Molecular Therapy
Original Article

Adoptive Transfer of IL13Rα2-Specific Chimeric Antigen Receptor T Cells Creates a Pro-inflammatory Environment in Glioblastoma

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In order to fully harness the potential of immunotherapy with chimeric antigen receptor (CAR)-modified T cells, pre-clinical studies must be conducted in immunocompetent animal models that closely mimic the immunosuppressive malignant glioma (MG) microenvironment. Thus, the goal of this project was to study the *in vivo* fate of T cells expressing CARs specific for the MG antigen IL13Rα2 (IL13Rα2-CARs) in immunocompetent MG models. Murine T cells expressing IL13Rα2-CARs with a CD28.ζ (IL13Rα2-CAR.CD28.ζ) or truncated signaling domain (IL13Rα2-CAR.Δ) were generated by retroviral transduction, and their effector function was evaluated both *in vitro* and *in vivo*. IL13Rα2-CAR.CD28.ζ T cells' specificity toward IL13Rα2 was confirmed through cytokine production and cytolytic activity. *In vivo*, a single intratumoral injection of IL13Rα2-CAR.CD28.ζ T cells significantly extended the survival of IL13Rα2-expressing GL261 and SMA560 glioma-bearing mice; long-term survivors were resistant to re-challenge with IL13Rα2-negative and IL13Rα2-positive tumors. IL13Rα2-CAR.CD28.ζ T cells proliferated, produced cytokines (IFNγ, TNF-α), and promoted a phenotypically pro-inflammatory glioma microenvironment by inducing a significant increase in the number of $CD4^+$ and $CD8^+$ T cells and $CD8\alpha^+$ dendritic cells and a decrease in $Ly6G^+$ myeloid-derived suppressor cells (MDSCs). Our data underline the significance of CAR T cell studies in immunocompetent hosts and further validate IL13Rα2-CAR T cells as an efficacious therapeutic strategy for MG.

INTRODUCTION

Malignant gliomas (MGs) are the most common and aggressive group of high-grade primary adult brain tumors. The current standard of care treatment, which includes surgical resection, radiation, and chemotherapy, extends the survival of patients to only 14.6 months.[1] MGs are virtually incurable due to their heterogeneity, immune evasion, infiltrative nature, and protection by the blood-brain barrier (BBB).[2–4] Thus, new therapeutic modalities directed at eliminating MGs are urgently needed. T cells engineered to express chimeric antigen receptors (CARs) have shown outstanding efficacy against hematological malignancies and offer great promise for the treatment of glioma.[5–7] This immunotherapy harnesses the ability of T cells to kill malignant cells by recognizing tumor-associated antigens (TAAs), or antigens overexpressed by cancer cells. CAR T cells recognizing interleukin 13 receptor α2 (IL13Rα2),[8–12] human epidermal growth factor receptor 2 (HER2),[13,14] epidermal growth factor variant III (EGFRvIII),[15] and erythropoietin-producing hepatocellular carcinoma A2 (EphA2)[16] have been evaluated already in pre-clinical models for glioblastoma or in phase I clinical trials.

CAR T cells targeting IL13Rα2 are emerging as an especially promising form of adoptive immunotherapy,[10] as IL13Rα2 is almost exclusively expressed in cancer and not in normal cells (with an exception of the testis).[17] In MG, the expression of IL13Rα2 is present in 50%–80% of cases.[18–20] IL13Rα2 is also directly associated with an increased malignancy grade and aggressive mesenchymal type and is inversely correlated with patient survival.[21] Furthermore, it has been shown that glioma-initiating cells express IL13Rα2.[22] While IL13Rα2 was previously thought to be a decoy receptor, the crucial role of IL13Rα2 in cancer invasiveness continues to emerge,[23–27] further validating IL13Rα2 as a target antigen.

One current IL13Rα2-targeted approach utilizes modified IL13 polypeptide variants known as zetakines for the CAR T cell-binding domain. Although zetakine CAR T cells demonstrate high affinity for IL13Rα2 binding, their interaction with the ubiquitously expressed IL13Rα1[8,28] remains a concern due to potential off-target toxicity. Therefore, we engineered a single-chain variable antibody fragment (scFv47)[29] derived from a well-characterized IL13Rα2-specific monoclonal antibody (mAb) developed in our laboratory.[30] scFv47 selectively binds IL13Rα2, avoids binding IL13Rα1, and successfully directs fiber-modified adenovirus or CAR T cells to IL13Rα2-expressing glioma cells.[9]

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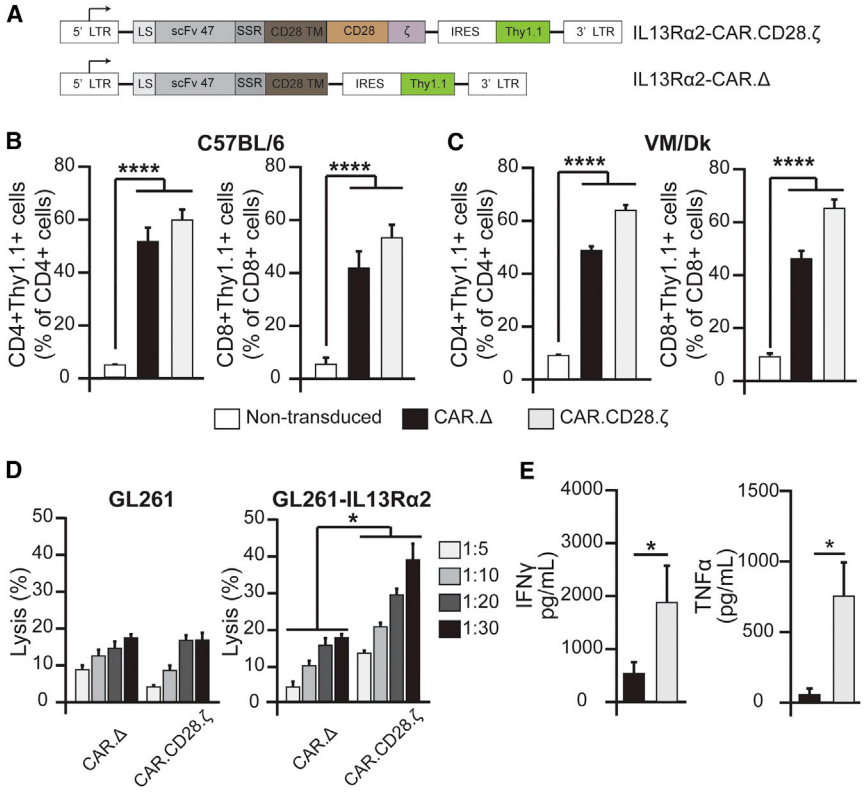

Figure 1. IL13Rα2-CAR.CD28.ζ T Cells Recognize and Kill GL261 Glioma Cells Expressing IL13Rα2

(A) IL13Rα2-CAR.CD28.ζ and IL13Rα2-CAR.Δ murine CAR constructs were designed as described in the Materials and Methods section. (B and C) Flow cytometry analysis of Thy1.1 expression in $CD3^+$ T cells from C57BL/6 (B) and VM/Dk (C) mice was performed at day 5 post-viral-transduction ($n \geq 3$,****$p \leq 0.0001$, one-way ANOVA). (D) IL13Rα2-CAR.CD28.ζ and IL13Rα2-CAR.Δ T cells were tested for their cytotoxic activity against Gl261 and GL261-IL13Rα2 glioma cells in a standard 4-hr ^{51}Cr release assay. IL13Rα2-CAR.CD28.ζ T cells killed GL261-IL13Rα2, but not GL261 glioma cells at all tested target:effector (T:E) ratios (1:5, 1:10, 1:20, 1:30) as compared to control IL13Rα2-CAR.Δ T cells ($n \geq 6$, *$p \leq 0.05$ for two-way ANOVA). (E) The IFNγ and TNF-α production were measured by ELISA after 24 hr with IL13α2-stumulation. ($n \geq 3$, *$p \leq 0.05$, Student's t test). CAR abbreviations: CAR.Δ, IL13Rα2-CAR.Δ; CAR.CD28.ζ, IL13Rα2-CAR.CD28.ζ. Data are presented as mean ± SEM.

CAR T cells directed with scFv47 to IL13Rα2 expressed in human glioma xenografts have demonstrated significant survival benefit in immune-deficient mice.[9] While it validated the functionality of different IL13Rα2 CAR constructs and their therapeutic potentials, this study did not address the behavior of CAR T cells in the context of the immunosuppressive microenvironment, a hallmark of MGs.[31] A recent phase I clinical trial of CAR T cells in patients with MGs suggested that the endogenous immune response significantly contributes to the therapeutic outcome of CAR T cell therapy, supporting the necessity of pre-clinical CAR T cell studies in syngeneic models of disease.[10] Understanding how CAR T cells interact with the host immune system to influence the glioma microenvironment is thus critical for the development of successful CAR T cell therapies.

Here, we analyzed IL13Rα2-CAR T cell-mediated cytotoxicity, their persistence within the tumor, and their influence on the tumor microenvironment in a syngeneic model of glioblastoma. We show that IL13Rα2-CAR T cells can efficiently kill MGs *in vitro* and *in vivo*. Further, IL13Rα2-CAR T cells positively modulate the immune landscape of glioma-bearing mice, likely improving their anti-tumor activity.

RESULTS

IL13Rα2-CAR T Cells Recognize and Kill IL13Rα2-Expressing Glioma Cells *In Vitro*

We have previously reported that mAb IL13Rα2 (clone 47) binds with a high affinity to human, but not murine, IL13Rα2.[30] The single chain (scFv47)[29] shares the epitope of the parental antibody and does not interact with murine IL13Rα2. In order to understand how murine T cells expressing IL13Rα2-CARs interact with the tumor microenvironment in an immune-competent glioma model, we generated IL13Rα2-CARs with a murine CD28 transmembrane and CD28.ζ endodomain (Figure 1A). An IL13Rα2-CAR with a truncated intracellular signaling domain, IL13Rα2- CAR.Δ, was generated as a control CAR. Activated $CD3^+$ T cells were transduced with VSV-G-pseudotyped retroviral particles encoding the respective CAR and Thy1.1. Transduction efficiency was determined by staining T cells for Thy1.1 expression. On average, 40%–60% of $CD3^+CD4^+$ and 40%–65% of $CD3^+CD8^+$ T cells were genetically modified as judged by Thy1.1 expression (Figures 1B and 1C). To determine the functionality of IL13Rα2-CAR.CD28.ζ T cells, we performed cytotoxicity and co-culture assays. IL13Rα2-CAR.CD28.ζ T cells readily killed GL261 modified to express human IL13Rα2 (GL261-IL13Rα2; Figure S1A), but not parental GL261 cells in a standard 4-hr chromium-51 (^{51}Cr) release assay. In contrast, IL13Rα2-CAR.Δ T cells killed neither GL261-IL13Rα2 nor GL261 (Figure 1D). IL13Rα2-CAR.CD28.ζ or IL13Rα2-CAR.Δ T cells were stimulated with IL13Rα2-expressing cells to determine cytokine production by ELISA. After 24 hr, production of IFNγ and TNF-α was significantly higher in IL13Rα2-CAR.CD28.ζ T cells than IL13Rα2-CAR.Δ T cells (Figure 1E), demonstrating antigen-specific CAR T cell activation.

Generation of Immunocompetent Mouse Models of Glioblastoma Expressing Human IL13Rα2

In order to conduct our studies in an immunocompetent host, we developed two syngeneic MG mouse models in which murine GL261 and SMA560 glioma cell lines were modified to stably express human IL13Rα2. Flow cytometric analysis of IL13Rα2 expression on the cell

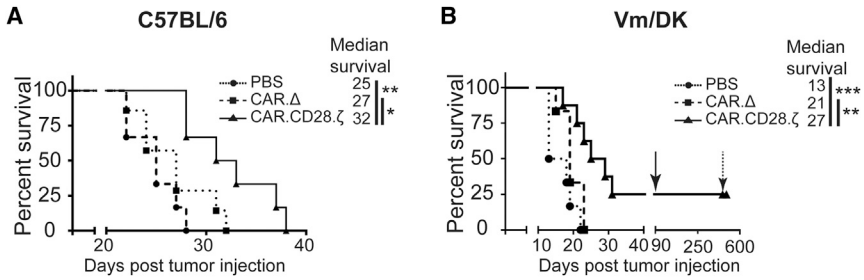

Figure 2. IL13Rα2-CAR.CD28.ζ T Cells Have Anti-glioma Activity in Mice Bearing IL13Rα2-Expressing Glioma

4.0×10^5 GL261-IL13Rα2 or 7.5×10^4 SMA560-IL13Rα2 glioma cells were intracranially injected into C57BL/6 and VM/Dk mice, respectively. Seven days later, animals were treated with a single intratumoral (i.t.) transplantation of PBS, 1.5×10^6 IL13Rα2-CAR.Δ T cells, or 1.5×10^6 IL13Rα2-CAR.CD28.ζ T cells. (A) IL13Rα2-CAR.CD28.ζ T cells significantly extended the survival of C57BL/6 animals from 25 to 32 days. ($n \geq 6$–8, $^*p \leq 0.05$, $^{**}p \leq 0.01$, Mantel-Cox test). (B) IL13Rα2-CAR.CD28.ζ T cells significantly extended the survival of VM/Dk glioma-bearing mice as compared to PBS and control IL13Rα2-CAR.Δ T cells ($n \geq 6$–8, $^*p \leq 0.05$, $^{**}p \leq 0.01$, Mantel-Cox test). Twenty-five percent of animals treated with IL13Rα2-CAR.CD28.ζ T cells survived for a prolonged time period and were re-challenged with 0.75×10^5 SMA560-IL13Rα2 glioma cells by an injection contralateral to the original tumor implantation hemisphere at day 90 as indicated by arrow. Eleven months later, animals were re-challenged again with 0.75×10^5 SMA560 cells as indicated by dashed arrow. While control animals ($n = 4$) injected in parallel with SMA560 cells succumbed to the disease (data not shown) within 3 weeks, none of the re-challenged animals developed tumors, suggesting the development of immunity against glioma ($n \geq 6$–8, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$, Mantel-Cox test).

surface revealed that 92% ± 0.3% of GL261 and 91% ± 0.9% of SMA560 cells were positive for IL13Rα2 *in vitro* (Figure S1A). Furthermore, analysis of excised tumor tissue confirmed that the expression of IL13Rα2 is maintained *in vivo*, albeit at lower levels than in *in vitro* (Figure S1B). Next, we determined the growth of GL261-IL13Rα2 and SMA560-IL13Rα2 tumors after intrancranial injection into their syngeneic hosts (C57BL/6 and VM/Dk mice, respectively). There were no differences observed in the survival of animals for either cell line in comparison to control parental cell lines, supporting the suitability of these models for our studies (Figure S1C). Collectively, these data indicate that both GL261-IL13Rα2 and SMA560-IL13Rα2 are viable and complementary syngeneic models of MG to study IL13Rα2-CAR T cells.

IL13Rα2-CAR T Cells Have Anti-glioma Activity in Two Immune-Competent Glioma Models

The anti-glioma activity of IL13Rα2-CAR.CD28.ζ T cells was evaluated in the GL261-IL13Rα2 and SMA560-IL13Rα2 immune-competent glioma models. On day 7 post-intracranial glioma cell injection, mice received an intratumoral (i.t.) injection of IL13Rα2-CAR.CD28.ζ or IL13Rα2-CAR.Δ T cells; PBS-injected mice served as controls. Mice that received IL13Rα2-CAR.CD28.ζ T cells had a significant survival benefit compared to PBS and IL13Rα2-CAR.Δ T cell-treated mice (Figures 2A and 2B). In addition, IL13Rα2-CAR.Δ T cell-treated mice had a survival advantage in comparison to PBS-treated mice, indicating that IL13Rα2-CAR.Δ T cells have limited therapeutic benefit. In the SMA560-IL13Rα2 model, 25% of mice survived long-term following IL13Rα2-CAR.CD28.ζ T cell therapy, while no mice survived in the GL261-IL13Rα2 model. Long-term survivors were re-challenged by injecting SMA560-IL13Rα2 cells into the contralateral brain and, after 11 months, injected again with SMA560 cells. While control animals succumbed to the disease, none of the re-challenged mice developed glioma, indicating the development of sustained long-term anti-glioma immunity.

IL13Rα2-CAR T Cells Persist and Expand in IL13Rα2-Expressing GL261 Glioma-Bearing Mice

In order to clearly differentiate between adoptively transferred and host T cells and determine if IL13Rα2-CAR.CD28.ζ T cells persist in the glioma environment, we utilized $CD3^+CD45.1^+$ cells to generate IL13Rα2-CAR.CD28.ζ and control transduced T cells for subsequent analysis in CD45.2 C57BL/6 mice bearing GL261-IL13Rα2 glioma. We observed robust persistence of adoptively transferred CAR T cells at 3 and 7 days in the brain after i.t. injection of T cells, corresponding to 10 and 14 days of tumor development (Figure 3A). While the number of IL13Rα2-CAR.CD28.ζ T cells was significantly higher than control IL13Rα2-CAR.Δ $CD3^+CD8^+$ T cells at 3 days (respectively, $5.1 \pm 2.7 \times 10^3$ versus $1.5 \pm\pm 0.8 \times 10^3$) and at 7 days (respectively, $6.7 \pm 4.0 \times 10^3$ versus $3.9 \pm 3.1 \times 10^3$), there was no statistical difference determined in the persistence of $CD3^+CD4^+$ IL13Rα2-CAR.CD28.ζ and IL13Rα2-CAR.Δ T cells (Figure 3A). Furthermore, we were able to detect only a small number of $CD3^+CD8^+$, but not $CD3^+CD4^+$ CAR T cells in the brain of animals prior to euthanasia (Figure S2). Next, we determined if the persistence of IL13Rα2-CAR.CD28.ζ T cells was the result of antigen-dependent proliferation. Indeed, IL13Rα2-CAR.CD28.ζ CAR T cells demonstrated more robust proliferation than IL13Rα2-CAR.Δ T cells at 3 days post-i.t.-delivery (Figure 3B). By day 7, the vast majority of IL13Rα2-CAR.CD28.ζ T cells were in their highest proliferative state (Figure 3B). These data demonstrate that IL13Rα2-CAR.CD28.ζ T cells are capable of surviving and expanding in the immunosuppressive glioblastoma environment.

IL13Rα2-CAR $CD8^+$ T Cells Produce Pro-inflammatory Cytokines in an Antigen-Dependent Fashion and Are Not Exhausted within the Glioma Environment

To determine the production of pro-inflammatory cytokines, IFNγ and TNF-α, by IL13Rα2-CAR.CD28.ζ T cells *in vivo* in response to antigenic stimulation, CAR T cells were isolated and analyzed at days 3 and 7 post-i.t.-injection in animals bearing GL261-IL13Rα2 tumors. A robust difference in cytokine production between therapeutic and control CAR T cells was observed at both time points (Figures 4A and 4B). Cytotoxic T cells characterized by the secretion of pro-inflammatory cytokines are likely to contribute an additional mechanism of CAR T cell anti-glioblastoma functionalities *in vivo*.

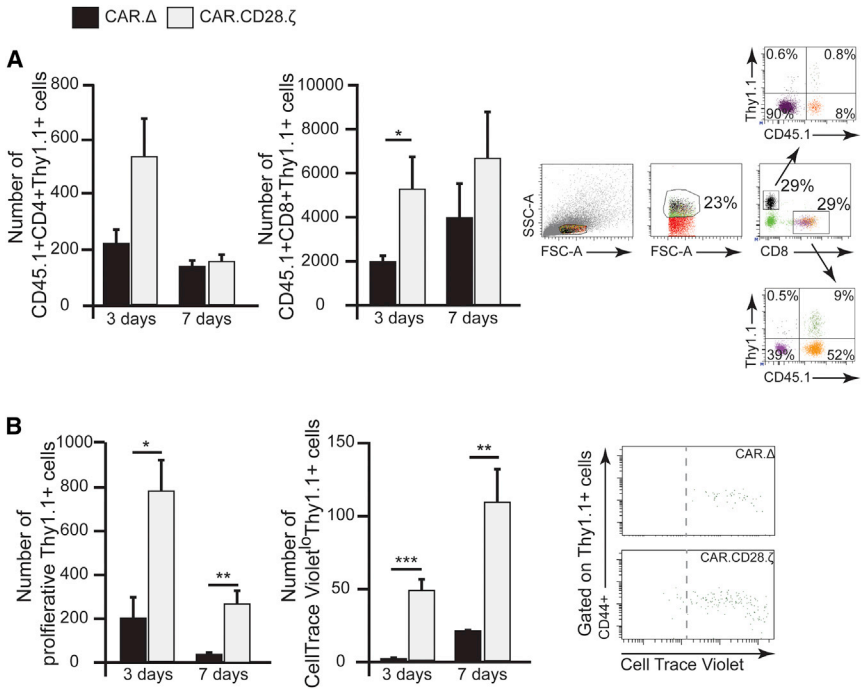

Figure 3. IL13Rα2-CAR.CD28.ζ T Cells Persist and Proliferate in the Brains of Glioma-Bearing Mice

(A) In order to clearly distinguish between host and adoptively transferred cells, $CD3^+$ T cells from CD45.1 mice were utilized to generate IL13Rα2-CAR.CD28.ζ and control IL13Rα2-CAR.Δ T cells and i.t. injected into CD45.2 mice bearing GL261-IL13Rα2 glioma. Gating strategy is presented in the right panel. Quantitative analysis of $CD4^+CD45.1^+Thy1.1^+$ and $CD8^+CD45.1^+$ $Thy1.1^+$ T cells' populations revealed a 2.5-fold higher number of $CD8^+$ CARs at day 3, but not at day 7, in the brain of animals treated with therapeutic CAR T cells as compared to control CAR T cells ($n \geq 4$, *p = 0.02, Student's t test). A similar trend was observed for $CD4^+$ CARs but did not reach significance. (B) For analysis of proliferation, CAR T cells were stained with CellTrace Violet prior to i.t. implantation. Consistent with a higher number of T cells treated with IL13Rα2-CAR.CD28.ζ, the total number of proliferating $CD8^+$ cells was (1) four times higher in animals treated with IL13Rα2-CAR.CD28.ζ than those treated with IL13Rα2-CAR.Δ T cells at 3 days and (2) eight times higher at 7 days after i.t. injection. A more robust proliferation in IL13Rα2-CAR.CD28.ζ compared to IL13Rα2-CAR.Δ T cells is noticeable, and the vast majority of IL13Rα2-CAR.CD28.ζ T cells were in the highest proliferative state, as determined by Cell Trace Violet detection using flow cytometry ($n \geq 6$, *p = 0.02, **p = 0.001, ***$p < 0.001$, Student's t test). The right panel is an example of the gating strategy. Data are presented as mean ± SEM.

Repeated antigenic stimulation has been shown to trigger exhaustion of CAR T cells, limiting their therapeutic activity. However, to date, it remains unknown whether CAR T cells become exhausted within gliomas in immunocompetent hosts. Thus, we analyzed the expression of the following exhaustion markers in CAR T cells at 3 and 7 days after i.t. injection: programmed cell death 1 (PD-1) receptor, lymphocyte-activation protein 3 (LAG-3), and T cell Ig- and mucin-domain-containing molecule–3 (Tim-3). At day 3, we found that only 11.2% ± 1.7% and 13.6% ± 4.3% were $PD\text{-}1^{hi}$ in the $Thy1.1^+$ population of IL13Rα2-CAR.CD28.ζ and IL13Rα2-CAR.Δ T cells, respectively. Similarly, at day 7, 12.5% ± 1.7% and 16.1% ± 3.4% cells were $PD\text{-}1^{hi}$ in $Thy1.1^+$ IL13Rα2-CAR.CD28.ζ and IL13Rα2-CAR.Δ T cells, respectively (Figure 4C). Since activated T cells naturally upregulate PD-1 expression, we further analyzed $PD\text{-}1^{hi}$ populations for the expression of LAG3 and Tim-3, which are well-known negative regulators of T cell activity and markers of exhaustion.[32] In contrast to tumor-infiltrating host $CD8^+$ T cells, of which 41.2% ± 4.8% of $PD\text{-}1^{hi}$ cells also expressed LAG3 and Tim-3, IL13Rα2-CAR.CD28.ζ T cells did not acquire an exhausted phenotype as judged by low LAG3 and Tim-3 expression (Figure 4C). In conjunction with the observed proliferation of CAR T cells within the tumor, these data further support the finding that CAR T cells can survive and expand in the immunosuppressive glioblastoma environment.

IL13Rα2-CAR T Cells Influence the Repertoire of Tumor-Infiltrating Immune Cells in the GL261-IL13Rα2 Model

At present, it is largely unknown if CAR T cells can convert the immunosuppressive MG environment into an immunostimulatory one, resulting in activation of endogenous, glioma-specific immune responses. To investigate this, we analyzed tumor-infiltrating immune cells at days 3 and 7 following CAR T cell injection. We observed significant changes in the frequency of host $CD4^+$ and $CD8^+$ cells after CAR T cell treatment in the brain, but not in the draining lymph node (dLN) or spleen (Figures 5A and 5B). Treatment with IL13Rα2-CAR.CD28.ζ T cells did not change the frequency of the host's regulatory T cells (Treg) or contribute to the pool of Tregs, as verified by analysis of FoxP3 expression in $CD4^+$ host's or CAR T cells (Figure 5C). However, treatment with IL13Rα2-CAR.CD28.ζ T cells resulted in a 2.3-fold decrease in $Ly6G^+$ MDSCs in the brain and spleens at day 3, but not at day 7 (Figure 5D). There were no significant changes in the number of $Ly6C^+$ MDSCs (Figure 5E), macrophages, or microglia (Figure S3) between groups of mice treated with control and IL13Rα2-CAR.CD28.ζ T cells. Intriguingly, we observed a 2.6-fold increase in $CD45^{hi}CD11c^+$ DCs in the brains of tumor-bearing mice treated with IL13Rα2-CAR.CD28.ζ T cells at day 3 compared to control CAR T cells (Figure 6A). Similarly, in these mice, we detected a 3.6-fold increase in the $CD8\alpha^+$ subset of DCs at day 3 (Figure 6B). The increased presence of DCs in the brain was maintained at 7 days post-injection of CAR T cells, with a noticeable decrease in DC numbers in dLN at day 7, but not in the spleen. To understand whether the increase in $CD8\alpha^+$ DCs lead to antigen presentation to $CD8^+$ T cells, we analyzed the brains of mice bearing GL261-IL13Rα2 tumors engineered to overexpress the surrogate antigen ovalbumin (OVA) after control and IL13Rα2-CAR.CD28.ζ T cell treatment. We found a significant increase in the percentage

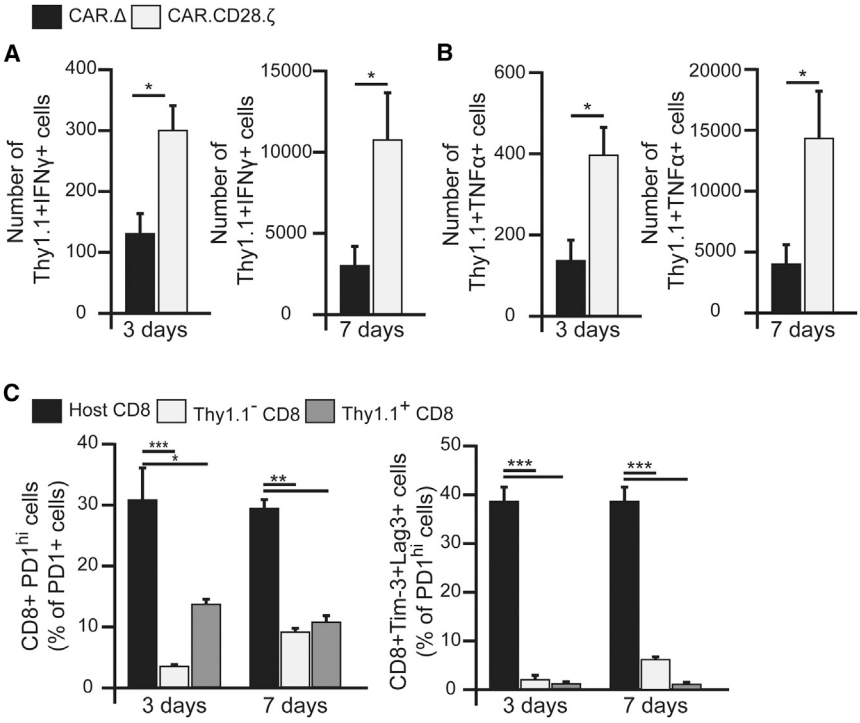

Figure 4. IL13Rα2-CAR.CD28.ζ T Cells Produce Cytokines in the Brains of Mice Bearing GL261-IL13Rα2 Glioma and Show a Non-exhausted Phenotype

(A) Functional analysis of Th1.1$^+$ T cells harvested from the brain of animals shows a significantly higher IFNγ production by IL13Rα2-CAR.CD28.ζ T cells at 3 and 7 days after i.t. injection than in control IL13Rα2-CAR.Δ T cells, as determined by flow cytometry ($n \geq 4$, $^*p \leq 0.05$, Student's t test). (B) Similarly, IL13Rα2-CAR.CD28.ζ T cells produced significantly more TNF-α compared to IL13Rα2-CAR.Δ T cells at 3 and 7 days ($n \geq 4$, $^*p \leq 0.05$, Student's t test). (C) IL13Rα2-CAR.CD28.ζ T cells significantly upregulate the expression of PD-1, as compared to IL13Rα2-CAR.Δ T cells within tumor. However, CAR T cells do not show an exhausted phenotype, as judged by a low expression of Tim-3 and LAG-3 expression in the PD-1^{hi} population compared to the host's CD8$^+$ T cells ($n \geq 5$, $^*p = 0.02$, $^{**}p = 0.001$, $^{***}p < 0.001$, Student's t test). Data are presented as mean ± SEM.

and absolute number of tetramer$^+$ CD8$^+$ T cells for OVA (Figure 6C) in mice treated with therapeutic CAR T cells. Collectively, these data demonstrate that therapeutic CAR T cells not only directly kill IL13Rα2-expressing glioma cells but also influence the tumor immune landscape by decreasing the number of immunosuppressive Ly6G$^+$ MDSCs and increasing the number of CD4$^+$, CD8$^+$, and CD8α$^+$ DCs in the tumors. The increase in the number of tetramer$^+$ CD8$^+$ T cells for OVA, along with lack of tumor development upon re-challenge with both IL13Rα2-negative and IL13Rα2-positive SMA560 glioma cells, also suggests that animals can acquire an anti-tumor immunity in response to CAR T cell therapy.

DISCUSSION

In the present work, we characterized the effects of IL13Rα2-CAR T cells in two immunocompetent models of glioblastoma. Our results demonstrated that, similarly to human CAR T cells, murine CAR T cells were activated upon engagement with IL13Rα2-expressing glioma cells, as judged by their cytolytic activity and the production of IFNγ and TNF-α in the presence of IL13Rα2-positive glioma cells. *In vivo*, IL13Rα2-CAR T cells had anti-glioma activity in two syngeneic glioma models and created a pro-inflammatory tumor microenvironment.

We show that IL13Rα2-CAR T cells retain the potential to produce IFNγ and TNF-α *in vivo* up to 7 days post-i.t.-injection in immunocompetent animal models. These cytokines have the potential to influence the tumor microenvironment by multiple mechanisms. For instance, IFNγ and TNF-α are known to promote anti-tumor immunity by upregulating the recruitment and maturation of antigen-presenting cells[33,34] and enhancing CD8$^+$ T cell responses.[35–37] We also observed an increased number of CD8α$^+$ DCs in the brains of glioma-bearing mice treated with IL13Rα2-CAR.CD28.ζ CAR T cells. The CD8α-expressing subset of DCs has been shown to efficiently cross-present both cell-bound and soluble antigens in the MHC class I context and strongly promote CD8$^+$ T cell responses.[38] In our studies, surviving VM/Dk animals developed long-lasting immunity, as determined by resistance to glioma re-challenge. No long-term survivors were observed in C57BL/6 mice bearing GL261-IL13Rα2 tumors. Although the reason for observed differences between both models is not clear, these findings are in line with reports from Sampson et al.,[39] who observed the development of a lasting immunity using CAR T cells directed against EGFRvIII in a syngeneic glioma mouse model. Thus, CAR T cells may stimulate the development of immune memory against glioma antigens.

Since the targeting of a single antigen invariably leads to escape variants, either through receptor downregulation or the selection of cells not expressing the targeted antigen(s), antigenic spread is important for the development of anti-glioma therapies.[10,13,40] In order to determine if CARs indeed potentiate immune responses against non-targeted antigens via bystander effect and/or antigen spreading, we also treated GL261-IL13Rα2-OVA glioma-bearing mice with IL13Rα2-CAR.CD28.ζ or IL13Rα2-CAR.ΔT cells. We observed a significant increase in OVA-specific T cell response in the therapeutic, but not control CAR T cells group, demonstrating that the interaction of CARs with their cognate antigen potentiates immunity against other tumor-expressed antigens.

Antigen escape is one limitation of immunotherapeutic approaches for MGs.[10,12,41] One way to overcome this obstacle is to engineer

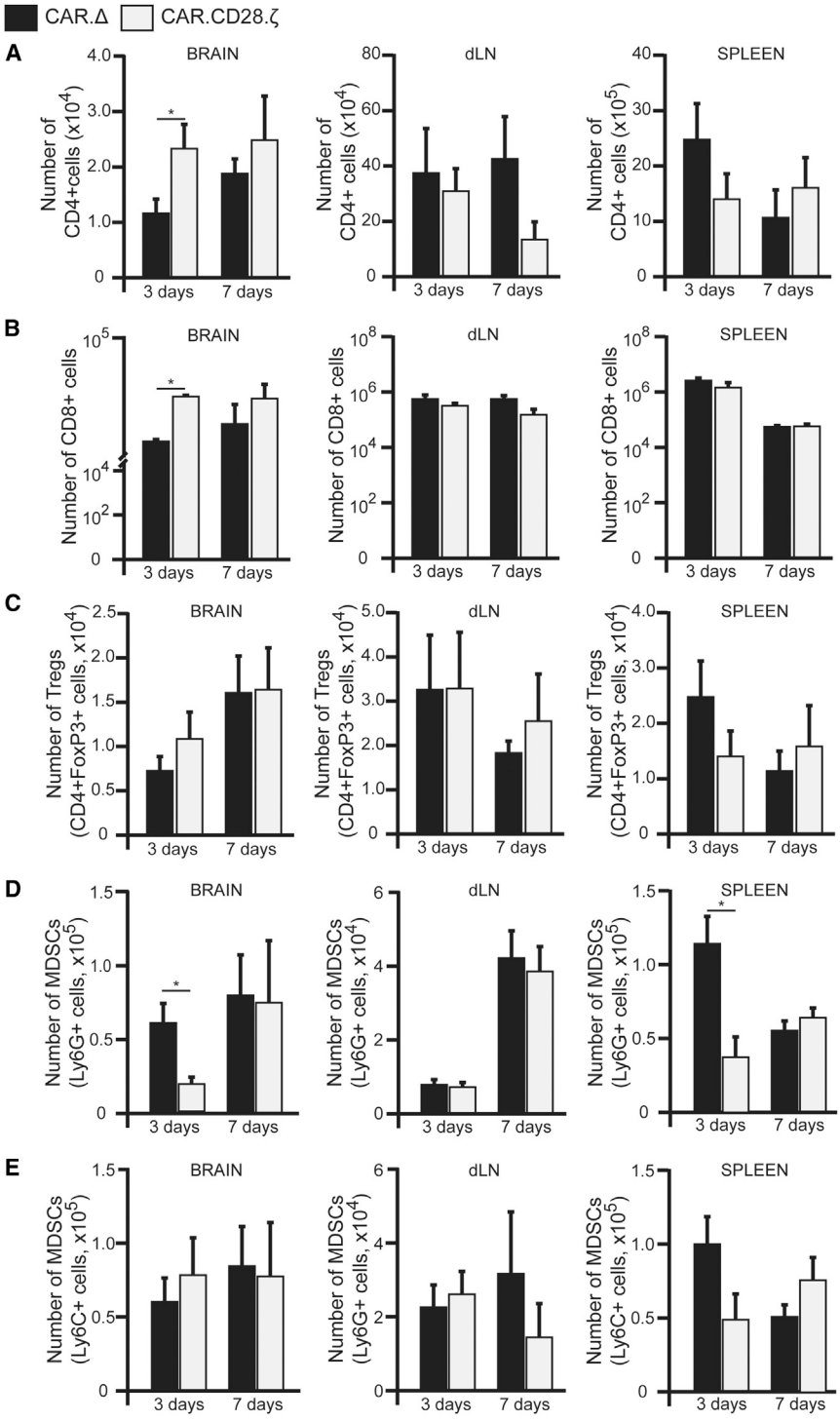

Figure 5. The Immune Landscape of Glioma-Bearing Mice after IL13Rα2-CAR.CD28.ζ T Cell Treatment

Analyses of host $CD4^+$, $CD8^+$, Treg, and MDSC subsets were performed at 3 and 7 days after i.t. injection of CAR T cells in animals bearing GL261-IL13Rα2 glioma. At 3 days, higher numbers of (A) $CD4^+$ and (B) $CD8^+$ cells were observed in the animals treated with IL13Rα2-CAR.CD28.ζ T cells when compared with animals treated with control IL13Rα2-CAR.Δ T cells ($n \geq 4$, $^*p \leq 0.05$, Student's t test). This difference was not statistically significant on day 7. There were no significant changes in these T cell compartments at either time point in analyzed dLN and spleen. (C) There was no change in the frequency of $CD4^+FoxP3^+$ regulatory T cells (Tregs) at any analyzed time point ($n \geq 4$, $^*p \leq 0.05$, Student's t test). (D) There was a significant drop in the number of $Ly6G^+$ myeloid-derived suppressor cells (MDSCs) after 3 days in the brain parenchyma of animals treated with IL13Rα2-CAR.CD28.ζ T cells. This drop was associated with decrease presence of $Ly6G^+$ MDSCs in the spleen at the same time point ($n \geq 4$, $^*p \leq 0.05$, Student's t test). (E) There was no change in the frequency of monocytic $Ly6C^+$ MDSCs at any analyzed time point ($n \geq 4$, $^*p \leq 0.05$ by Student's t test). Data are presented as mean ± SEM.

CAR T cells to target multiple TAAs. Indeed, several pre-clinical studies evaluating CAR T cells targeting two or three antigens (e.g., HER2, IL13Rα2, and EphA2) have demonstrated that it is feasible to prevent immune escape and also overcome heterogeneous TAA expression by MGs.[13,42,43]

The immunosuppressive microenvironment is a hallmark of many solid cancers, including MGs, and is highly influenced by MDSCs and Tregs.[44–47] Increased numbers of these cells positively correlates with poorer prognosis in patients with MGs and is one of the biggest obstacles to the success of immunotherapy. Our studies revealed that i.t. treatment with IL13Rα2-CAR.CD28.ζ T cells did not alter the number of tumor-infiltrating Tregs in mice, which is significant given the established inhibitory effects of Tregs on antigen presentation[48] and the anti-tumor efficacy of adoptively transferred T cells.[49,50] Our study suggests that depleting Tregs[39,47,51] prior to CAR T cell administration may enhance the efficacy of this therapy. Even though we did not observe sustained changes in the MDSC compartment, a decrease in presence of $Ly6G^+$ MDSCs in the brain was associated with a significant decrease of these cells in the spleen at 3 but not 7 days following CAR T cell administration. Reducing the negative effects of MDSCs on antigen-presentation and T cell proliferation has been the subject of intense study in the context of glioma treatment.[44,52] In the framework of CAR T cell therapies, reducing granulocytic and monocytic MDSCs could contribute to additional anti-glioma efficacy. Treg infiltration of tumor tissue, along with an increase in expression of inhibitory molecules, was observed after a single

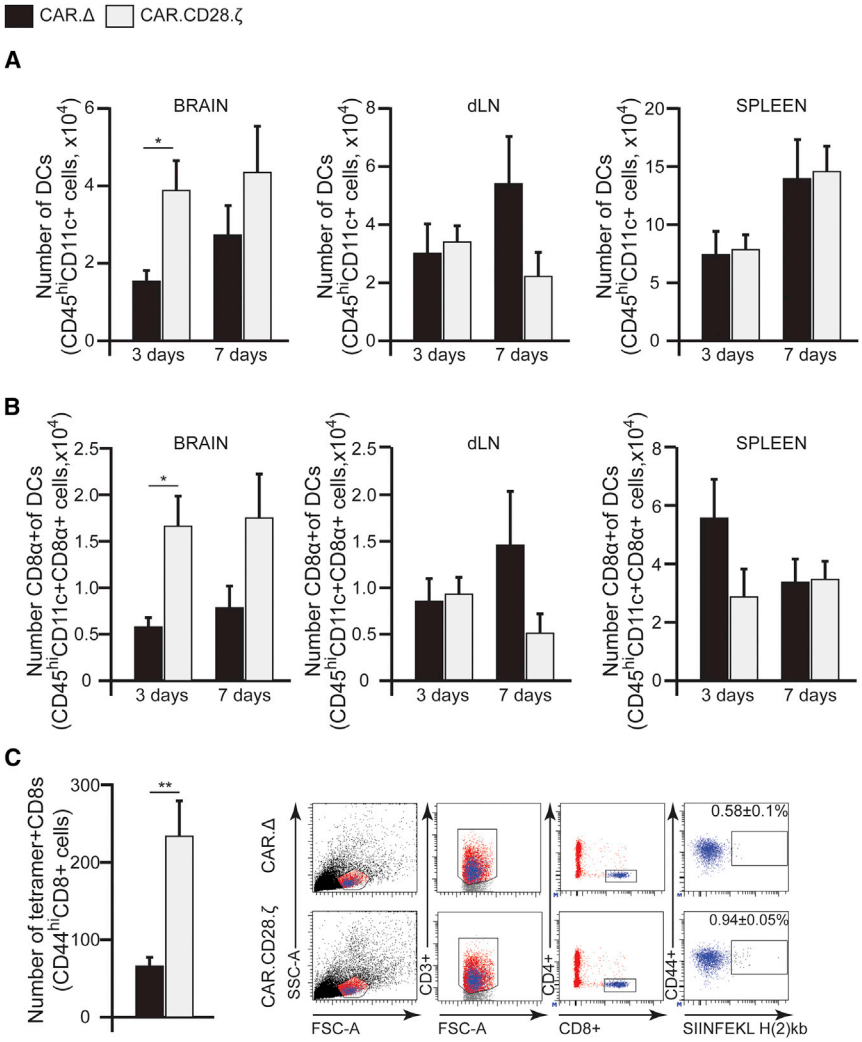

Figure 6. T Cells Modified with IL13Rα2-CAR.CD28.ζ Construct Mobilize Dendritic Cells to the Brain of Glioma-Bearing Mice

(A) Analysis showed a 3-fold higher number of DCs in the brain of mice treated with IL13Rα2-CAR.CD28.ζ T cells at day 3, as compared to control IL13Rα2-CAR.Δ T cells, but not in the dLNs or spleens ($n \geq 4$, $^*p \leq 0.05$, Student's t test). (B) Increased DC infiltration to the brain parenchyma was associated with an increased presence of the CD8α$^+$ subset of DCs at day 3 ($n \geq 4$, $^*p \leq 0.05$, Student's t test). (C) Animals bearing GL261-IL13Rα2 glioma tumors also expressing a surrogate antigen, OVA (GL261-IL13Rα2-OVA), were analyzed for the presence of OVA-specific CD8$^+$ cells on day 7 after i.t. transplantation of CAR T cells. There were significantly more OVA-reactive CD8$^+$ cells present in the brains of animals treated with IL13Rα2-CAR.CD28.ζ T cells, as compared to IL13Rα2-CAR.Δ T cells-treated mice ($n \geq 5$, $^{**}p \leq 0.01$, Student's t test). The panel on the right is an example of the gating strategy. Data are presented as mean ± SEM.

infusion of CAR T cells targeting EGFRvIII in patient with MG.[41] The data from this clinical study and our findings suggest that combinatorial therapies targeting the immunosuppressive MG environment has the potential to enhance the anti-glioma activity of CAR T cells.

Although less benefit to survival was seen in these models compared to in immunodeficient mice treated with the human version of these CAR T cells,[9] our results are consistent with the survival benefit of EGFRvIII-CAR T cells in the SMA560-EGFRvIII glioma model.[39] Despite lack of exhaustion, the limited proliferation and persistence of our CAR T cells likely decreased their efficacy in these models. Indeed, IL13Rα2-CAR T cell proliferation peaked in response to antigenic stimulation 3 days after i.t. injection. Furthermore, as in our xenograft glioma model, only a fraction of the injected CAR T cells survived, highlighting their limited persistence within the tumor environment.[9] Several approaches are being developed to render CAR T cells resistant to the inhibitory tumor microenvironment. These include transgenic expression of cytokines, expression of so-called switch receptors that convert an inhibitory into a stimulatory signal, and/or silencing or knocking out inhibitory genes.[53–57] For example, we recently have demonstrated that expression of IL15 in CAR T cells enhanced their effector function *in vitro* and improved their anti-glioma activity in xenograft models.[12]

Finally, we recognize that animal models of MGs utilized in our previous and current reports carry inherit limitations. Nevertheless, we were able to recapitulate the heterogeneity of IL13Rα2 expression normally present in human MG and demonstrated the therapeutic potential of IL13Rα2-CAR.CD28.ζ T cells in two complementary murine models of MGs. Results observed in these models complement our pre-clinical studies of human CAR T cells in a glioma xenograft model[9] and a case report from a phase I clinical trial,[10] demonstrating that IL13Rα2-targeted CAR T cells have significant therapeutic efficacy against MG. As seen here, these studies showed a significantly lower expression of the targeted antigen in recurrent tumor tissue, highlighting the importance of developing CAR T cell therapies targeting additional TAAs, a process already underway in our laboratories. However, in order to fully realize the potential of such an approach,[13,43] it has to be first thoroughly evaluated in immunocompetent models of disease such as the ones developed here.

In conclusion, our data from pre-clinical CAR T cell therapies in mouse models show great promise for immunotherapy for IL13Rα2-positive glioblastomas. We demonstrated that IL13Rα2-CAR T cells efficiently kill glioma cells both *in vitro* and *in vivo*.

For the first time, we showed that CAR T cell therapy positively modulates the immune landscape by creating a pro-inflammatory microenvironment in glioma-bearing mice, likely enhancing the anti-tumor activity of these CAR T cells and warranting further future studies. All together, our work provides strong rationale for further development and rapid clinical translation of this highly promising and selective therapeutic strategy for MGs.

MATERIALS AND METHODS

Detailed information is provided in the Supplemental Materials and Methods.

Cell Culture

293T (ATCC), murine parental GL261 (NIH), and SMA560 glioma cell lines (a generous gift from Dr. Sampson, J.H., Duke University), and their modified sublines, GL261-hIL13Rα2, GL261-hIL13Rα2-OVA, and SMA560-hIL13Rα2, were maintained in complete DMEM (Invitrogen, Grand Island, NY). T cells were maintained in RPMI 1640 (Invitrogen, Grand Island, NY). Cells were screened for mycoplasma with MycoAlert Mycoplasma Detection Kit (Lonza, Walkersville, MD) every 3–6 months.

Generation of Retroviral Chimeric Antigen Receptor Constructs

The codon-optimized cDNA encoding the CARs was synthesized by Thermo Fisher Scientific (Waltham, MA) and sub-cloned into a multiple cloning site (MCS) of the pRV2011(M) vector. The sequences of all final CAR constructs were verified by sequencing.

Generation of CAR T Cells

$CD3^+$ T cells were isolated from the spleens of 6- to 8-week old C57BL/6 (syngeneic for GL261) or VM/Dk (syngeneic for SMA560) male or female mice using a Mouse T Cell Isolation Kit (eBioscience Affymetrix, San Diego, CA) according to the manufacturer's directions. T cell cultures were transduced with replication-deficient retrovirus encoding for CARs for 3 consecutive days starting 24 hr after T cell stimulation. CAR and Thy1.1 marker gene expression was assessed on day 5 or 6 using flow cytometry.

Generation of Glioblastoma Cell Lines Expressing Human IL13Rα2 and/or OVA

GL261-hIL13Rα2 and SMA560-hIL13Rα2 were generated by transducing parental GL261 and SMA560 cell lines with a previously developed pEF6myc,his vector encoding human IL13Rα2[30] using Lipofectamine 2000 transfection reagent (Invitrogen, Grand Island, NY) according to the manufacturer's specifications. Transfected cells were selected with 5 μg/mL and maintained with 2 μg/mL of blasticidin S HCl (Invitrogen, Grand Island, NY). GL261-hIL13Rα2 expressing OVA (GL261-hIL13Rα2-OVA) were produced by transducing GL261-hIL13Rα2 cells with pAc-Neo-OVA (Addgene, Cambridge, MA) using Lipofectamine 2000 transfection reagent. Transfected cells were selected with 400 μg/mL and maintained in 200 μg/mL of geneticin (G418, Invitrogen, Grand Island, NY) in complete culture medium.

Intracranial Implantation of Murine Glioma Cells and Treatment with CAR T Cells

CD45.1 and CD45.2 C57BL/6 mice were obtained from Jackson Laboratory. VM/Dk mice were bred in-house in accordance with a study-specific animal protocol approved by the Northwestern University Institutional Animal Care and Use Committee (IACUC). We utilized mixed-gender animals, 6–12 weeks of age, for glioma implantation. The following coordinates were used: 2 mm from bregma, 3 mm right of the cranial midline suture, and 3.5 mm depth below the dura. On day 7 after implantation of glioma cells, animals received an i.t. injection of 1.5×10^6 CAR T cells. Treated animals were randomly assigned to housing cages, separated by gender. Animals were monitored for survival according to Northwestern University IACUC-approved protocols.

Flow Cytometry

Flow cytometric analysis was carried out using the BD Fortessa flow cytometer (Becton Dickinson, Franklin Lakes, NJ) at the Robert H. Lurie Comprehensive Cancer Center Flow Cytometry Core Facility. Single-cell isolates were blocked with PBS supplemented with 2% fetal bovine serum (FBS) and mouse CD16/32 blocking reagent (BioLegend, San Diego, CA). Following the Fc blockade, flourochrome-conjugated antibodies (all from BioLegend, San Diego, CA, unless specified) were added and incubated on ice for 20 min. For detection of intracellular proteins, cells were fixed, permeabilized, and subsequently incubated with antibodies. Labeled cells were then washed and analyzed.

In Vitro and *In Vivo* Functional Assays

The cytotoxicity of CAR T cells against control and IL13Rα2-expressing glioma cells was determined in a standard ^{51}Cr release assay with increasing ratios of the effector (CAR T cells) to target (glioma) cells. Antigen stimulation of CAR T cells was performed for the intracellular detection of cytokines by flow cytometry.

Statistical Analysis

All statistical analyses of collected data were performed with GraphPad 7 Software (Prism, La Jolla, CA). Student's t test was used to compare the two groups. Significance, defined as p less than 0.05 in all statistical tests, was calculated with an unpaired Mann-Whitney test or an unpaired two-tailed Student's t test, as indicated. Multiple groups were analyzed with either a one- or two-way ANOVA, followed by a Tukey's multiple comparisons test. Kaplan-Meier plots were generated using GraphPad 7 Prism, and p values for curve comparisons were calculated using the log-rank method. All experiments, besides survival analyses, were carried out in at least triplicates. All survival studies were carried out at least twice. Data are presented as mean ± SEM.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Materials and Methods and three figures and can be found with this article online at https://doi.org/10.1016/j.ymthe.2018.02.001.

AUTHOR CONTRIBUTIONS

K.C.P., I.V.B., and J.M. designed the experiments. K.C.P., J.M., G.K., W.K.P., G.L., T.R.-C., Y.H., and S.G. generated data, developed protocols, and performed experiments. K.C.P. and M.W. performed statistical analyses. K.C.P., J.M., M.S.L., S.G., and I.V.B. wrote and reviewed the manuscript. All authors reviewed, edited, and commented on the manuscript.

CONFLICTS OF INTEREST

M.S.L., I.V.B., and S.G. have patent applications in the field of T cell and gene therapy for cancer and/or IL13Rα2-targeted therapies.

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Supplemental Information

Adoptive Transfer of IL13Rα2-Specific Chimeric Antigen Receptor T Cells Creates a Pro-inflammatory Environment in Glioblastoma

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SUPPLEMENTAL METHODS

Cell culture

293T (ATCC), murine parental GL261 (NIH), and SMA560 glioma cell lines (a generous gift from Dr. John Sampson, Duke University), and their modified sublines, GL261-hIL13Rα2, GL261-hIL13Rα2-OVA and SMA560-hIL13Rα2, were maintained in complete Dulbecco's Modified Eagle Medium (DMEM; Invitrogen, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS; GE Healthcare Life Sciences, Pittsburgh, PA), streptomycin (100 mg/ml), and penicillin (100 U/ml) (Invitrogen, Grand Island, NY). T cells were maintained in RPMI 1640 (Invitrogen, Grand Island, NY), supplemented with 10% FBS, 0.025% GlutaMax (Invitrogen, Grand Island, NY), 0.001% penicillin, 0.002% streptomycin, and 50 μg/mL of mIL-2 (Peprotech, Rocky Hill, NJ), supplemented with 2-mercaptoethanol (Sigma-Aldrich, St. Louis, MO) to a final concentration of 45 μM. All cell cultures were maintained in a standard humidified incubator at 37°C in 5% CO_2 atmosphere.

Generation of glioblastoma cell lines expressing human IL13Rα2 and ovalbumin

GL261-hIL13Rα2 and SMA560-hIL13Rα2 were produced by transducing parental GL261 and SMA560 cell lines with a previously developed pEF6myc,his vector encoding human IL13Rα2[1] using Lipofectamine 2000 Transfection Reagent (Invitrogen, Grand Island, NY) according to the manufacturer's specifications. Transfected cells were selected with 5μg/mL of blasticidin S HCl (Invitrogen, Grand Island, NY) for 7 days. The expression of hIL13Rα2 was validated by staining with anti-human IL13Rα2 mAbs (clone 47) conjugated to PE and isotype control IgG1-PE (BioLegend, San Diego, CA). Cells were sorted using a BD FACSAria II cell sorter to enrich the

positive cell population. Sorted cells were routinely maintained in the presence of blasticidin S HCl at a concentration of 2 μg/mL with periodic validation of hIL13Rα2 expression on the surface of the modified cells by flow cytometry using a BD LSRFortessa Analyzer. GL261-hIL13Rα2 expressing ovalbumin (GL261-hIL13Rα2-OVA) were produced by transducing GL261-hIL13Rα2 cells with pAc-Neo-OVA (Addgene, Cambridge, MA) using Lipofectamine 2000 Transfection Reagent. Transfected cells were selected with 400 μg/mL and maintained in 200 μg/mL of geneticin (G418, Invitrogen, Grand Island, NY) in complete culture medium.

Generation of retroviral chimeric antigen receptor constructs

The codon-optimized cDNA encoding for CARs was synthesized by Thermo Fisher Scientific (Waltham, MA) and subcloned into a multiple cloning site (MCS) of the pRV2011(M) vector. pRV2011 (M) was derived from pRV2011 oFL,[2] which encodes an MSCV-based retroviral vector optimized for expression in murine T cells, the firefly luciferase gene, an internal ribosomal entry site (IRES), and Thy1.1, by replacing the firefly luciferase gene with an MCS. The IL13Rα2.CAR.CD28.ζ CARs consisted of 1) a murine IgG leader peptide, 2) the IL13Rα2-specific scFv47;[1,3] 3) the hinge region of murine IgG1, 4) the CD28 transmembrane domain, and 5) the CD28 and CD3ζ signaling domains. IL13Rα2.CAR.Δ was generated by deleting the CD28.ζ signaling domain of IL13Rα2.CAR.CD28.ζ. The sequences of all final CAR constructs were verified by sequencing.

Generation of CAR T cells

$CD3^+$ T cells were isolated from the spleens of 6-8-week old C57BL/6 (syngeneic for GL261) or VM/Dk (syngeneic for SMA560) male and female mice using a Negative Selection Mouse T Cell

Isolation Kit (eBioscience Affymetrix, San Diego, CA) according to the manufacturer's directions. Pure $CD3^+$T cells were activated in plates covered with anti–mouse CD3 at 5 μg/mL (eBioscience, Affymetrix, San Diego, CA) and anti–mouse CD28 antibodies at 2 μg/mL (eBioscience, Affymetrix, San Diego, CA) in RPMI 1640 media containing 10% FBS, antibiotics, and 50 ng/mL mouse recombinant IL2. T cell cultures were transduced with replication-deficient retrovirus encoding for CAR constructs after 24 hours of stimulation. Retroviral supernatants were prepared by co-transfection of 293T cells with plasmids encoding for CAR constructs, VSV-G, and Peg-Pam-e plasmid encoding MoMLV gag-pol genes using a GeneJuice transfection reagent (EMD Biosciences, San Diego, CA), as specified by the manufacturer. The supernatant was collected after 48 and 72 hours, filtered through 0.22 μm-pore filters (Invitrogen, Grand Island, NY), and used to spinoculate T cells for 3 consecutive days starting 24 hours after T cell stimulation. Spinoculation was carried out with 1 mL of viral supernatant, supplemented with 50 ng/mL of IL-2 in 24 well plates pre-coated with 10 μg/mL RetroNectin (Takara, Mountain View, CA). T cells were added to viral supernatant, spun at 2,000*g*, 21°C for 90 minutes, and recovered at 37°C in 5% CO_2 atmosphere each time. Additional freshly prepared 1 mL aliquots of RPMI-1640 medium containing 10% FBS, antibiotics, and IL2 at 50 ng/mL were added to each well 4 hours later. CAR and Thy1.1 tag protein expression was assessed on day 5 or 6 using flow cytometry.

Intracranial implantation of murine glioma cells and treatment with CAR T cells

CD45.1 and CD45.2 C57BL/6 mice were obtained from Jackson Laboratory. VM/Dk mice were bred in-house in accordance with a study-specific animal protocol approved by the Northwestern University Institutional Animal Care and Use Committee (IACUC). We utilized mixed gender animals, 6-12 weeks of age for glioma implantation. Mice were anesthetized with a

ketamine/xylazine mixture at 115/17 mg/kg, hydrated with 0.5 mL of lactated Ringer's electrolyte solution and prophylactically treated with meloxicam (0.2 mg/kg every 24 hours) and buprenorphine (0.05 mg/kg every 8 hours) for 3 days post-procedure.

Glioma cells were harvested using 0.5% trypsin-EDTA, neutralized by serum-containing medium, and washed twice with Dulbecco's phosphate-buffered saline (DPBS). We assessed cell viability using trypan blue dye exclusion and resuspension in DPBS (GL261) or 1.5% methylcellulose in Zinc Option Modified Eagle's medium (MEM; Invitrogen, Grand Island, NY) (SMA560). Glioma cells were injected into mice at $4x10^5$ GL261, GL261-hIL13Rα2, or GL261-hIL13Rα2-OVA cells or $7.5x10^4$ SMA560 or SMA560-hIL13Rα2 cells per animal in 2.5μL of sterile saline using a 25 μL syringe equipped with a 25-gauge needle (Hamilton, Reno, NV). The following coordinates were used: 2 mm from bregma, 3 mm right of the cranial midline suture, and 3.5 mm depth below the dura. On day 7 after implantation of glioma cells, animals received an intratumoral (i.t) injection of $1.5x10^6$ CAR T cells. Treated animals were randomly assigned to housing cages, separated by gender. *In vivo* survival experiments were repeated at least twice. Animals were monitored for survival according to Northwestern University IACUC-approved protocols.

Flow Cytometry

Immunophenotypic analyses of immune cells in tumor-bearing animals were done on days 3 and 7 after i.t. injection with IL13Rα2.CAR.Δ or IL13Rα2.CAR.CD28.ζ. CAR T cells. Brains, cervical draining lymph nodes (dLNs), and spleens were dissected from sacrificed mice and processed in a single cell suspension by pulverization through a sterile 70 μm cell strainer (Invitrogen, Grand Island, NY) using a 3 mL syringe plunger (BD, Franklin Lakes, NJ) into ice-cold PBS. Cell pellets

were treated with Ammonium-Chloride-Potassium buffer (ACK; Lonza, Walkersville, MD) to remove red blood cells. Brain cell pellets were mixed in a PBS/30% percoll (GE Healthcare Life Sciences, Pittsburgh, PA) solution and placed over a 70% percoll gradient. Samples were centrifuged at 1200*g* for 30 minutes without brakes. The top layer was aspirated, and the leukocyte interphase was collected into 20 mL of ice-cold PBS and washed twice. Cells were stained with antibodies according to the assay's protocol. Flow cytometric analysis was carried out using the BD Fortessa flow cytometer (Becton Dickinson, Franklin Lakes, NJ) at the Robert H. Lurie Comprehensive Cancer Center Flow Cytometry Core Facility. The general protocol used for surface staining is as follows: single cell isolates were incubated in PBS supplemented with 2% FBS and blocked with mouse CD16/32 blocking reagent (BioLegend, San Diego, CA). Following the Fc blockade, flourochrome-conjugated antibodies (all from BioLegend, San Diego, CA unless specified) were added and incubated on ice for 20 minutes. Cells were then washed and analyzed for surface staining. The surface staining antibody panels are detailed as follows:

Surface T-cell panel for ICCS: CD3-Alexa 700, CD4- Percp-Cy5.5, CD8-BV605, Thy1.1-APC-Cy7, CD45.1 PE-Cy7.

Exhaustion analysis: CD3-Alexa 700, CD4-Percp-Cy5.5, CD8-BV605, Thy1.1-APC-Cy7, CD45.1-PE-Cy7, Tim3-PE, Lag3-APC, PD1-FITC. This keeps the 450 laser open for *in-vivo* Cell Trace Violet experiments and Regulatory T-cell determination using Foxp3 Efluor-450.

Myeloid analysis: CD3-PE, CD11b-Efluor 450, CD11c-APC, CD8α-BV605, Ly6G-Percp-Cy5.5, Ly6C-Alexa-700.

Surface Panel for Tetramer Analysis: CD3-PE-Cy7, CD4-APC-Cy7, CD8-PE (Clone KT15 from MBL Life Science, Woburn, MA), CD44-Percp-Cy5.5, MHC-Class I Tetramer H2-

k(b) (produced by the NIH Tetramer core facility, Atlanta, GA). Tetramer staining was performed for 1 hour at 37°C before surface staining for respective CD3, CD4, and CD8 antigens.

In order to determine Foxp3 expression, cells were permeabilized, fixed overnight at 4°C, and subsequently stained using anti-Foxp3 Efluor450 utilizing the Foxp3 / transcription factor staining buffer set (eBioscience, Affymetrix, San Diego, CA).

Cell surface expression of CARs was determined using 10 μg/mL of hrIL13Rα2Fc protein (R&D Systems, Minneapolis, MN) with subsequent detection by fluorescein isothiocyanate (FITC)-conjugated anti-human Fc secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA). Intracellular cytokine staining was done on single-cell isolates incubated for one hour in complete RPMI medium before the addition of a protein transport inhibitor cocktail (eBioscience, Affymetrix, San Diego, CA). Thirty minutes later, a cell stimulation cocktail was added and incubated for 6-8 more hours. Cells were subsequently surface stained and fixed with 2% paraformaldehyde (Sigma-Aldrich, St. Louis, MO). Intracellular staining was then performed using flourochrome-conjugated anti-interferon gamma (IFNγ)-PE and anti-tumor necrosis factor alpha (TNFα)-BV421 antibodies (BioLegend, San Diego, CA). Cellular frequency and mean fluorescent intensity values were determined using FlowJo analysis software (TreeStar, Cupertino, CA).

The leukocytes were obtained from a Percoll gradient centrifugation of brain tissue. The cell number in each sample was determined using the Bio-Rad Cell Counter. These values were used to calculate the number of analyzed cells present within the brains.

***In vitro* functional assays**

The cytotoxicity of CAR T cells against control and IL13Rα2-expressing glioma cells was determined in a standard ^{51}Cr release assay with increasing ratios of the effector (CAR T cells) to target (glioma) cells. First, $1x10^6$ glioblastoma cells were labeled with 50 µCi ^{51}Cr, as we previously described[4] and mixed with CAR T cells at 1:5, 1:10, 1:20, and 1:30 target to effector (T:E) ratios. Maximum ^{51}Cr release was measured on cells treated with 1% Triton X-100 (Sigma-Aldrich, St. Louis, MO). Spontaneous ^{51}Cr release was determined in cells incubated in complete medium without CAR T cells. After 4 hours of co-culture at 37°C in a 5% CO_2 atmosphere, 20 µl of supernatant was placed in a 96-well LumaPlate (PerkinElmer, Akron, OH) and dried for 24 hours. Plates were then read using a Packard TopCount NXT Gamma Counter (PerkinElmer, Akron, OH). Specific lysis was determined using the following formula: (experimental release–spontaneous release)/(maximal release–spontaneous release) × 100. CAR T cells were stimulated with IL13Rα2-expressing cells for 24 hours. The analysis of IFNγ and TNFα production by CAR T cells was analyzed by ELISA per manufacturer's recommendations (R&D Systems, Minneapolis, MN).

***In vivo* functional assays**

In order to determine if CAR T cells proliferate in the tumor environment *in vivo*, CAR T cells were labeled with CellTrace™ Violet (Invitrogen, Grand Island, NY) prior to intratumoral injection according to the manufacturer's specifications. At days 3 and 7 after implantation, immune cells were extracted from brains, dLNs, and spleens and analyzed for persistence and proliferation by flow cytometry. A degranulation assay of CAR T cells for the intracellular detection of cytokines by flow cytometry were carried out after 12 hours in culture. In the last 6 hours of the culture, cells were stimulated with a stimulation cocktail, followed by treatment with

protein transport inhibitors (eBioscience, Affymetrix, San Diego, CA). CAR T cells were then removed from cultures and processed for flow cytometric analysis of IFNγ and TNFα expression and lineage markers using specific antibodies.

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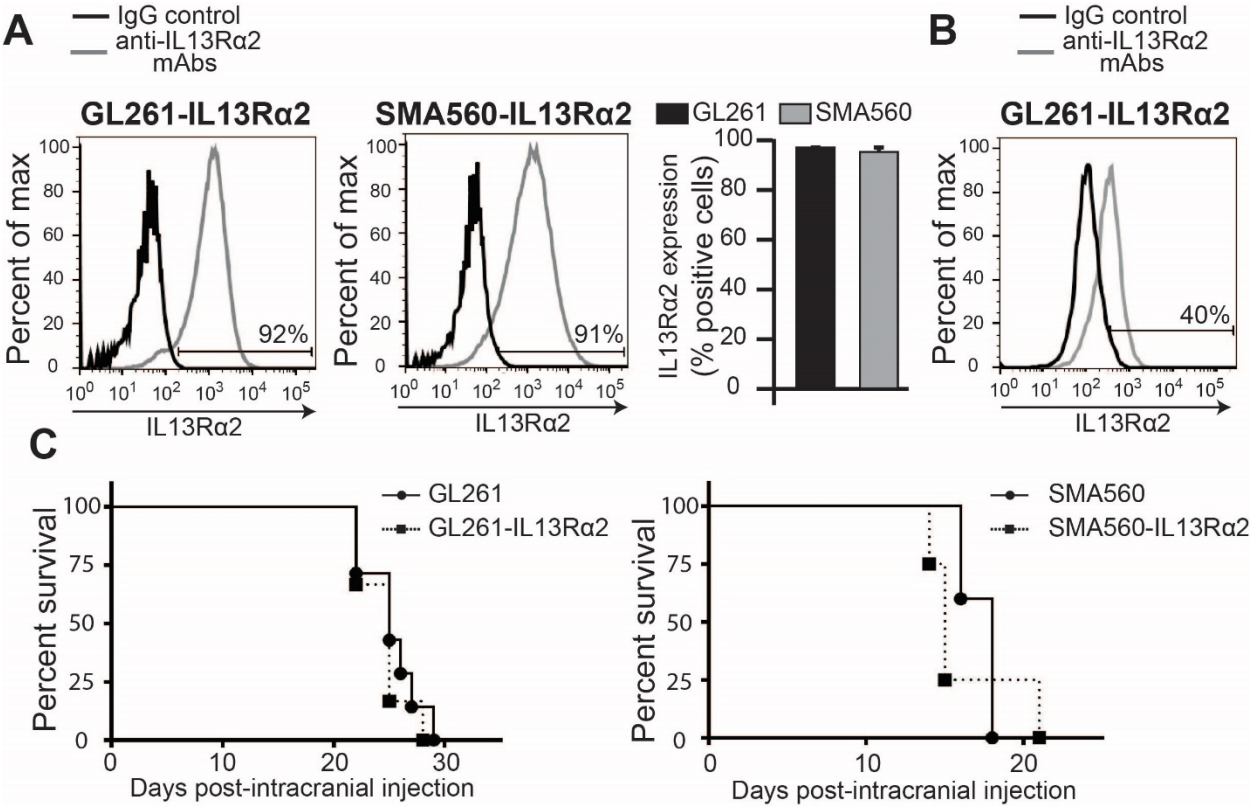
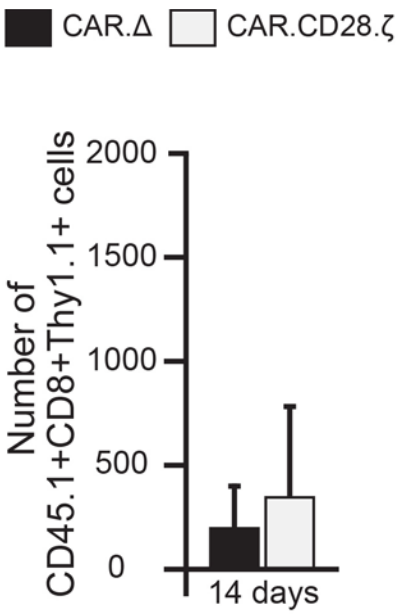
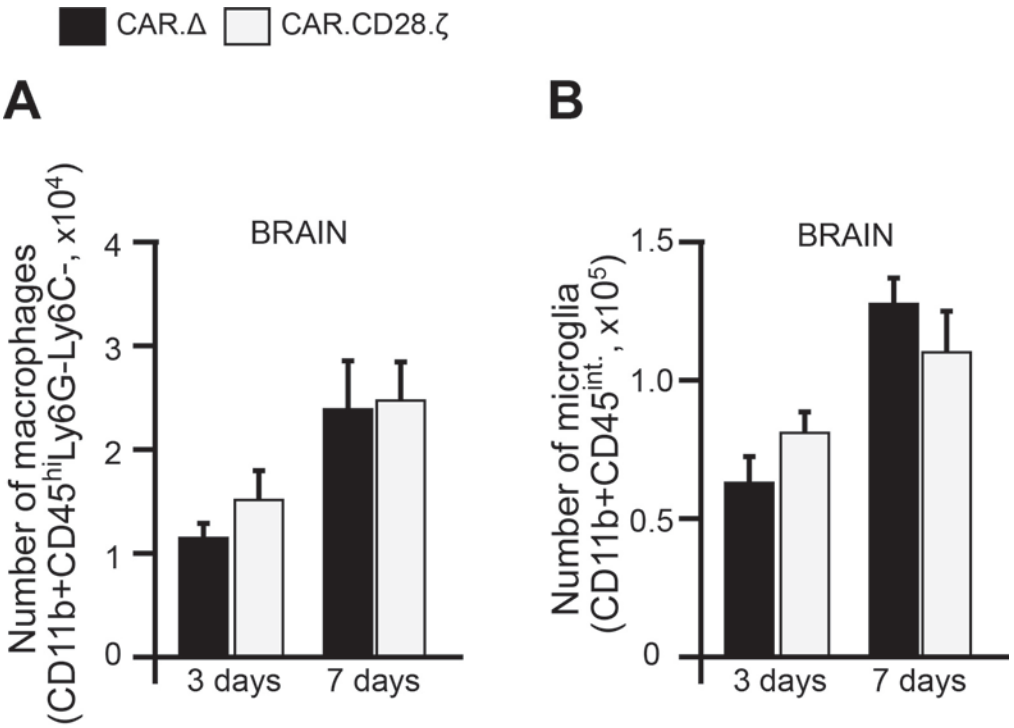


Supplemental Figure 1. Expressing human IL13Rα2 in murine glioma cells does not alter their tumorigenicity *in vivo*. A) Example of flow cytometry histograms of mouse glioma cell lines GL261 and SMA560 modified to express human IL13Rα2. Staining with IgG1 isotype control is shown in black, and staining with anti-IL13Rα2 mAb (clone 47) is shown in gray. The expression levels of IL13Rα2 were 92±0.3% for GL261 and 91±0.9% for SMA560 (n≥3). **B)** Example of a flow cytometry histogram showing that 40% of cells express hIL13Rα2 in GL261-IL13Rα2 tumors, excised from glioma-bearing animals 7 days post-intracranial injection. **C)** Kaplan-Meier survival plots of C57BL/6 and VM/Dk mice. GL261 or GL261-IL13Rα2 cells were intracranially injected to congenic C57BL/6 mice. Median survival of animals was 25 days in both groups (n≥8). SMA560 or SMA560-IL13Rα2 cells were intracranially injected to congenic Vm/DK mice. Median survival of animals was 15 days for SMA560 and 18 days for the SMA560-IL13Rα2 group (n≥5). Data are presented as mean±SEM.



Supplemental Figure 2. The persistence of CD8+ IL13Rα2-CAR.CD28.ζ T cells in the brains at the end point of glioma-bearing mice. CD3+ T cells from CD45.1 mice were utilized to generate IL13Rα2-CAR.CD28.ζ and control IL13Rα2-CAR.Δ T cells and i.t.-injected into CD45.2 mice bearing GL261-IL13Rα2 glioma. Quantitative analysis of CD45.1+Thy1.1+ T cells' populations was performed on day 14, which represented the end point of glioma-bearing mice treated with control IL13Rα2-CAR.Δ T cells. Data are presented as mean±SEM.



Supplemental Figure 3. Figure 5. The immune landscape of glioma-bearing mice after IL13Rα2-CAR.CD28.ζ T cell treatment. Analyses of macrophages **A)** and microglia **B)** in the brains of glioma-bearing mice was performed at 3 and 7 days after i.t. injection of CAR T cells. Data are presented as mean±SEM. Hi-high; int-intermediate.