

# IL-13R $\alpha$ 2 humanized scFv-based CAR-T cells exhibit therapeutic activity against glioblastoma

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**Chimeric antigen receptor (CAR)-modified T cells have exhibited impressive anti-tumor effects in both B cell malignancies and some types of solid tumors. However, single-chain variable fragment (scFv) of a murine monoclonal antibody will induce immune responses, limit CAR-T cell persistence, and thus increase the risk of relapse. This study successfully constructed a CAR-targeting interleukin-13 receptor  $\alpha$ 2 (IL-13R $\alpha$ 2) according to a murine antibody, and then humanized the scFv sequence to generate another CAR. T cells expressing any of these two CARs demonstrated superior tumor inhibitory effects *in vitro* and in two xenograft mouse models. However, T cells transduced with humanized CAR have an increased expansion and reduced cytokines, including interleukin-6 and interferon- $\gamma$ . The top expressed genes clustered in leukocyte-mediated cytotoxicity, and T cell migration and immunological synapse formation contributed to the anti-glioblastoma (GBM) activity of the humanized CAR. In conclusion, we successfully generated a humanized third-generation CAR-targeting IL-13R $\alpha$ 2 and confirmed its anti-GBM efficacy, which provide a candidate method for clinical GBM treatment.**

## INTRODUCTION

Glioblastoma (GBM) is the most common and aggressive primary brain malignancy in adults.<sup>1</sup> Due to its high morbidity, high mortality, and low cure rates, GBM has caused a great social and medical burden worldwide, and is still incurable with conventional therapies.<sup>2</sup> Therefore, developing novel therapies for GBM is necessary to improve the outcome and prognosis of GBM patients.

Adoptive T cell immunotherapy is a promising strategy for cancer treatment, especially chimeric antigen receptor (CAR)-modified T cell therapy.<sup>3</sup> CARs are synthetic molecules that redirect T cells to eradicate tumors through specific recognition of surface proteins expressed on tumor cells, which consist of an extracellular tumor antigen-binding domain linked to hinge, transmembrane, and intracellular signaling domains.<sup>4–6</sup> T cells expressing CAR (CAR-T cells) can directly identify tumor-associated antigens through single-chain variable fragment (scFv) of the extracellular domain and then, activated by intracellular signal transduction, release multiple cytokines, such as perforin, granzyme, interferon- $\gamma$  (IFN- $\gamma$ ), and tumor necrosis factor (TNF), and induce tumor cell apoptosis.<sup>7,8</sup> Therefore, CAR-T cells act in an MHC-independent manner and ingeniously combine the

abilities of antibody-specific recognition of antigens and cytotoxicity of T lymphocytes.

CAR-T cell therapy was first applied in treatment of hematologic B cell malignancies and exhibited effective and encouraging results.<sup>9,10</sup> Two products (Kymrial and Yescata) have been available for the treatment of hematologic malignancies in the United States and Europe.<sup>11,12</sup> However, CAR-T cell treatment for solid tumors have shown limited anti-tumor activity and is still in the experimental stage. Until recently, several groups have developed different CARs for GBM treatment, including CARs targeting IL-13 receptor subunit  $\alpha$ 2 (IL-13R $\alpha$ 2), human epidermal growth factor receptor 2 (HER2), or epidermal growth factor receptor variant III (EGFRvIII), and these have exhibited encouraging anti-tumor effects.<sup>13–15</sup>

IL-13R $\alpha$ 2 is a monomeric high-affinity interleukin-13 (IL-13) receptor, which is overexpressed by the majority of GBM tumors (>60%) and not expressed at significant levels on normal brain tissue.<sup>16–18</sup> IL-13R $\alpha$ 2 expression is closely associated with that of the mesenchymal GBM subtype and is a prognostic indicator of poor patient survival.<sup>19</sup> This favorable expression profile provides the rationale for continued development of CAR-T cells targeting IL-13R $\alpha$ 2.<sup>20,21</sup> Previous CARs interacting with IL-13R $\alpha$ 2 were mostly derived from murine antibodies.<sup>22</sup> However, murine-derived CARs could induce human anti-mouse antibody responses, which further limit the persistence of the CAR-T cells and increase the risk of relapse.<sup>23</sup>

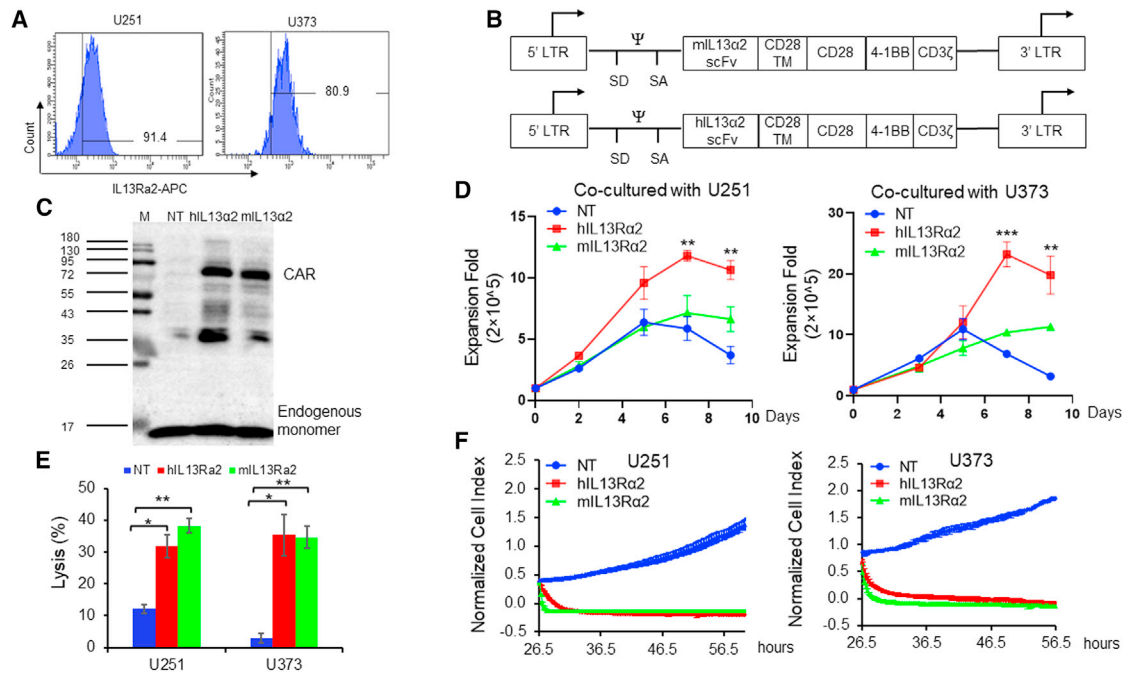
In this study, we generated a CAR derived from a murine antibody targeting IL-13R $\alpha$ 2 and humanized the sequence to form a humanized anti-IL-13R $\alpha$ 2 CAR. We hypothesized that T cells expressing humanized CAR-targeting IL-13R $\alpha$ 2 would exhibit similar tumor cell killing activity, but better expansion and lower cytokine production. We evaluated the expansion, anti-GBM efficacy, and cytokine release of T cells transfected with these two CARs *in vitro* and in xenograft mouse models.

Received 4 August 2021; accepted 7 January 2022;  
<https://doi.org/10.1016/j.omto.2022.01.002>.

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**Figure 1. Developing IL-13R $\alpha$ 2-specific CAR-T cells and evaluating the anti-GBM activity *in vitro***

(A) Flow cytometry examining the cell surface IL-13R $\alpha$ 2 expression of GBM cells. (B) Schematic diagram of the CAR constructions. (C) Immunoblotting examined the CAR transduction. (D) T cell expansion after co-culturing with GBM cells at E:T = 1:1. (E) *In vitro* killing assay at E:T = 1:1. The tumor cell lysis was examined by detecting luciferase activity. (F) Tumor cell killing activity of CAR-T cells was evaluated by real-time cell analysis. Results were analyzed by Student's *t* test. Statistical significance was set at  $p < 0.05$ . \* $p < 0.05$ , \*\* $p < 0.01$ .

## RESULTS

### T cells expressing the humanized third-generation CAR-targeting IL-13R $\alpha$ 2 inhibit GBM cell growth and have a better expansion

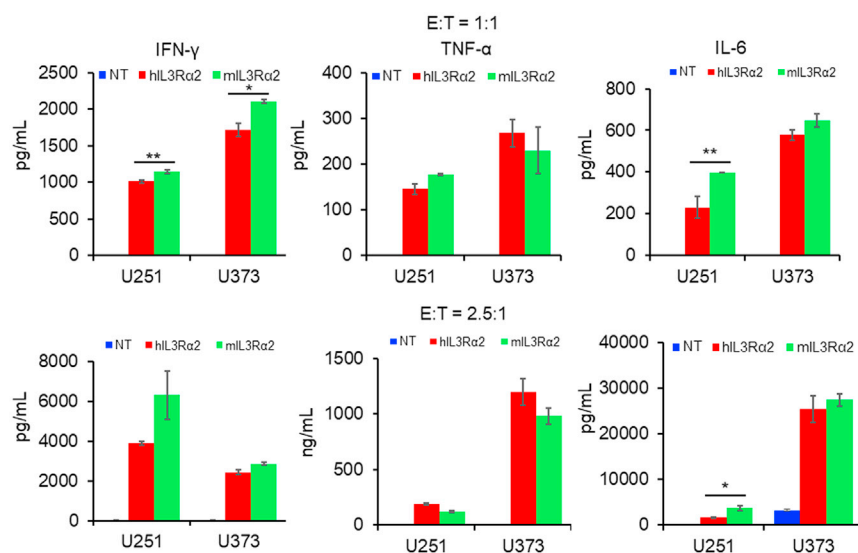
To develop GBM-specific CARs for adoptive T cells therapy, we first confirmed the expression of IL-13R $\alpha$ 2 on the GBM cell surface by flow cytometry. We observed that more than 91.4% U251 and more than 80.9% U373 cells were IL-13R $\alpha$ 2 positive (Figure 1A). Subsequently, we generated the humanized IL-13R $\alpha$ 2 CAR using a typical third-generation design (Figure 1B). The scFv derived from mouse monoclonal antibody targeting IL-13R $\alpha$ 2 or the humanized scFv was connected with the CD28 transmembrane domain. The intracellular signaling domain contains the co-stimulation domain consisting of CD28 and 41BB, and the CD3 $\zeta$  domain (Figure 1B). The CAR transduction was processed using retrovirus, and the efficiency was confirmed by immunoblotting (Figure 1C).

T cell expansion was examined via calculating the cell number after co-culturing with IL-13R $\alpha$ 2-positive GBM cells. As shown in Figure 1D, T cells transduced with humanized CAR-targeting IL-13R $\alpha$ 2 had a 12.3- or 24.6-fold expansion after being co-cultured with U251 or U373 cells separately, compared with mIL-13R $\alpha$ 2 CAR-T cells that had an 8.1- or 11.6-fold expansion. Subsequent *in vitro* kill assays verified the anti-GBM activity of humanized IL-13R $\alpha$ 2 CAR-T cells. As shown in Figure 1E, both kinds of IL-

13R $\alpha$ 2 CAR-T cells lysed more than 30% tumor cells compared with NT cells that lysed 12.1% U251 cells and 2.82% U373 cells at an effector to target (E:T) cell ratio = 1:1. The GBM cell killing activities were similar between these two CAR-T cells at four different E:T ratios (Figure S1). The complete tumor cell killing processes were recorded using a real-time cell growth monitoring system (Figure 1F). The tumor cell growth was repressed by both kinds of CAR-T cells, but not by NT cells. Significantly increased IFN- $\gamma$ , TNF- $\alpha$ , and IL-6 levels were found in the medium from the IL-13R $\alpha$ 2 CAR-T cells compared with NT cells (Figure 2), whereas hIL-13R $\alpha$ 2 CAR-T cells released relatively lower levels of IFN- $\gamma$  and IL-6, when compared with mIL-13R $\alpha$ 2 CAR-T cells (Figure 2).

### T cells expressing CAR-targeting IL-13R $\alpha$ 2 inhibit GBM growth in xenograft mouse models

To further examine the anti-GBM activity of humanized IL-13R $\alpha$ 2 CAR-T cells *in vivo*, we generated the GBM xenograft mouse model with subcutaneous injection of U251-eGFP-Luc into non-obese diabetic/severe combined immunodeficient (NOD-SCID) mice with peritumoral administration of T cells 9 days later (Figure 3A). The tumor growth was then monitored and quantified by using an *in vivo* imaging system (IVIS). As shown in Figures 3B and 3C, the tumor growth was significantly repressed by IL-13R $\alpha$ 2 CAR-T cells compared with the NT group. Meanwhile, IL-13R $\alpha$ 2 CAR-T cell treatment significantly prolonged the survival of tumor-bearing



**Figure 2. T cells expressing humanized CAR-targeting IL-13Rα2 release reduced cytokines**

The *in vitro* killing assay was processed at E:T = 1:1 and 2.5:1 for 8 h and the medium was subjected to ELISA for cytokine detection. Results were analyzed by Student's *t* test. Statistical significance was set at  $p < 0.05$ . \* $p < 0.05$ , \*\* $p < 0.01$ .

mice ( $p = 0.006$ ). Although T cells expressing humanized CAR have better tumor repressing activity, the results were not statistically significant ( $p = 0.13$ ) (Figures 3B–3D).

Subsequently, we evaluated the anti-GBM activity of CAR-T cells by using an intracranial GBM mouse model via U251-eGFP-Luc intracranial implantation, followed by intravenous T cell administration (Figure 4). IL-13Rα2 CAR-T cell treatment repressed the tumor growth (Figures 4B and 4C) and significantly prolonged the survival of tumor-bearing mice (Figure 4D). A similar phenotype has been observed in a U373 cell-generated GBM mouse model (Figure S2A). Intravenous administration of IL-13Rα2 CAR-T cells inhibits the tumor growth in the first 40 days (Figures S2B and S2C). However, the tumors relapsed after day 40, and the survival of tumor-bearing mice was not significantly improved (Figure S2D).

#### Top expressed genes in the humanized third-generation IL-13Rα2 CAR-T cells were functionally enriched in T cell proliferation, migration, and cytotoxicity

To understand the origin of powerful anti-GBM activity of IL-13Rα2 CAR-T cells, we examined the gene expression profile by high-throughput sequencing. There were 23,577 detectable genes in IL-13Rα2 CAR-T cells. The top 100 expressed genes were subjected to GO analysis for three categories: biological process, molecular function, and cellular component. As shown in Figure 5A, five genes (SOS1, CALM1, IL2RB, IL2RG, and UBC) were enriched in MAPK cascade, five genes (CD74, SOS1, ITGAL, MSN, and MYH9) were enriched in leukocyte migration and ten genes were enriched in immune response (CD74, ETS1, TNFRSF1B, GZMA, IL2RG, IL32, LTB, HLA-B, HLA-E, and HLA-DRA). We also observed that five genes (ARL4C, ARHGDI1B, EEF1A1, EEF2, and RAC2) functionally enriched in GTPase activity that may contribute to activated T cell activity (Figure 5B). The subcellular localization analysis indicated that proteins encoded by these

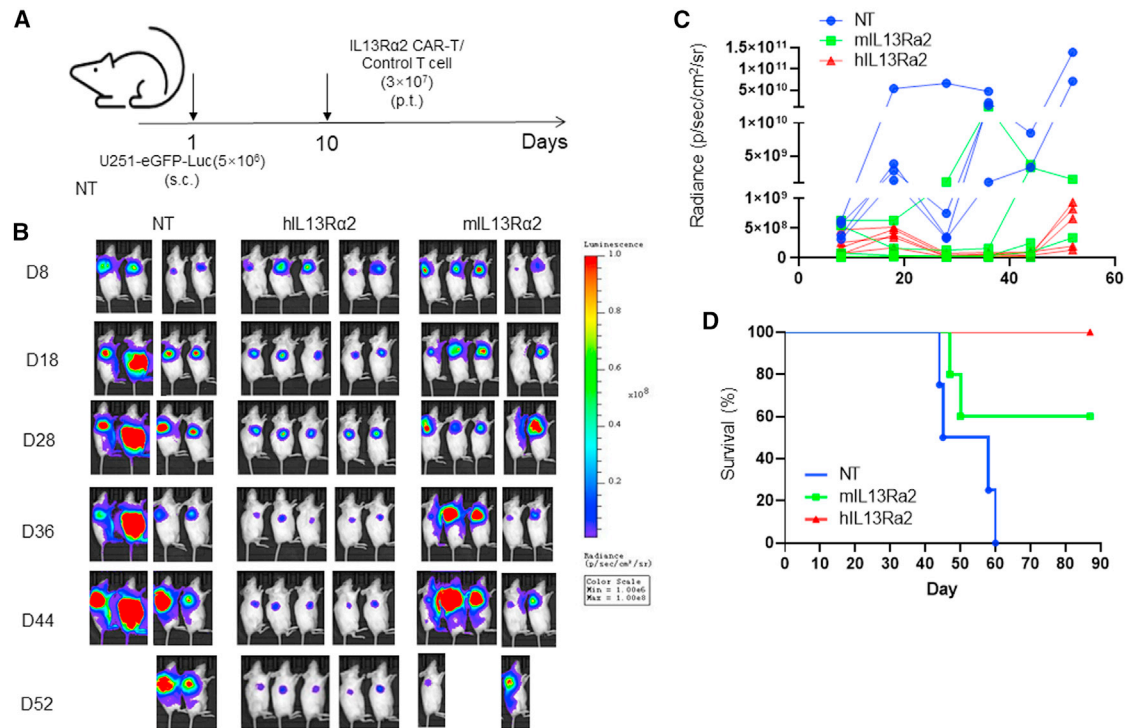
genes evenly distributed in the cell nucleus, cytosol, extracellular matrix, and on the cell membrane (Figure 5C).

Subsequently, we analyzed the interrelations of different pathways in immune system processes. We observed that genes enriched in T cell proliferation, activation, toxicity, migration, and synapse formation pathways (Figure 6). While genes, such as MSN, HLA-E, SOS1, CD74, PTPRC, and RAC2, were key hubs connected these pathways (Figure 6). Finally, we analyzed the interaction of these top expressed genes, and found four main clusters (Figure 7). These clusters include cytoskeleton genes (cluster 1), cytotoxicity-related genes (cluster 2), protein folding and modification genes (cluster 3), and mitochondrial function genes (cluster 4).

#### DISCUSSION

GBM is the most aggressive primary brain malignancy in adults, and is still incurable with conventional therapies.<sup>1,2</sup> Recently, the emerging immune-based technologies exhibited encouraging anti-GBM activity including adoptive T cell therapies. Brown and colleagues reported for the first time that a second-generation autologous CAR-T cells targeting IL-13Rα2 mediated a transient complete response in a patient with recurrent multifocal glioblastoma, with dramatic improvements in quality of life.<sup>13</sup> In this study, we generated a humanized third-generation CAR-targeting IL-13Rα2 and then examined the anti-GBM efficacy *in vitro* and in xenograft mouse models. To our knowledge, this is the first research of the humanized third-generation CAR-targeting IL-13Rα2 for GBM treatment that provides a candidate tool for clinical use.

The first-generation CAR without a co-stimulatory signal domain was first developed to treat cancer, but T cells transduced with these CARs were found to be poorly persistent *in vivo*, which promoted the later incorporation of co-stimulatory signal domains from CD28 or 4-1BB into the CAR intracellular structure. Since the two domains determine different functional properties of CAR-T cells, the third-generation of CARs having these two different functional properties were established. Because mouse antibody-derived CARs will induce immune responses, limit CAR-T cell persistence, and increase the risk of relapse, in this study we developed the humanized third-generation CAR for GBM treatment. Our *in vitro* cell killing result indicated that this humanized third-generation CAR-targeting IL-13Rα2 did not secrete increased IL-6 and expressed dramatic low levels of IL-10.



**Figure 3. T cells expressing humanized CAR-targeting IL-13R $\alpha$ 2 exhibit impressive anti-GBM efficacy in a subcutaneous xenograft mouse model**

(A) Schematic diagram of the generation of GBM xenograft mouse model and the ministration of CAR-T cells. (B) A xenograft mouse model was generated via U251-eGFP-Luc cells subcutaneous injection and followed by the treatment of T cells. The tumor growth was monitored by using the IVIS system. (C) The luciferase signal was quantified by using Living Image® 3.2 software. (D) The overall survival was measured using Kaplan-Meier method, with Cox proportional hazard regression analysis for group comparison. Statistical significance was set at  $p < 0.05$ .

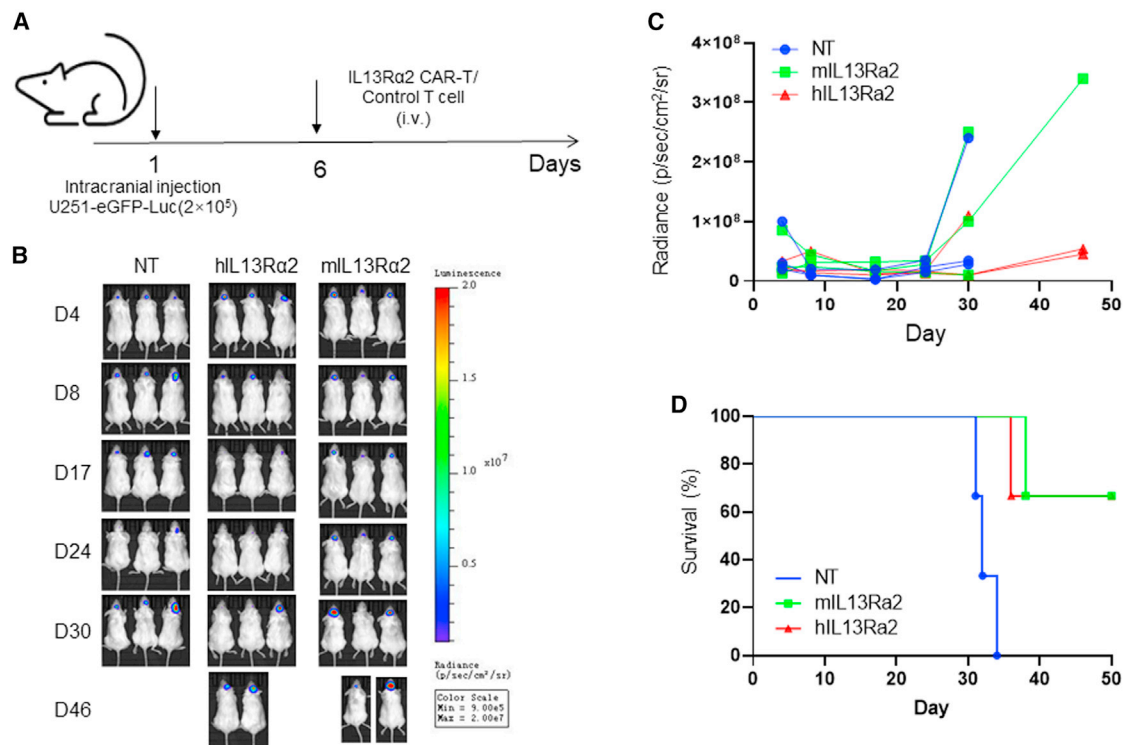
Since IL-6 was identified to be a key driver of CRS,<sup>24</sup> while IL-10 was found to induce T cell dysfunction,<sup>25</sup> our results suggest a powerful anti-GBM potential in patients.

One key reason for lack of progress in the treatment of glioblastoma is extensive intra- and inter-tumor heterogeneity.<sup>26</sup> In this study, the humanized third-generation CAR-targeting IL-13R $\alpha$ 2 had an impressive anti-GBM efficacy in U251 cell-generated mouse models but did not exhibit significant anti-tumor effect in a U373-generated mouse model. This may be a result of lower IL-13R $\alpha$ 2 expression that will escape from the T cell surveillance.

CAR-T cell therapy has been successfully applied in the treatment of hematologic B cell malignancies. However, CAR-T cell treatment for solid tumors has limited anti-tumor activity. One of the main causes is the suppressive tumor microenvironment, because activated T cells must adapt their metabolism to meet the energetic demands associated with rapid proliferation and effector function. The aberrant metabolic milieu in tumor microenvironments, a result of the high metabolic activity of tumor cells and dysfunctional tumor vasculature, inhibits T cell effector functions and promotes a defective T cell state.<sup>27,28</sup> The blood-brain barrier (BBB) is also a key factor that limits the efficacy of CAR-T cells. In this study, both U251

and U373 generated intracranial tumors relapsed 40 days after treatment, which may be the result of tumor microenvironment and reduced T cell persistence. So, further study will be focused on optimizing the CAR structure to increase the persistence of T cells and combination with other treatments to overcome the BBB for GBM treatment.

High-throughput sequence data unveiled the origin of powerful anti-GBM activity of this kind of humanized third-generation CAR-T cells. Genes consisting of a cytoskeleton, contributing to cytotoxicity, mitochondrial function, and protein folding, were found highly expressed in CAR-T cells, such as MYH9 and FLNA. MYH9 was the sole class II myosin expressed in T cells and required for T cell motility but is dispensable for synapse formation.<sup>29</sup> FLNA provides an important link between the integrin and the actin cytoskeleton, which is required for T cell adhesion and trafficking.<sup>30</sup> These highly expressed genes represent the high migration ability of T cells. The initiation of T cell activation results in the reprogramming of cellular metabolic processes to facilitate proliferation, differentiation, and production of effector molecules, such as cytokines.<sup>31</sup> The highly expressed mitochondrial genes and protein folding-related genes contribute to the activation of T cells.



**Figure 4. T cells expressing humanized CAR-targeting IL-13R $\alpha$ 2 exhibit impressive anti-GBM efficacy in an intracranial mouse model**

A xenograft mouse model was generated via U251-eGFP-Luc cell intracranial injection. Tumor growth was monitored using the IVIS system. The overall survival was measured using the Kaplan-Meier method, with Cox proportional hazard regression analysis for group comparison. Statistical significance was set at  $p < 0.05$ .

In conclusion, we successfully constructed a humanized third-generation CAR-targeting IL-13R $\alpha$ 2 and then examined its anti-GBM activity *in vitro* and in xenograft mouse models, which provide a candidate tool for clinical GBM treatment.

## MATERIALS AND METHODS

### Cell lines

The human GBM cell lines U251 and U373 were kindly provided by Dr W.K. Cavenee. The retrovirus packaging cell line PG13 was purchased from the American Type Culture Collection. U251 and U373 cells expressing eGFP and firefly luciferase were generated by retroviral infection. All these cells were maintained in Dulbecco's modified Eagle's medium (Lonza), containing 10% fetal bovine serum (Biosera) and 10,000 IU/mL penicillin/10,000  $\mu$ g/mL streptomycin (EallBio Life Sciences).

### Generation of retroviral vectors encoding humanized IL13-BB $\zeta$ CAR-T cells

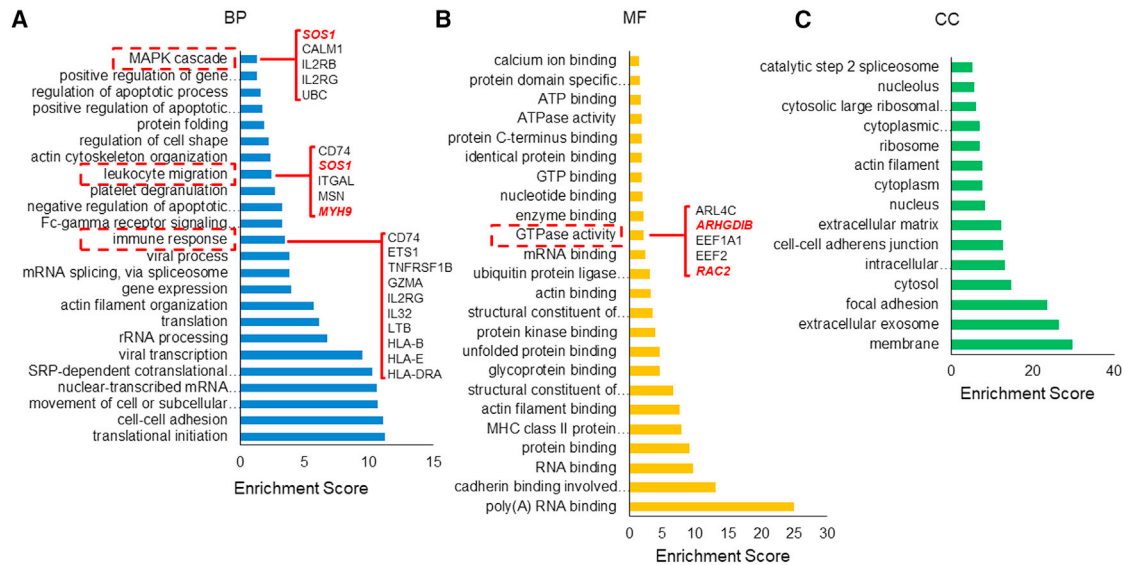
IL-13R $\alpha$ 2-scFv coding sequence was synthesized by GeneArt (Invitrogen) and then subcloned into the SFG retroviral vector. Transfection experiments were performed using a Calcium Phosphate Transfection Kit (Sigma) according to the instructions with minor modifications.

### Generation of CAR-T cells

Human peripheral blood mononuclear cells (PBMCs) from healthy donors were isolated by gradient centrifugation using Ficoll solution (GE Healthcare). T cells in PBMCs were stimulated with anti-CD3/CD28 T cell Activator Dynabeads (Invitrogen). After 48 h of bead activation, T cells were transduced with retroviral supernatants by centrifugation on Retronectin (Takara)-coated plates. On day 7, the transduction efficiency was measured through detecting CD3 $\zeta$  level by immunoblotting. T cells were cultured in X-VIVO-15 medium with 5% human AB serum (Sigma), 100 U/mL IL-2, 100 U/mL penicillin, and 100  $\mu$ g/mL streptomycin (EallBio Life Sciences). This study was approved by the institutional review committee of Beijing Shijitan Hospital and informed consent was obtained from all participants.

### Xenograft mouse model with subcutaneous glioma cells injection

Six- to 8-week-old NOD-SCID mice were purchased from Charles River Laboratories. The right flanks of female NOD-SCID mice were injected with  $5 \times 10^6$  U251-eGFP-Luc cells to construct the xenograft mouse model. Ten days after tumor cell injection,  $3 \times 10^7$  CAR-T cells were injected directly into the tumor. Tumor development was monitored using IVIS (IVIS, Xenogen, Alameda, CA, USA), and the mice were sacrificed when the diameter of the tumor reached 20 mm.



**Figure 5. GO analysis of the top 100 expressed genes in CAR-T cells**

T cells transfected with humanized CAR-targeting IL-13R $\alpha$ 2 were subjected to high-throughput sequencing to examine the gene expression profile. The top 100 expressed genes in CAR-T cells were subjected to GO analysis using an online bioinformatics tool: DAVID Bioinformatics Resources 6.8: <https://david.ncifcrf.gov>. Fisher's exact test was used for the gene-enrichment analysis. The GO terms of the three categories, BP(A), CC(B) and MF(C) were shown separately. BP, biological process; CC, cellular components; MF, molecular function.

### Xenograft mouse model with glioma cells intracranial implantation

Six- to 8-week-old NOD-SCID mice were anesthetized with a ketamine/xylazine cocktail solution. Animals were secured in a stereotaxic head frame, a 1 cm midline scalp incision was made, and  $2 \times 10^5$  U373-eGFP-Luc cells in 5  $\mu$ L PBS were injected into the left striatum (coordinates: 2.5 mm lateral and 0.5 mm posterior to the bregma) through a burr hole in the skull using a 10- $\mu$ L BD syringe to deliver tumor cells to a 3.5-mm intraparenchymal depth. The burr hole in the skull was sealed with bone wax, and the incision was closed using medical adhesive glue (COMPONT). Two weeks after the tumor cells injection,  $3 \times 10^7$  CAR-T cells were injected through the tail vein and tumor growth was monitored using the IVIS *in vivo* imaging system (IVIS, Xenogen). All experiments with mice were approved by the Beijing Shijitan Hospital institutional review board.

### Flow cytometry analysis

Flow cytometry was performed on a FACSCanto Plus instrument (BD Biosciences) and FlowJo v.10 (FlowJo, LLC) was used for data analysis.

Antibody list: APC-conjugated CD3 antibody (BD Pharmingen 555340); V450-conjugated CD4 antibody (BD Pharmingen 560650); PE-Cy7-conjugated CD8 antibody (BD Pharmingen 560960); FITC-labeled goat anti-mouse IgG(H+L) antibody (Sigma); FITC-conjugated IL13Ra antibody (BD Biosciences); and APC-labeled anti-CD107a-APC antibody (BD Biosciences).

### Cytotoxicity assay

IL-13R $\alpha$ 2 CAR-T cells were co-cultured with GBM cells at an E:T ratio from 1:1 to 10:1 in a 24-well plate. Non-transduced T cells were used as negative control. After 24 h, cells were collected and stained with the tumor cell surface marker to evaluate the cytotoxicity of CAR-T cells by flow cytometry.

### Cytokine production assays

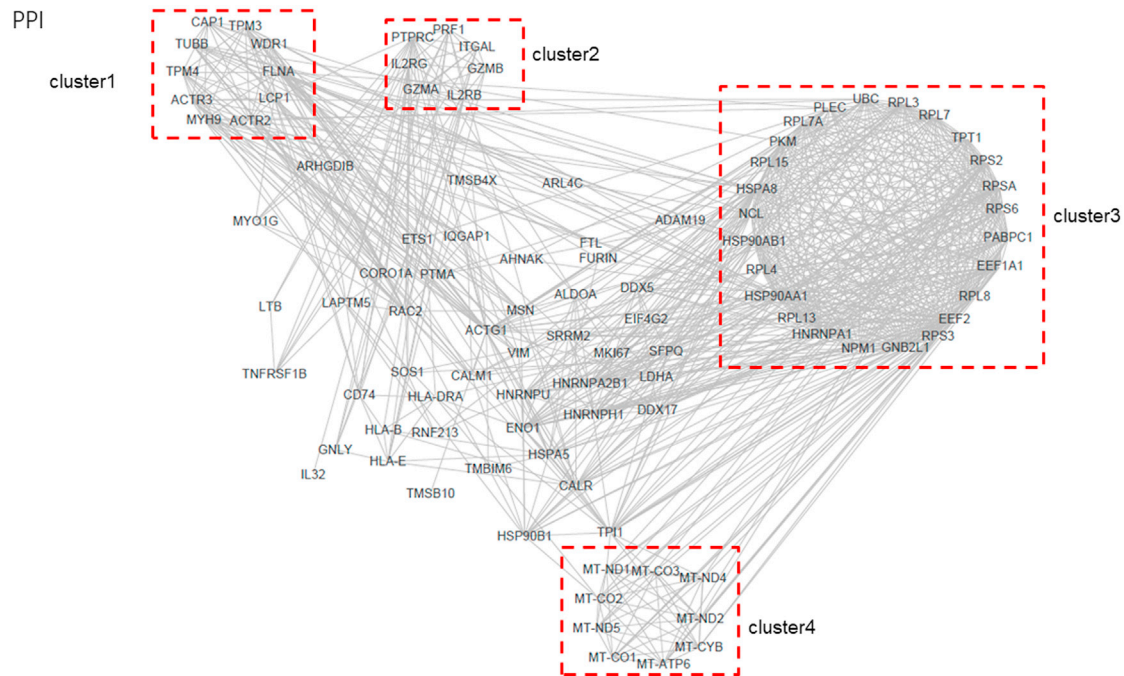
CAR-T cells were co-cultured with the human GBM cell lines at an E:T = 1:1 for 24 h. The expression of human IFN- $\gamma$ , TNF- $\alpha$ , IL-6, and IL-10 in the supernatant from co-culture cells was assessed using commercial Cytometric Bead Array Kits (BD Biosciences) according to the procedure of the manufacturer. The level of human IFN- $\gamma$  was also evaluated using DuoSet ELISA kits (R&D, Minneapolis, MN, USA) following the manufacturer's instructions.

For intracellular cytokine staining assay, effect T cells were co-cultured with target cells at a 10:1 ratio in 24-well microplates. Every well contained 1 mL T cell medium and 1  $\mu$ L Golgi Stop (BD cat no. 554724). After 6 h, the cells were harvested and incubated with anti-human IFN- $\gamma$  (BD Pharmingen, clone B27) antibodies and then measured through flow cytometry analysis.

### Real-time cell analysis

The xCELLigence real-time cell analysis (RTCA) system (Roche Applied Science, Basel, Switzerland) was used to evaluate the proliferation/cytotoxicity of CAR-T cells. This system is based on electrical impedance readings of a gold plate sensor electrode at the bottom of





**Figure 7. rotein-protein interaction analysis of the top 100 expressed genes**

Protein-protein interaction analysis of the top 100 expressed genes in CAR-T cells.

## AUTHOR CONTRIBUTIONS

Conception and design, X.Z.; data acquisition, C.X., Z.A., Y.H., Y.B., and C.Z.; data analysis, C.X., Y.H., and Y.B.; funding acquisition, X.Z.; resources, C.X.; software, Y.H. and Z.A.; writing – original draft, C.X. and Y.H.; writing – review & editing, X.Z.; final approval, C.X., Y.B., Y.H., C.Z., Z.A., and X.Z.

## DECLARATION OF INTERESTS

The authors declare no competing interests.

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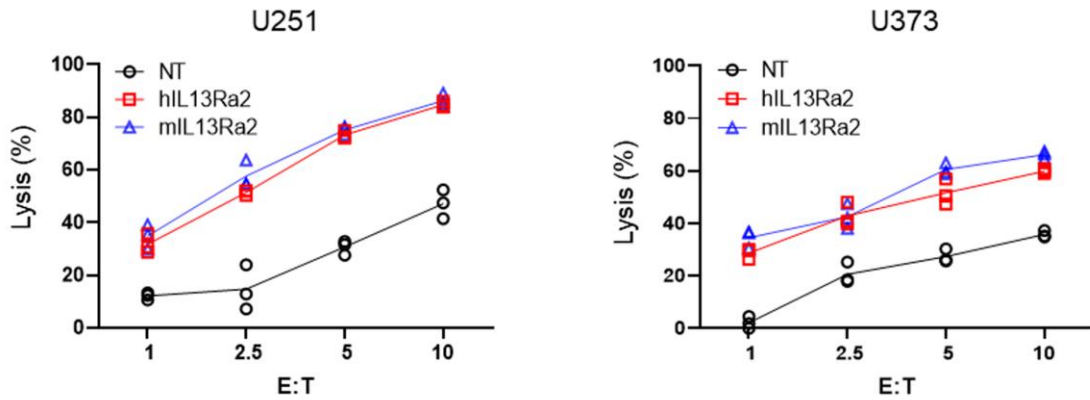
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**Supplemental information**

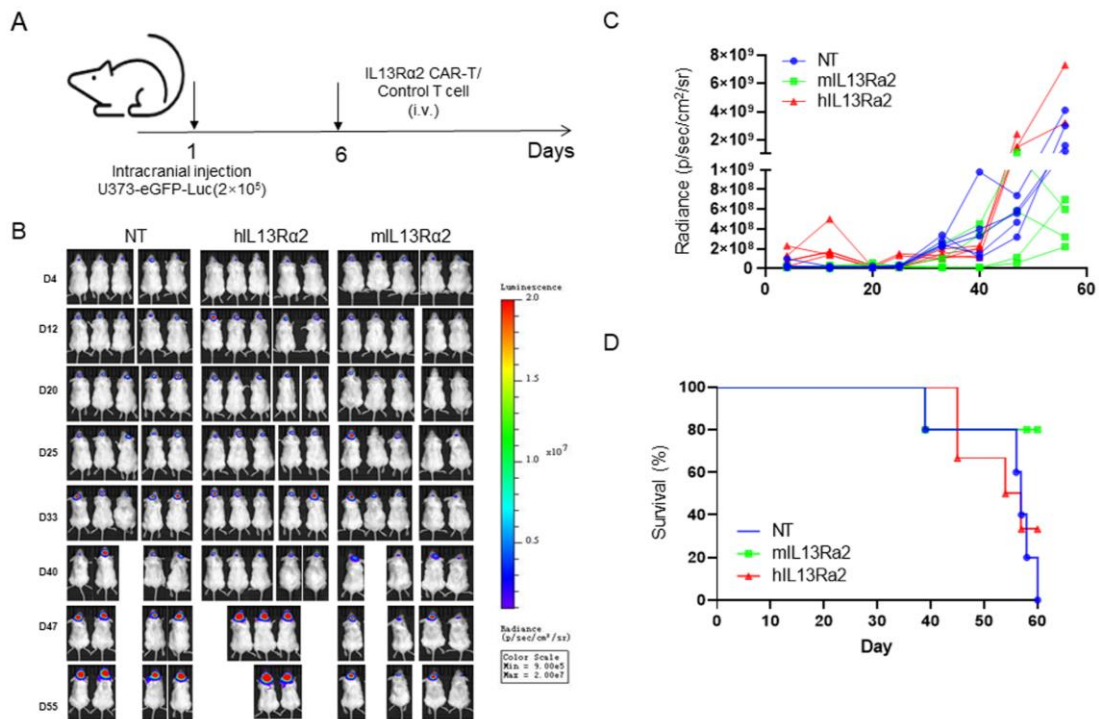
**IL-13R $\alpha$ 2 humanized scFv-based CAR-T cells exhibit  
therapeutic activity against glioblastoma**

**Chang Xu, Yue Bai, Zhijing An, Yi Hu, Can Zhang, and Xiaosong Zhong**



**Fig. S1. In vitro killing assay**

In vitro killing assays were processed at E:T ratio from 1:1 to 10:1 with non-transduced T cells as negative control. The tumor cell lysis was examined by detecting luciferase activity.



**Fig. S2. T cells expressing humanized CAR targeting IL13Ra2 do not show significant suppression activity in U373 cell generated intracranial mouse model.**

Xenograft mouse model was generated via U373-eGFP-Luc cell intracranial injection. The tumor growth was monitored by IVIS system. The overall survival was measured using the Kaplan–Meier method, with Cox proportional hazard regression analysis for group. comparison. Statistical significance was set at  $p < 0.05$ .