α 2-CAR.SSR. Δ , and IL13R α 2-CAR.LSR. Δ T-cell lines, it was ~1.5:1 for IL13R α 2-CAR.LSR.CD28. ζ (Supplementary Figure S1).

IL13R α 2-CAR T cells recognize IL13R α 2 but not IL13R α 1

To initially determine the specificity of IL13R α 2-CARs, we cultured T cells expressing IL13R α 2-CAR.SSR.CD28. ζ , IL13R α 2-CAR.LSR.CD28. ζ , IL13R α 2-CAR.SSR. Δ , or IL13R α 2-CAR.LSR. Δ on tissue culture plates that were uncoated or coated with recombinant proteins IL13R α 1, IL13R α 2, or IL4R α . Nontransduced (NT) T cells and T cells expressing an IL13mucin-CAR.LSR.CD28. ζ ¹⁰ that recognizes IL13R α 1 and IL13R α 2 served as controls. T cells expressing IL13R α 2-CAR.SSR.CD28. ζ or IL13R α 2-CAR.LSR.CD28. ζ produced significant levels of IFN γ ($n = 4$, $P < 0.001$) when stimulated with recombinant IL13R α 2 proteins in comparison to IL13R α 1- or IL4R α -stimulated T cells (Figure 2a). In contrast, T cells expressing IL13R α 2-CAR.SSR. Δ or IL13R α 2-CAR.LSR. Δ produced no IFN γ in response to all three proteins, indicating that IFN γ production depends on an intact IL13R α 2-CAR signaling domain. IL13R α 2-CAR.LSR.CD28. ζ T cells also produced low levels of IFN γ without activation, indicating “baseline” T-cell activation, which was confirmed by intracellular staining for

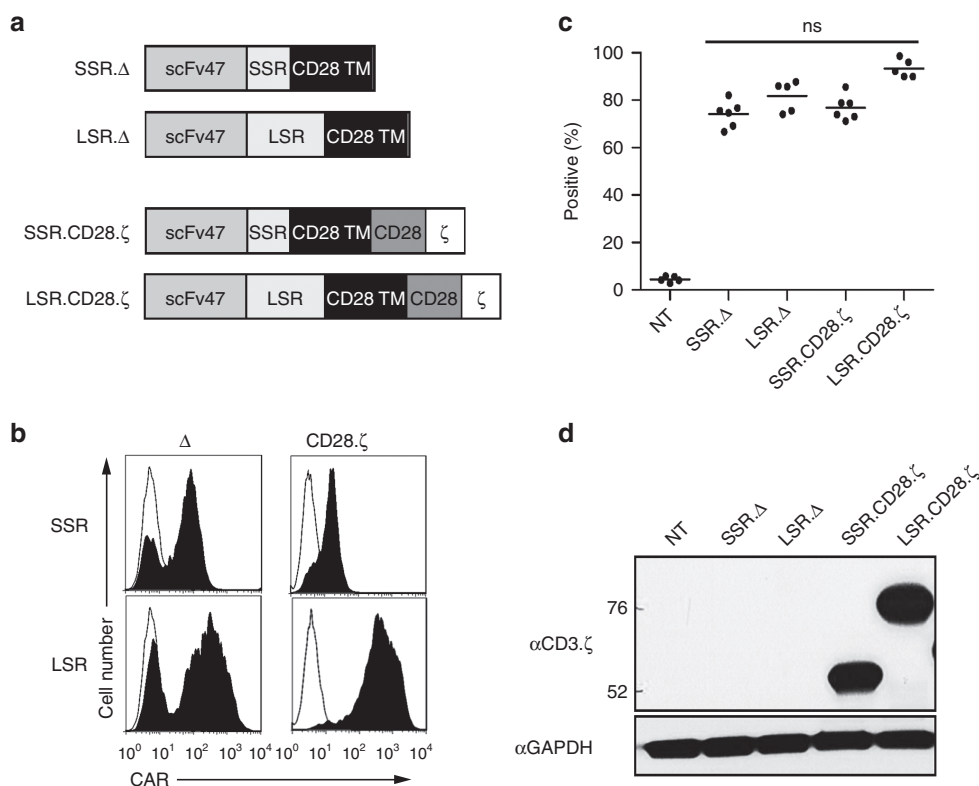


Figure 1 Generation of IL13R α 2 CAR T cells. (a) Scheme of IL13R α 2 CARs. All CARs contained an N-terminal leader sequence, a codon-optimized synthetic gene encoding for scFv47, a spacer region, a CD28 transmembrane domain, and signaling domains derived from CD28 and CD3. ζ . Spacer region was either the IgG1 hinge (16 amino acids; short spacer region; IL13R α 2-CAR.SSR.CD28. ζ) or the IgG1-CH2CH3 domain (239 amino acids; long spacer region; IL13R α 2-CAR.LSR.CD28. ζ). LSR. Δ and SSR. Δ IL13R α 2-CARs without signaling domains were constructed and served as controls. (b,c) CAR expression was confirmed using FACS analysis. Representative plots (b) and summary data (c) is shown (mean 74.1–93.3%, $n = 5$ –6 per CAR construct). (d) Expression of full-length IL13R α 2-CAR.SSR.CD28. ζ and IL13R α 2-CAR.LSR.CD28. ζ by western blot analysis using a CD3- ζ antibody. CAR, chimeric antigen receptor.

phosphorylated CD3.ζ (**Supplementary Figure S3**). IL13mucin-CAR.LSR.CD28.ζ T cells produced significant levels of IFNγ in the presence of IL13Rα1 ($n = 4$, $P < 0.001$) and IL13Rα2 ($n = 4$, $P < 0.05$) in comparison to NT T cells.

We next confirmed the specificity of IL13Rα2-CAR T cells using cell lines that were negative for IL13Rα1 and IL13Rα2 (Raji), positive for IL13Rα1 (293T-GFP cells), or positive for IL13Rα1 and IL13Rα2 (U373, 293T-GFP/IL13Rα2; **Supplementary Figure S4**). T cells expressing IL13Rα2-CAR.SSR.CD28.ζ, IL13Rα2-CAR.LSR.CD28.ζ, IL13Rα2-CAR.SSR.Δ, or IL13Rα2-CAR.LSR.Δ were cocultured with Raji, 293T-GFP, or 293T-GFP/IL13Rα2 cells. NT T cells served as controls. After 24 hours, media was collected and the concentration of IFNγ and IL2 was determined by ELISA. IL13Rα2-CAR.SSR.CD28.ζ and IL13Rα2-CAR.LSR.CD28.ζ T cells

produced significant amounts of IFNγ only in the presence of U373 or 293T-GFP/IL13Rα2 cells (**Figure 2b**) with SSR.CAR T cells producing significant more IFNγ than LSR.CAR T cells ($n = 6$, $P < 0.001$). IL13Rα2-CAR.SSR.CD28.ζ T cells produced also significant amounts of IL2 in the presence of 293T-GFP/IL13Rα2 and U373 cells, while IL13Rα2-CAR.LSR.CD28.ζ T cells did not (**Figure 2c**). NT T cells and T cells expressing IL13Rα2-CAR.SSR.Δ or IL13Rα2-CAR.LSR.Δ produced no IFNγ or IL2 in response to any target cells. Finally, we confirmed the specificity of IL13Rα2-CAR T cells in standard cytotoxicity assays using Raji, 293T-GFP, 293T-GFP/IL13Rα2, U373 (**Figure 2d**). In addition, IL13Rα2-CAR T cells killed the IL13Rα2-positive glioma cell line U87 and primary glioma cells, while IL13Rα2-negative primary glioma cells were not killed (**Supplementary Figure S5**).

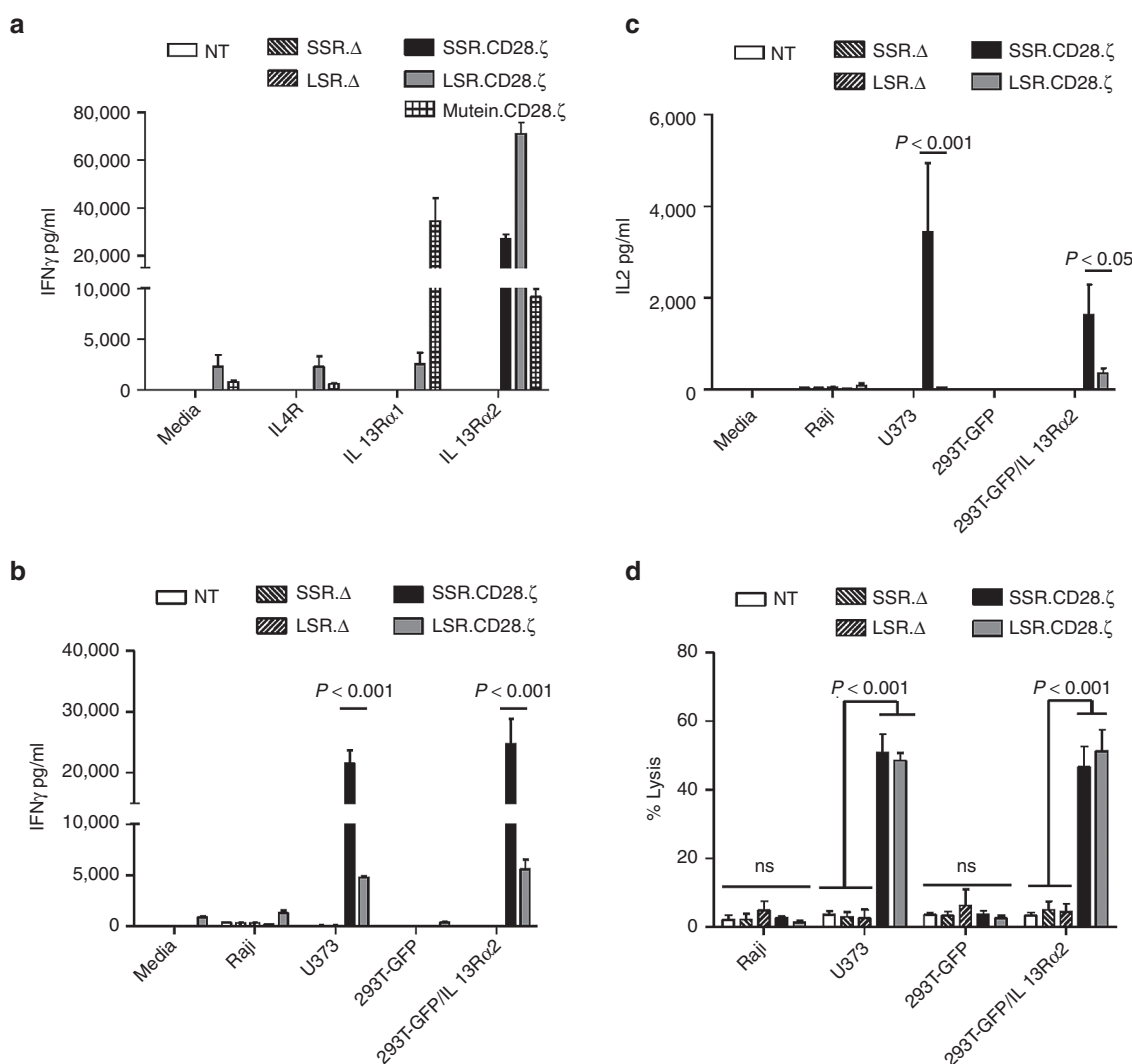


Figure 2 IL13Rα2-CAR T cells release cytokines after stimulation with recombinant IL13Rα2 protein or IL13Rα2-positive cells. IL13Rα2-CAR or nontransduced (NT) T cells were stimulated with recombinant IL13Rα1, IL13Rα2, or IL4Rα proteins. After 24 hours, IFNγ (**a**) was measured by ELISA ($n = 4$). T cells expressing IL13Rα2-CAR constructs, but not controls, expressed significant levels of IFNγ ($P < 0.001$) when stimulated with recombinant IL13Rα2 protein in comparison to IL13Rα1 and IL4Rα stimulated T cells. IL13Rα2-CAR T cells were cocultured with Raji, U373 cells, 293T-GFP, and 293T-GFP/IL13Rα2 at a 2:1 E:T ratio. NT and CAR.Δ T cells served as controls. (**b,c**) After 24h cytokines (IFNγ, IL2) were measured by ELISA. (**b**) U373 and 293T-GFP-IL13Rα2 (IFNγ); SSR.Δ versus SSR.CD28.ζ: $n = 6$, $P < 0.001$; LSR.Δ versus LSR.CD28.ζ: $n = 6$, $P < 0.05$. (**c**) U373 and 293T-GFP-IL13Rα2 (IL2); SSR.Δ versus SSR.CD28.ζ: $n = 4$, $P < 0.01$; LSR.Δ versus LSR.CD28.ζ: $n = 4$, NS. (**d**) Four hours cytotoxicity assay at an E:T ratio of 10:1 ($n = 4$). CAR, chimeric antigen receptor.

Generation of SSR IL13R α 2-CARs with CD28.OX40. ζ , CD28.41BB. ζ , or 41BB. ζ endodomains

While the results described above demonstrated that IL13R α 2-CAR T cells only recognize IL13R α 2 as judged by cytokine production and cytolytic activity, they also highlighted differences between LSR and SSR IL13R α 2-CARs. Since only IL13R α 2-CAR.SSRs produced IL2 in the presence of IL13R α 2-positive target cells, we focused in the next set of experiments on IL13R α 2-CARs with SSRs, and generated additional CARs with CD28.OX40. ζ , CD28.41BB. ζ , or 41BB. ζ endodomains (Figure 3a). CAR T cells were generated by retroviral transduction and CAR expression on the cell surface was determined by FACS analysis (Figure 3b,c) and western blot (Figure 3d). While all CARs were expressed by T cells as judged by western blot analysis, IL13R α 2-CAR.SSR.CD28.41BB. ζ was not expressed on the cell surface, and was excluded from further analysis.

Functional comparison of IL13R α 2-CAR.SSR.CD28. ζ , IL13R α 2-CAR.SSR.41BB. ζ , and IL13R α 2-CAR.SSR.CD28.OX40. ζ T cells

To compare the ability of IL13R α 2-CAR.SSR T cells to produce IFN γ and IL2 in response to antigen exposure, we performed coculture assay with U373 cells. NT and T cells expressing IL13R α 2-CAR.SSR. Δ served as controls. All IL13R α 2-CAR.SSRs with functional endodomains induced IFN γ and IL2 production in the presence of U373 cells, however IL13R α 2-CAR.SSR.41BB. ζ T cells produced significantly less ($n = 5$, $P < 0.05$) IFN γ in comparison to IL13R α 2-CAR.SSR.CD28. ζ and IL13R α 2-CAR.SSR.CD28.OX40. ζ T cells (Figure 4a). IL13R α 2-CAR.SSR.CD28. ζ

T cells produced the highest amount of IL2, followed by IL13R α 2-CAR.SSR.41BB. ζ and IL13R α 2-CAR.SSR.CD28.OX40. ζ T cells. In cytotoxicity assays, no significant difference was observed between all three constructs using Raji, 293T-GFP, 293T-GFP/IL13R α 2, and U373 as targets (Figure 4b).

Since all three IL13R α 2-CAR.SSRs T cells with functional endodomains produced IL2, we tested all three constructs in our orthotopic U373 glioma xenograft mouse model in which T cells are directly injected into tumors.⁶ The model allows for serial bioluminescence imaging since U373 are genetically modified to express an eGFP.flLuc fusion protein (U373.eGFP.flLuc). On day 0, U373.eGFP.flLuc cells were injected stereotactically into brains of SCID mice, and on day 7 T cells expressing IL13R α 2-CAR.SSR.CD28. ζ , IL13R α 2-CAR.SSR.41BB. ζ , IL13R α 2-CAR.SSR.CD28.OX40. ζ , or IL13R α 2-CAR.SSR. Δ were injected intratumorally. While mice treated with IL13R α 2-CAR.SSR. Δ T cells showed continuous tumor growth within 4 days of T-cell injection, mice treated with IL13R α 2-CAR.SSR T cells that had functional endodomains did not (Figure 5a,b). Comparison of bioluminescence imaging results revealed no significant difference between IL13R α 2-CAR.SSR. Δ T cells and the IL13R α 2-CAR.SSR T cells groups on the day of T-cell injection. However, mice treated with IL13R α 2-CAR.SSR.CD28. ζ or IL13R α 2-CAR.SSR.CD28.OX40. ζ T cells had significantly lower tumor signals as early as 1 day post-treatment in comparison to mice treated with IL13R α 2-CAR.SSR. Δ T cells (day 8, $P = 0.012$ and $P = 0.023$; Table 1). This resulted in a significant survival advantage of IL13R α 2-CAR.SSR.CD28. ζ or IL13R α 2-CAR.SSR.CD28.OX40. ζ T-cell-treated mice ($P = 0.0002$ and $P = 0.0092$; Figure 5c). While IL13R α 2-CAR.

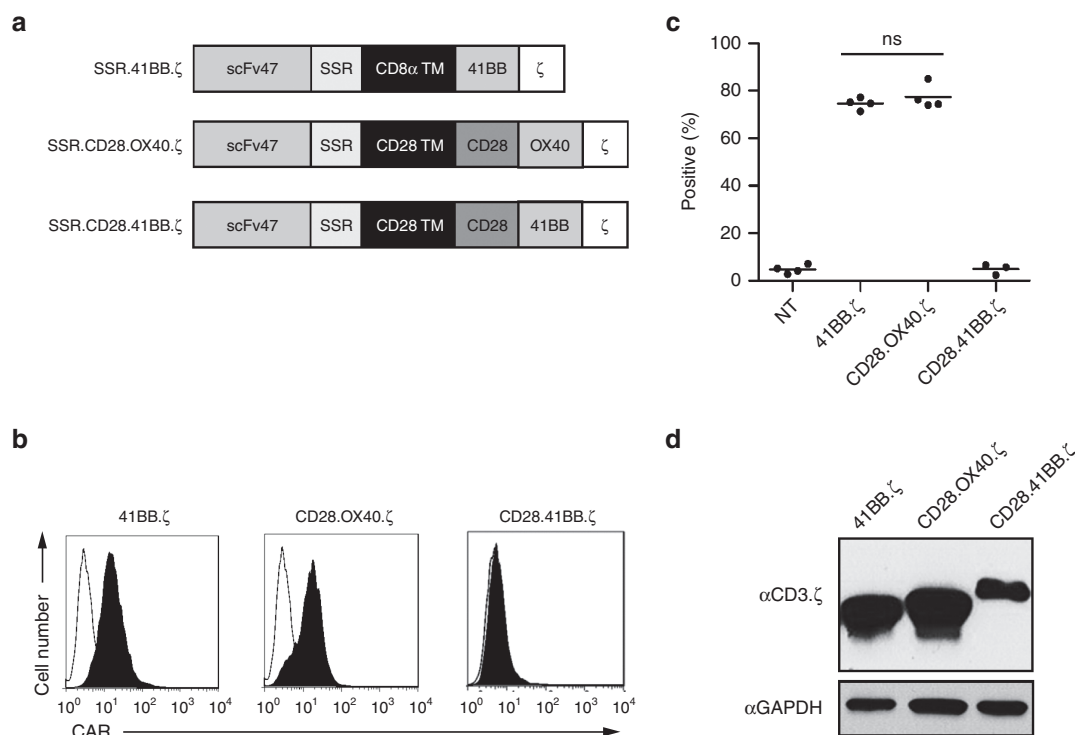


Figure 3 Generation of SSR IL13R α 2-CARs with CD28.OX40. ζ , CD28.41BB. ζ or 41BB. ζ endodomains. (a) Scheme of SSR IL13R α 2-CARs. (b,c) CAR expression was confirmed using FACS analysis. Representative plots (b) and summary data (c) is shown. IL13R α 2-CAR.SSR.CD28.OX40. ζ and IL13R α 2-CAR.SSR.CD28.41BB. ζ : mean: 74.6–77.5% ($n = 4$); IL13R α 2-CAR.SSR.CD28.41BB. ζ : mean: 4.9% ($n = 3$). (d) Expression of IL13R α 2-CAR.SSR.41BB. ζ , IL13R α 2-CAR.SSR.OX40.CD28. ζ , and IL13R α 2-CAR.SSR.41BB.CD28. ζ by western blot analysis. CAR, chimeric antigen receptor.

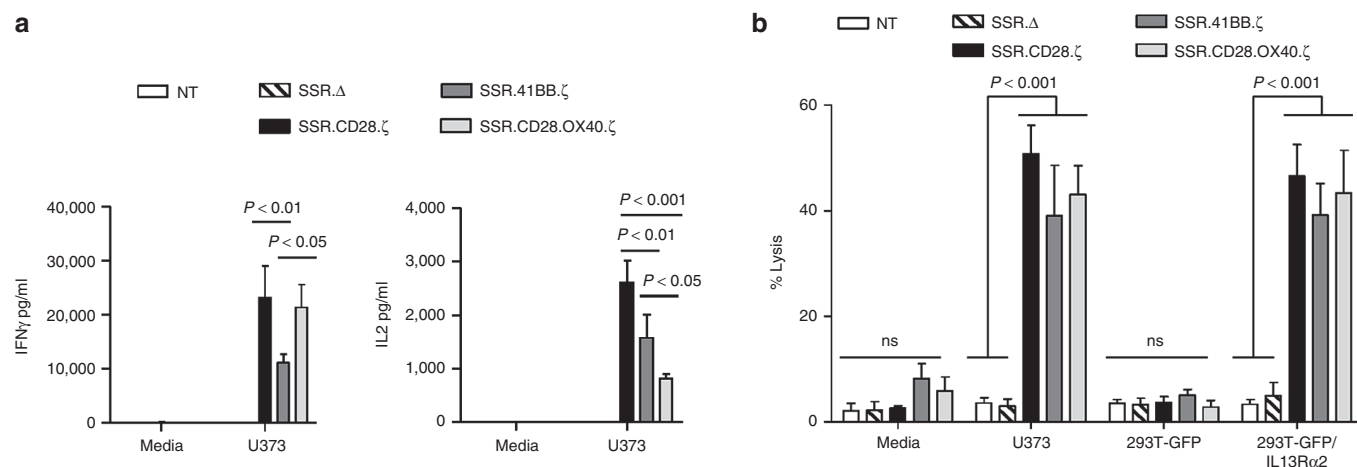


Figure 4 Comparison of IL13R α 2-CAR.SSR.CD28. ζ , IL13R α 2-CAR.SSR.41BB. ζ , and IL13R α 2-CAR.SSR.CD28.OX40. ζ T cells. **(a)** IL13R α 2-CAR T cells were cocultured with U373 cells at a 2:1 E:T ratio. NT and CAR. Δ T cells served as controls. After 24 hours, IFN γ and IL2 was measured by ELISA ($n = 5$); SSR. Δ versus SSR.CD28. ζ (U373; IFN γ): $P < 0.001$; SSR. Δ versus SSR.41BB. ζ (U373; IFN γ): $P < 0.05$; SSR. Δ versus SSR.CD28.OX40. ζ for (U373; IFN γ): $P < 0.001$; SSR. Δ versus SSR.CD28. ζ (U373; IL2): $P < 0.001$; SSR. Δ versus SSR.41BB. ζ (U373; IL2): $P < 0.001$; SSR. Δ versus SSR.CD28.OX40. ζ (U373; IL2): $P < 0.01$. **(b)** Four hours cytotoxicity assay at an E:T ratio of 10:1 ($n = 4$). CAR, chimeric antigen receptor; ELISA, enzyme-linked immunosorbent assay.

SSR.41BB. ζ T-cell-treated mice responded slower resulting in a significant difference between IL13R α 2-CAR.SSR. Δ T-cell-treated on day 14 ($P = 0.005$; **Table 1**), treatment also resulted in a significant survival advantage ($P = 0.0039$; **Figure 5c**). IL13R α 2-CAR.SSR.CD28. ζ T-cell-treated mice had the longest median survival (84 days). However, there was no statistical difference in comparison to the median survival of IL13R α 2-CAR.SSR.41BB. ζ (63 days) or IL13R α 2-CAR.SSR.CD28.OX40. ζ (56 days) T-cell-treated mice.

While IL13R α 2-CAR T cells had potent anti-glioma activity, mice eventually developed recurrent gliomas. To investigate the etiology of tumor recurrence, U373 cells were isolated from two tumor-bearing mice that had been treated either with IL13R α 2-CAR.SSR.CD28. ζ or IL13R α 2-CAR.SSR.CD28.OX40. ζ T cells. FACS analysis after short-term culture revealed cell surface expression of IL13R α 2, and these cells were readily killed by IL13R α 2-CAR T cells in cytotoxicity assay (**Figure 6**). We next determined T-cell persistence by bioluminescent imaging of genetically modified T cells expressing IL13R α 2-CAR.SSR.CD28. ζ and eGFP. β Luc (Luc/IL13R α 2-CAR T cells) injected into U373 tumors. T cells were detected for about 6 days making limited persistence the most likely explanation for tumor recurrence (**Figure 7**).

DISCUSSION

Here, we describe the development and characterization of a new scFv-based CAR, IL13R α 2-CAR that is specific for IL13R α 2. We show that T cells expressing this CAR can effectively target and kill IL13R α 2, but not IL13R α 1-positive target cells and that only IL13R α 2-CARs with a SSR induce IL2 production in a strictly IL13R α 2-dependent manner. Finally, we demonstrate that IL13R α 2-CAR.SSR T cells have potent antitumor activity *in vivo*.

IL13R α 2, a cancer testis antigen, is aberrantly expressed in GBM and other malignancies such as melanoma, adrenocortical carcinoma, colorectal, pancreatic, ovarian cancers.^{15,25–27} It is a promising immunotherapy target since it is not expressed in

most normal tissues and in one study, using nanostring digital RNA counting, belonged to the top 10% of 73 evaluated tumor associated antigens that were differentially expressed between tumors and normal tissues.²⁵ While IL13R α 2 has been targeted with immunotoxins, vaccines, and antigen-specific T cells with encouraging results,^{10,28,29} few approaches so far have used IL13R α 2-specific mAbs or scFvs as targeting moieties for therapeutics.^{24,30} We constructed IL13R α 2-specific, scFv-based CARs and determined the influence of long and short spacer regions, as well as endodomains on their function. While IL13R α 2-CAR.SSR.CD28. ζ and IL13R α 2-CAR.LSR.CD28. ζ recognized target cells as judged by IFN γ production, only IL13R α 2-CAR.SSR.CD28. ζ induced IL2 production, indicating better T-cell activation. We confirmed the inability to induce IL2 expression for one additional LSR IL13R α 2-CAR containing a CD28.41BB. ζ endodomain (**Supplementary Figure S6**). Other groups have also reported that the length of the spacer region contributes to CAR function.^{31,32} For example, scFvs that bind to an epitope in close proximity to the cancer cell membrane, require long spacer regions for optimal CAR function in contrast to scFvs that bind to epitopes distal to the cell membrane. While the precise epitope within the IL13R α 2 molecule for scFv47 is not defined,^{23,24} it binds to the same epitope as the parental MAb 47 that competes with IL13 for its binding site.²⁴ Our finding that a SSR confers optimal CAR activity suggests that the epitope is located distal to the cell membrane.

We constructed four SSR IL13R α 2-CARs with different endodomains, CD28. ζ , 41BB. ζ , CD28.OX40. ζ , and CD28.41BB. ζ . While all four CARs were expressed as judged by western blot analysis, however no significant cell surface expression was observed for IL13R α 2-CAR.SSR.CD28.41BB. ζ . We explored if changing the transmembrane domain from CD28 to CD8 α in IL13R α 2-CAR.SSR.CD28.41BB. ζ would result in better cell surface expression, however no increased expression was observed (**Supplementary Figure S7**). Since IL13R α 2-CARs.LSR.

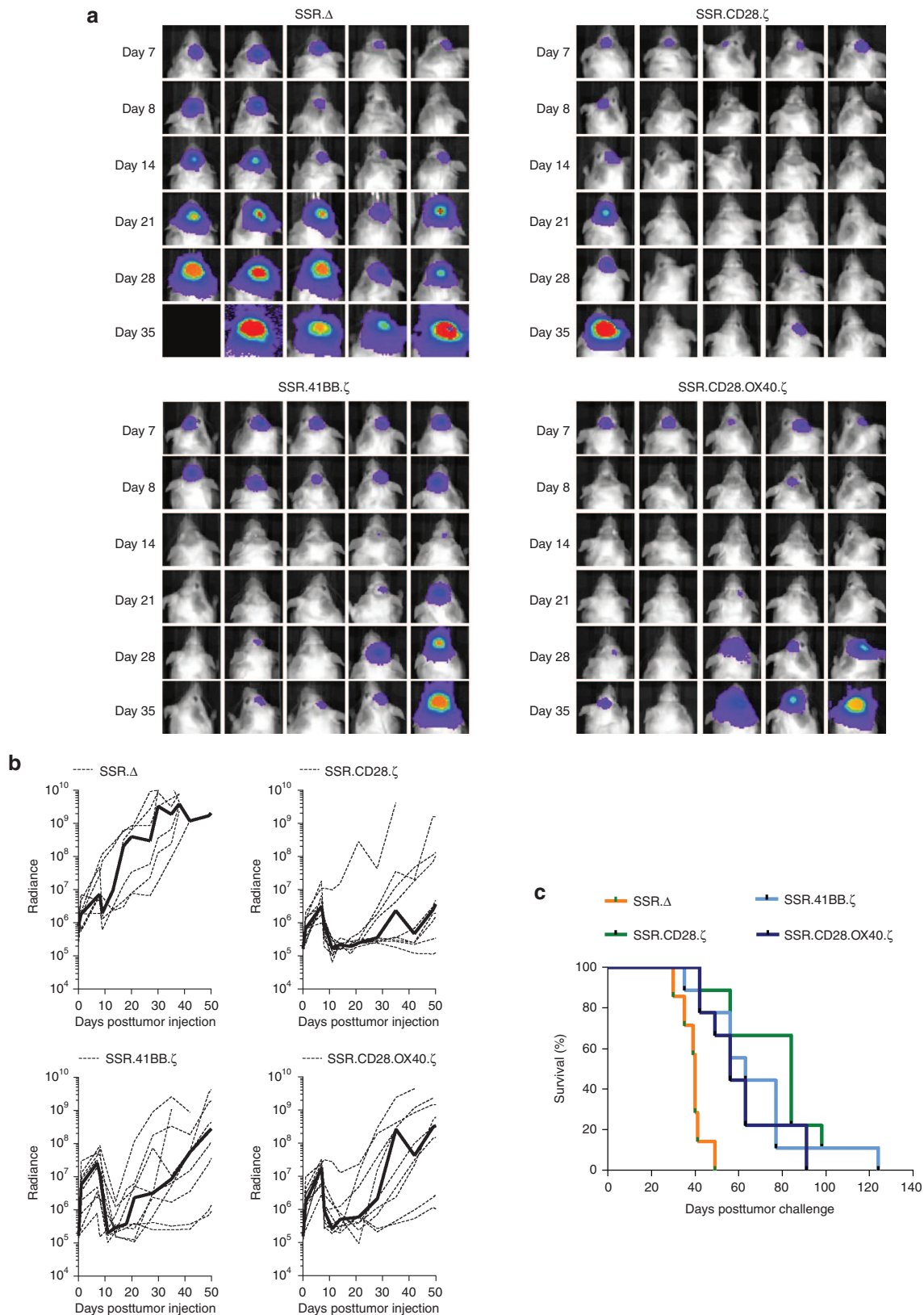


Figure 5 Treatment of glioma xenograft with T cells expressing IL13R α 2-CARs results in tumor regression and improved overall survival. U373 glioma-bearing mice were treated on day 7 with SSR.CD28. ζ ($n = 9$), SSR.41BB. ζ ($n = 9$), or SSR.OX40.CD28. ζ ($n = 9$) T cells. SSR. Δ CAR T cells ($n = 7$) served as controls. **(a)** Representative images for each group and **(b)** quantitative bioluminescence (radiance = photons/sec/cm²/sr) imaging data for all mice are shown (dotted lines: individual mice; solid lines: median). **(c)** Kaplan-Meier survival analysis (SSR. Δ versus SSR.CD28. ζ : $P = 0.0002$; SSR. Δ versus SSR.41BB. ζ : $P = 0.0039$; SSR. Δ versus SSR.OX40.CD28. ζ : $P = 0.0092$; SSR.CD28. ζ versus SSR.41BB. ζ : $P = 0.4723$; SSR.CD28. ζ versus SSR.OX40.CD28. ζ : $P = 0.3582$; SSR.41BB. ζ versus SSR.OX40.CD28. ζ : $P = 0.8374$). CAR, chimeric antigen receptor.

CD28.41BB. ζ were expressed on the cell surface (**Supplementary Figure S6**), our results suggests that the SSR interferes with efficient CAR trafficking to the cell surface.

Local or systemic injections of conventional as well as CAR T cells are actively being explored for high-grade glioma.^{10,33,34} We focused on local injections since in our previous study with human EphA2-CAR T cells in SCID mice we only observed antitumor activity after local and not after tail vein injection,⁵ most likely reflecting the inability of human T cells to transverse the murine blood brain barrier. IL13R α 2-CAR.SSR.CD28. ζ , IL13R α 2-CAR.SSR.41BB. ζ , and IL13R α 2-CAR.SSR.CD28.OX40. ζ T cells had potent antitumor *in vivo* resulting in a significant survival advantage. While mice treated with IL13R α 2-CAR.SSR.CD28. ζ T cells

had the longest median survival in comparison to IL13R α 2-CAR.SSR.41BB. ζ or IL13R α 2-CAR.SSR.CD28.OX40. ζ T-cell-treated mice, this difference did not reach significance. Our finding that addition of a second costimulatory endodomain does not improve antitumor activity *in vivo* is in agreement with recent findings by others.³⁵ While we favor one of our second-generation CARs to move forward for further preclinical testing, we are conducting additional experiments to establish the minimal effective dose of each CAR T-cell population, test their efficacy in additional glioma models, and evaluate their ability to kill glioma cells after repeated stimulations.

Our data suggest that tumor recurrence is most likely due to limited T-cell persistence in the tumor milieu *in vivo*. This limitation may be overcome by modifying the glioma cells to secrete IL-2.²² While effective, this strategy is difficult to translate into the clinical setting. We therefore are exploring additional genetic modifications of IL13R α 2-CAR T cells to enhance their expansions and persistence like transgenic expression of cytokines^{36,37} and/or silencing negative regulators. For example, gliomas express PD-L1,^{38,39} and U373 expresses PD-L1, which is upregulated in the presence of IFN γ (**Supplementary Figure S8**), and could be targeted in future studies.

While we did not observe immune escape, targeting a single antigen is associated with the risk of selecting tumor cells that have down regulated the expression or have deleted the targeted antigen.^{40,41} In the context of CAR T cells several groups including ours have developed strategies to overcome this limitation by infusing two T-cell products with different specificity, expressing multiple CARs in T cells or expressing a single CAR with dual specificity.⁴²⁻⁴⁴

We evaluated IL13R α 2-CAR T cells in a immune-deficient mice, which are widely used to study the efficacy of CAR T cells.¹⁹ While ideal to study the interaction between human T cells and human tumor cells, immune deficient mice do not recapitulate realistically the complex interactions between adoptively transferred T cells, tumor cells, and the resident immune system, that often contributes to the immunosuppressive tumor microenvironment. To overcome this limitation, several groups have developed immune-competent mouse models to study CAR T cells.^{11,45} For our IL13R α 2-CAR T cells, such studies would require the genetic modification of glioma cells to express human IL13R α 2, since scFv47-based CAR T cells do not recognize murine IL13R α 2

Table 1 Tumor signal comparison

Tumor signal comparison	P
Day 7	
SSR. Δ versus SSR.41BB. ζ	0.917
SSR. Δ versus SSR.CD28 ζ	0.111
SSR. Δ versus SSR.CD28.OX40 ζ	0.917
Day 8	
SSR. Δ versus SSR.41BB ζ	0.835
SSR. Δ versus SSR.CD28 ζ	0.012
SSR. Δ versus SSR.CD28.OX40 ζ	0.023
Day 14	
SSR. Δ versus SSR.41BB ζ	0.005
SSR. Δ versus SSR.CD28 ζ	0.015
SSR. Δ versus SSR.CD28.OX40 ζ	0.015
Day 21	
SSR. Δ versus SSR.41BB ζ	0.010
SSR. Δ versus SSR.CD28 ζ	0.010
SSR. Δ versus SSR.CD28.OX40 ζ	0.012
Day 28	
SSR. Δ versus SSR.41BB ζ	0.051
SSR. Δ versus SSR.CD28 ζ	0.008
SSR. Δ versus SSR.CD28.OX40 ζ	0.034

*Wilcoxon rank-sum test.

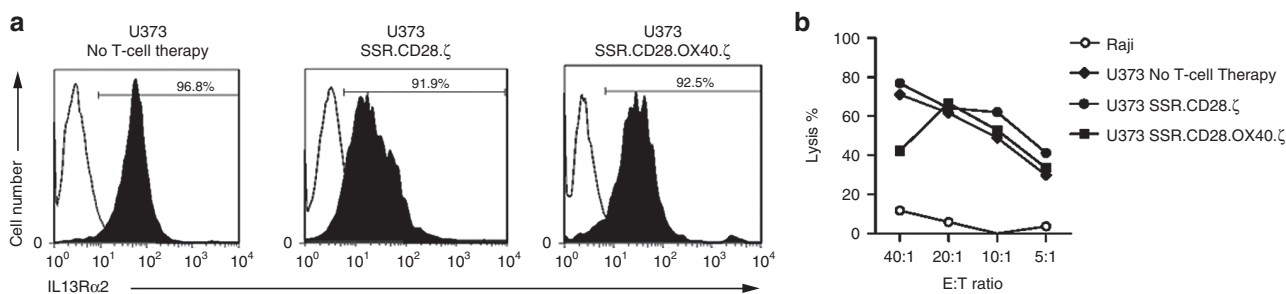


Figure 6 Analysis of U373 cells isolated from recurrent tumors. U373 cells were isolated from recurrent tumor of mice that were treated with IL13R α 2-CAR T cells. After short-term culture (2–7 days), FACS analysis and cytotoxicity assays were performed. **(a)** FACS analysis for IL13R α 2. **(b)** IL13R α 2-CAR T cells killed U373 tumor cells isolated from recurrent tumors in contrast to Raji cells in a standard 4-hour cytotoxicity assay. CAR, chimeric antigen receptor.

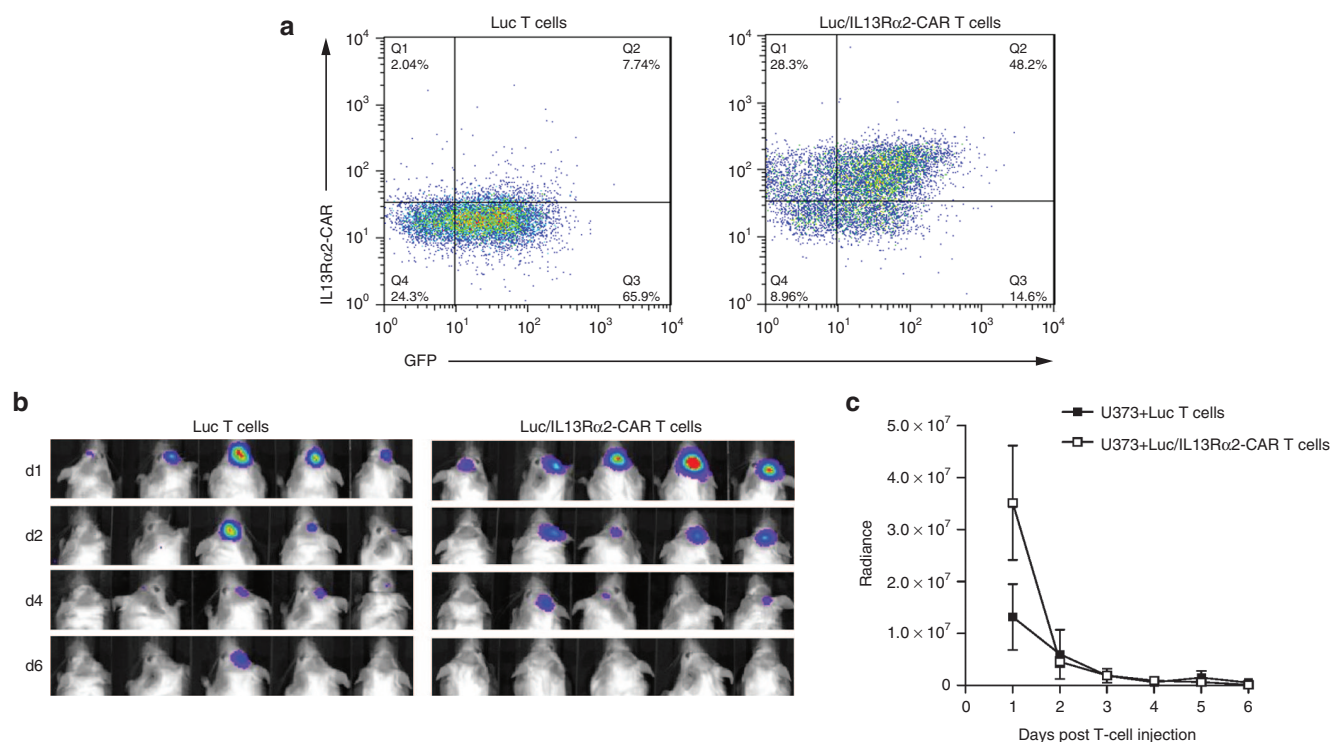


Figure 7 Limited persistence of IL13R α 2-CAR T cells *in vivo*. SSR.CD28. ζ -CAR T cells were transduced to express eGFP.ffLuc. **(a)** FACS analysis confirmed the expression of CAR and eGFP.ffLuc transgenes. **(b,c)** 1×10^5 unmodified U373 cells were injected intracranially into mice. On day 7, mice received 2×10^6 SSR.CD28. ζ -eGFP.ffLuc CAR T cells intracranially using the same tumor coordinates. Bioluminescence imaging was used to monitor T-cell persistence. CAR, chimeric antigen receptor.

(Krenciute *et al.*, unpublished data), or the generation of a murine IL13R α 2-specific scFv.

In conclusion, T cells redirected to IL13R α 2 with scFv47-based CARs have potent antitumor activity against glioma cells *in vitro*, and induce the regression of established GBM xenografts *in vivo*. Our study adds to the growing literature^{31,32} that there is an intricate interplay between scFvs, spacer region, transmembrane domain, and endodomain that determines CAR function, and that there is no single optimal configuration that is “one size fits all”. IL13R α 2-CAR T cells may be of value in the treatment of not only IL13R α 2-positive GBMs but also other malignancies in which IL13R α 2 is expressed.

MATERIALS AND METHODS

Cell lines. U373 (GBM), U87 (GBM), 293T (human embryonic kidney), and Raji (Burkitt’s lymphoma) cell line were purchased from the American Type Culture Collection (ATCC; Manassas, VA). GBM4687 and GBMR031 are primary pediatric GBM cell lines,⁴⁶ and GBM6 and GBM39 are primary adult GBM cell lines.⁴⁷ The generation of U373 cells expressing enhanced green fluorescent protein and firefly luciferase (U373.eGFP.ffLuc), 293T cells expressing green fluorescent protein (293T.GFP) or IL13R α 2 and GFP (293T.IL13R α 2.GFP) was previously reported.⁵⁷ Cell lines were grown in Roswell Park Memorial Institute or Dulbecco’s Modified Eagle Medium (GE Healthcare Life Sciences HyClone Laboratories, Logan, UT) with 10% fetal bovine serum (FBS; GE Healthcare Life Sciences HyClone) and 2 mmol/l GlutaMAX-I (Invitrogen, Carlsbad, CA). The “Characterized Cell Line Core Facility” at MD Anderson Cancer Center, Houston, Texas, performed cell line validation.

Generation of retroviral vectors encoding IL13R α 2-scFv-specific CARs. A codon-optimized gene was synthesized by GeneArt (Invitrogen)

containing the immunoglobulin heavy-chain leader peptide, and scFv47 flanked by 5’ NcoI and 3’ BamHI sites. This mini gene was subcloned into SFG retroviral vector containing IL13R α 2-specific CARs (IL13R α 2-CARs) with short or long spacer regions (SSRs, LSRs) and CD28. ζ , CD28.OX40. ζ , CD28.41BB. ζ , or 41BB. ζ endodomains.^{4,48,49} All CARs contained a CD28 transmembrane domain except for IL13R α 2-CAR.SSR.41BB. ζ , which had a CD8 α transmembrane domain. IL13R α 2-CAR.SSR. Δ and IL13R α 2-CAR.LSR. Δ without an endodomain were generated by PCR cloning. All cloning of the CARs were verified by sequencing (Seqwright, Houston, TX). RD114-pseudotyped retroviral particles were generated by transient transfection of 293T cells as previously described.⁵

Generation of CAR T cells. Human peripheral blood mononuclear cells from healthy donors were obtained under a Baylor College of Medicine Institutional Review Board-approved protocol, after informed consent was obtained in accordance to the Declaration of Helsinki. To generate IL13R α 2-CAR T cells, peripheral blood mononuclear cells were isolated by Lymphoprep (Greiner Bio-One, Monroe, NC) gradient centrifugation and then stimulated on treated nontissue culture 24-well plates, which were precoated with OKT3 (CRL-8001, ATCC) and CD28 (BD Bioscience, Mountain View, CA) antibodies. Recombinant human interleukin-7 and -15 (IL7, 10 ng/ml; IL15, 5 ng/ml; Proleukin; Chiron, Emeryville, CA) was added to cultures on day 2.⁵⁰ 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 IL7 and IL15. On day 5 or 6, T cells were transferred into new wells and subsequently expanded with IL-7 and IL15. Nontransduced (NT) T cells were activated with OKT3/CD28 and expanded in parallel with IL-7 and IL-15. IL13R α 2-CAR expression was determined 4 to 5 days post-transduction.

Flow cytometry. A FACSCalibur (BD Bioscience) or BC Gallios (Beckman Coulter, Brea, CA) instruments were used to acquire immunofluorescence data which were analyzed with CellQuest (BD Bioscience)

or BC Gallios (Beckman Coulter) respectively. FlowJo v.7 (FlowJo, LLC Ashland, OR) or Kaluza v1.2 (Beckman Coulter) were used for final data analysis and graphic representation. Isotype controls were immunoglobulin G1–fluorescein isothiocyanate (IgG1–FITC; BD Bioscience), IgG1–phycoerythrin (IgG1–PE; BD Bioscience). SSR IL13R α 2-CAR expression was detected by staining T cells with human IL13R α 2 chimera (R&D Systems, Minneapolis, MN) followed by Fc-FITC (Milipore, Billerica, MA) or Fc-PE (SouthernBiotech, Birmingham, AL). LSR IL13R α 2-CARs were detected using Fc-FITC or Fc-PE. U373 cells were analyzed for PD-L1 expression using a CD271 PE antibody (BD Bioscience). Forward and side scatter gating were used to discriminate live cells from dead cells. Cells were collected and washed once with PBS (Sigma, St. Louis, MO) 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 Bioscience) prior to analysis.

Western blot. Cells were dissociated with PBS + 3 mmol/l ethylenediaminetetraacetic acid and lysed in a buffer containing 50 mmol/l Tris, 150 mmol/l NaCl, 5 mmol/l Ethylenediaminetetraacetic acid, 1% Triton X-100 (all from Sigma), 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) with β ME (2-mercaptoethanol, Bio-Rad; reducing conditions) or without β ME (nonreducing condition) at 95 °C for 5 minutes. Cell lysate (5 μ g per lane) were run on a 10% SDS polyacrylamide gel and transferred to nitrocellulose membranes (BioRad). Membranes were blocked with 5% milk powder in Tris-buffered saline + 0.1% Tween-20 (all from Sigma) and then probed with anti-CD3 ζ (sc-1239, Santa Cruz Biotechnology, Santa Cruz, CA) or glyceraldehyde-3-phosphate dehydrogenase (sc-47724, Santa Cruz Biotechnology) 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).

Coculture assays for coculture assays, each CAR was expressed in T cells from the same donor. Biological repeats were done using different donors and data presented in the figures is the average of three to five donors.

Recombinant protein coculture assay. Nontissue culture 24-well plates were precoated with recombinant human IL13R α 1, IL13R α 2, or IL4R α proteins, (R&D Systems) at a final concentration of 500 ng/well. Plates were washed once using Roswell Park Memorial Institute, and CAR or NT T cells were plated. After 24 hours, supernatants were harvested and IFN γ and IL2 release was measured by ELISA per the manufacturer's instructions (R&D Systems).

Cell culture 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. NT T cells served as controls. After 24 hours, culture supernatants were harvested, and the presence of IFN γ and IL2 was determined by ELISA as per the manufacturer's instructions (R&D Systems).

Cytotoxicity assay. Standard chromium (^{51}Cr) release assays were performed as previously described.⁵ 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 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 ^{51}Cr 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: (test release–spontaneous release)/(maximal release–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 previously⁵ 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 7- to 9-week-old mice were anesthetized, the head was shaved and the mice were immobilized in a Cunningham Mouse/Neonatal Rat Adaptor (Stoelting, Wood Dale, IL) stereotaxic apparatus fitted into an E15600 Lab Standard Stereotaxic Instrument (Stoelting), and then scrubbed with 1% povidone-iodine. A 10-mm skin incision was made along the midline. The tip of a 30G $\frac{1}{2}$ 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. 1×10^5 U373.eGFP:ffLuc cells in 2.0 μ l 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 effector cells in 2 μ l 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. Mice were euthanized when the tumor radiance was greater than 1×10^9 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. All *in vitro* experiments were performed at least in triplicate, GraphPad Prism 5 software (GraphPad software, La Jolla, CA) was used for statistical analysis. Measurement data were presented as mean \pm standard deviation. The differences between means were tested by appropriate tests. The significance level used was $P < 0.05$. For the mouse experiments, changes in tumor radiance from baseline at each time point were calculated and compared between groups using *t*-test or Wilcoxon rank-sum test, whichever appropriate. Survival determined from the time of tumor cell injection was analyzed by the Kaplan-Meier method and differences in survival between groups were compared by the Wilcoxon test.

SUPPLEMENTARY MATERIAL

Figure S1. Phenotypic analysis of IL13R α 2-CAR T-cell lines.

Figure S2. Western blot of IL13R α 2-CAR T cells.

Figure S3. IL13R α 2-CAR.LSR.CD28. ζ induces constitutive CD3 ζ phosphorylation.

Figure S4. Cell surface expression of IL13R α 1 and IL13R α 2.

Figure S5. Cell surface expression of IL13R α 2 in brain tumor cell lines.

Figure S6. Generation and characterization of LSR.CD28.41BB. ζ CAR T cells.

Figure S7. Generation of SSR.CD28.41BB. ζ CAR T cells.

Figure S8. FACS analysis of PD-L1 expression on U373 cell surface with and without IFN γ stimulation.

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