Intracranially injected chimeric antigen receptor T cells eradicate glioblastoma cells but have limited potential to persist in the brain in a syngeneic mouse model

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Abbreviations

CAR, chimeric antigen receptor; GBM, glioblastoma multiforme; GFP, green fluorescent protein; FBS, fetal bovine serum; PBS, phosphate-buffered saline; EGFR, epidermal growth factor receptor; IVIS, In Vivo Imaging System

Abstract

Glioblastoma multiforme (GBM) is among the most malignant primary brain tumors, necessitating the development of novel therapies. Intracranial injection of chimeric antigen receptor (CAR)-T cell therapy is effective against GBM. However, whether intracranially injected CAR-T cells persist in the brain for long periods is unclear. In this study, we established a syngeneic murine model of GBM by injecting GL261 murine GBM cells expressing human B7-H3. After confirming tumor engraftment, murine T cells transduced with CAR-targeting human B7-H3 were injected intratumorally. Reduced tumor burden and enhanced survival were observed in mice injected with B7-H3 CAR-T cells compared with those injected with control T cells. CAR-T cells were distinctly detected in the brain 1 week after CAR-T cell injection, but disappeared 2 weeks after injection. In the syngeneic mouse model, intracranially injected CAR-T cells have the potential to eradicate GBM, but do not persist in the brain for long, suggesting that intracranial injections of CAR-T cells need to be repeatedly administered, as has been done in several clinical trials. It will be important to develop strategies to enhance persistence of CAR T cells in the brain.

Keywords: glioblastoma, CAR-T cell therapy, B7-H3, persistence

Introduction

Glioblastoma multiforme (GBM) is among the most malignant primary brain tumors, with a median overall survival of approximately 15–20 months, despite intensive treatments, including surgery, radiotherapy, and chemotherapy [1, 2], necessitating novel therapies to improve patient prognosis. The development of chimeric antigen receptor (CAR)-T cell therapy for brain tumors is underway, and intracranial injection of CAR-T cells has shown significant anti-tumor effects against GBM in clinical trials [3-12].

In most clinical trials of CAR-T cell therapy against GBM, CAR-T cells have been repeatedly injected [4, 5, 8, 11-14]. In a few studies, single-dose CAR T administration has been tested, and no or only transient efficacy was observed [3, 7, 9, 15]. It remains unclear whether CAR T cells do not persist in the brain or they persist but lose their function. In xenograft models of immunodeficient mice and human CAR-T cells, human CAR-T cells elicit a xeno-reaction in response to murine antigens; therefore, the ability of CAR-T cells to persist in vivo could not be assessed. In CAR-T cell experiments using syngeneic mouse GBM models [16-23], the duration for which intracranially injected CAR-T cells persist in the brain is unclear, although CAR-T cells have been detected a few days after injection in some studies [19, 22, 23].

In this study, we established a syngeneic mouse model by injecting GBM cells expressing human B7-H3 as a tumor-specific antigen model. We intratumorally injected CAR-T cells targeting

human B7-H3 and examined their persistence in the brain.

Materials and Methods

Tumor cells and generation of B7-H3-expressing GL261 cells

The murine glioma cell line, GL261, was used. G261 cells were infected with a retroviral vector expressing green fluorescent protein (GFP)-luciferase and sorted to establish single-cell clones. These clones were transduced with retroviral vectors encoding human B7-H3 and sorted to generate single-cell clones.

Animal experiments

Six-week-old C57BL/6 albino mice were purchased from Jackson Laboratory Japan, Inc. (Yokohama, Japan). All animal experiments were approved by the Institutional Animal Care and Use Committee of The University of Osaka Graduate School of Medicine (approval numbers: 03-071-000 and 04-028-002) and were performed following the animal use guidelines of the Animal Experiment Committee of The University of Osaka Graduate School of Medicine.

Flow cytometry

Whole brain containing tumor tissues obtained from the mice were immediately dissociated by

enzymatic digestion using Brain Tumor Dissociation Kits (P) and a GentleMACS Octo Dissociator with Heaters (Miltenyi Biotec) following the manufacturer's instructions. The resultant cell suspensions were passed through a 40-µm filter with Hanks' Balanced Salt Solution containing Ca^{2+} and Mg^{2+} (Sigma Aldrich Corp.) and centrifuged for 5 min at 300 × *g*. Filtered cells were lysed with 1X RBC Lysis Buffer (Thermo Fisher Scientific Inc.) to remove red blood cells and resuspended in staining medium (2% fetal bovine serum (FBS) + phosphate-buffered saline (PBS)).

The general protocol for surface staining was as follows: single-cell isolates were incubated in PBS supplemented with 2% FBS and blocked with anti-mouse CD16/32 antibody (93; BioLegend, RRID: AB_312800). Fluorochrome-conjugated antibodies were added and incubated on ice for 30 min after the Fc blockade. The cells were washed and analyzed for surface staining.

The antibodies utilized for staining included anti-human CD276 (B7-H3) PE (MIH42; BioLegend, RRID: AB_10719959), anti-mouse CD3 PE-Cy7 (145-2C11; BioLegend, RRID: AB_312684), anti-mouse CD8a Brilliant Violet 421 (53-6.7; BioLegend, RRID: AB_10897101), anti-mouse CD4 Brilliant Violet 480 (RM4-5; BD Biosciences, RRID: AB_2739506), anti-mouse CD45 APC-Cy7 (30-F11; BioLegend, RRID: AB_312980), and goat anti-human F(ab')2 Alexa Fluor 647 (109–607-003; Jackson ImmunoResearch, West Grove, PA, USA, RRID: AB_2337903), Streptoavidin PE (BD Biosciences, RRID: AB_10053328). Cetuximab (Merck Biopharma,

Darmstadt, Germany), targeting the epidermal growth factor receptor (EGFR), was biotinylated using the Biotin Labeling Kit (Dojindo, Kumamoto, Japan) for staining purposes.

First, debris was excluded based on forward scatter (FSC) and side scatter (SSC) properties. Doublets were removed using FSC-A versus FSC-H gating. Live cells were then identified based on viability dye exclusion. Among live cells, $CD45^+$ hematopoietic cells were gated, followed by dentification of $CD3^+$ T cells. $CD4^+$ and $CD8^+$ T-cell subsets were subsequently defined, and CAR T cells were identified based on the expression of truncated EGFR (tEGFR) as indicated.

Cells were analyzed using a BD FACS Canto II, BD FACS Celesta, or FACS Aria II flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). Flow cytometry data were processed using FlowJo (BD Biosciences, RRID: SCR_008520).

Generation of CAR T cells for B7-H3

The anti-B7H3 chimeric antigen receptor (B7-H3 CAR) was generated using the human anti-B7-H3 single-chain variable fragment (scFv) BRCA84D (MG27A; US patent #8802091 B2) from MacroGenics Inc. (Rockville, MD, USA) and the CAR construct contained mouse CD8 alpha, CD28 and CD3ζ intracellular signaling domains. As the control, T cells expressing GFP were generated using the MigR1 retroviral vector. Isolated cDNAs for the variable regions of the κ light chain and heavy chain were combined with the transmembrane domain of CD8α, cytoplasmic

domains of CD28 and CD3ζ, using an overlapping polymerase chain reaction. In addition, for the detection of CAR T cells in brain tissue after mouse transplantation, T2A peptides and a truncated EGFR sequence were linked using an overlapping polymerase chain reaction as needed. The BRCA-CAR construct was inserted into the retroviral vector. “Phoenix” packaging cells (Allele; Phoenix Eco Cell Line No. ABP-RVC-10002) were co-transfected with retroviral vector plasmids and helper plasmids using Lipofectamine 2000 (Thermo Fisher Scientific) to obtain viral supernatants. Supernatants containing retroviruses were collected after 48 h.

Activated T cells were subjected to retroviral transduction using the BRCA-CAR construct. Murine T cells were isolated from the spleens of mice following the manufacturer’s protocol (no. 130-095-130; Miltenyi Biotec) and were cultured in RPMI 1640 supplemented with 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin sulfate, 1 mM pyruvate, and 50 μM β-mercaptoethanol. These cells were incubated in 12-well plates [2×10^6 cells/well in 2 mL of T cell medium with 50 U/mL mouse interleukin (IL)-2 (no. 11271164001; Roche) and Dynabeads Mouse T-Activator CD3/CD28 (no. 11453D; Gibco) at a 1:1 ratio].

Cells were harvested 2 days post-activation and subjected to retroviral transduction using RetroNectin (Takara Bio, Kusatsu, Japan).

Un-transduced T cells were generated through completely the same procedure except for the addition of retrovirus for CAR expression.

Cytokine release assays

B7-H3 CAR-T cells and mock-transduced (control) T cells were co-cultured with B7-H3-expressing GL261 cells for 16 h. Cytokine production was measured using Quantikine enzyme-linked immunosorbent assay kits (IL-2 and interferon-gamma [IFN-γ]; R&D Systems, Inc.). Co-cultures were performed in quintuplicate.

Immunohistochemistry

Brains were harvested from mice that had been injected with CAR-T cells and control T cells on day 7 and day 14. Harvested brains were formalin-fixed and six micrometers sections of frozen tissues were used for fluorescent immunohistochemical analysis. For each mouse, brains were sliced to obtain three cerebral coronal sections (using the Mouse Brain Slicer [Muromati Kikai Co., Tokyo, Japan]).

All dissected tissues were stained with DAPI, and immunostained for GFP and PE was used to identify tumor, or CAR-T or control T cells in the orthotopic mouse model. For immunohistochemical analysis, heat-induced antigen retrieval was performed using a pressure cooker in 0.01 M citrate buffer (pH 6.0) for 10 min. Sections were incubated with a primary antibody against GFP (Invitrogen, RRID: AB_221477) and Cetuximab (Merck Biopharma, Darmstadt, Germany), targeting the epidermal growth factor receptor (EGFR) at 4 °C overnight. Streptoavidin Alexa Fluor 546 (BD Biosciences, RRID: AB_10053328) was used and the antibody complexes were visualized with fluorescent microscope (All in One

Fluorescence Microscope, BZ-X800L, Keyence, Osaka, Japan)

In vivo* syngeneic murine *models

Orthotopic syngeneic murine allograft models were established using B7-H3-expressing GL261 cells and C57BL/6 albino mice (Jackson Laboratory Japan, Yokohama, Japan). Mice were anesthetized with isoflurane. The skull burr hole was opened using a drill, and 2×10^5 B7-H3 expressing GL261 cells labeled with GFP/luciferase were injected into the right cerebrum using a stereotactic injector (Muromachi Kikai, Osaka, Japan). The injection point in the cerebrum was 1 mm forward from the bregma, 2 mm to the right, and 2 mm deep. Tumor engraftment in mouse heads was confirmed using an In Vivo Imaging System (IVIS) (PerkinElmer, Inc., Waltham, MA, USA) and 200 μL of luciferin (Promega, Madison, WI, USA). Seven days after tumor injection, 2×10^6 B7-H3 CAR-T cells were injected at the same surgical coordinates as the tumor cell injections. Tumor volume was analyzed weekly using IVIS. Mice were euthanized when they exhibited neurological symptoms. To test whether a higher number of injected CAR-T cells would improve their persistence in the brain, we performed an additional dose-escalation experiment. In this experiment, 4×10^6 B7-H3 CAR-T cells, which was twice the dose used in the standard treatment experiment, were injected directly into the tumor using the same coordinates and procedures described above. Mice were euthanized 14 days after

CAR-T cell injection, and the presence of CAR-T cells in the brain was evaluated using the same detection methods described above. To examine whether the intracranially injected CAR-T cells migrated to the spleen, spleens were collected from mice 14 days after CAR-T cell injection. Single-cell suspensions were prepared and analyzed by flow cytometry using antibodies against CD3, CD4, CD8, and tEGFR. CAR-T cells were identified as tEGFR-positive cells among $CD3^+$ T cells, and $CD4^+$ and $CD8^+$ T-cell populations were separately evaluated.

Statistical analysis

Statistical analyses were performed using JMP (version 16.0; SAS Institute, Cary, NC, USA). An unpaired two-tailed Student *t*-test was used to compare between two groups. $p < 0.05$ indicated a statistically significant result. The survival of mice was analyzed using Kaplan–Meier curves, and differences were assessed using log-rank tests.

Results

Development of mouse CAR T cells targeting human B7-H3

B7-H3 CAR was constructed by fusing antigen recognition domains of the anti-human B7-H3 mAb BRCA84D (MG27A; US patent #8802091 B2) with cytoplasmic regions of CD28 and CD3ζ. B7-H3 CAR-T cells were established by transducing the CAR construct into T cells activated

with anti-CD3 and CD28 mAbs **(Figure 1A, B)**. B7-H3 CAR-T cells produced higher amounts of IFN-γ and IL-2 than the control T cells when co-cultured with human B7-H3-expressing GL261 cells **(Figure 1C)**.

Intracranial injection of CAR-T cells targeting human B7-H3 eliminates GBM cells expressing human B7-H3 in the orthotopic GL261 syngeneic murine allograft model

A syngeneic murine allograft model using GL261 cells was established by transplanting GL261 cells transduced with GFP, luciferase, and human B7-H3 into the right cerebrum of C57BL/6 albino mice. The engraftment of GL261 cells was confirmed using IVIS 6 days after tumor injection. B7-H3 CAR-T or control T cells were injected into the same surgical coordinates of the mouse brain **(Figure 2A)**. Treatment with B7-H3 CAR-T cells reduced tumor growth within 2 weeks after injection, whereas tumors rapidly grew in all mice injected with control T cells **(Figure 2B, C)**. In two of the seven mice treated with B7-H3 CAR-T cells, the tumors were completely eradicated **(Figure 2B, C)**. Survival of mice injected with B7-H3 CAR-T cells was longer than that of mice injected with control T cells ($p=0.00379$, **Figure 2D)**. These results indicate that intracranially injected CAR-T cells have substantial anti-tumor effects in vivo, and in some cases, eradicate GBM cells. However, tumor regrowth was observed in many mice that responded to B7-H3 CAR-T cell treatment **(Figure 2C)**.

Intracranially injected B7-H3 CAR-T cells are detected in the brain after 1 week, but disappear 2 weeks after the injection

We investigated the persistence of the intracranially injected CAR-T cells. Truncated EGFR (tEGFR) was co-expressed with B7-H3 CAR in T cells to identify T cells transduced with the CAR **(Figure 3A)**. The transduction efficiency of tEGFR expression ranged from 66.8% to 85.8%. After confirming the engraftment of GL261 cells using IVIS, B7-H3 CAR-T or control T cells were injected into the tumor region of the mouse brain.

Seven mice were euthanized 7 days and 4 mice were euthanized 14 days after CAR-T cell injection. Single-cell suspensions were generated from tumor regions in the brain and subjected to flow cytometry analysis using an anti-EGFR antibody. On day 7, CAR-T cells were distinctly identified as $CD4^+$ or $CD8^+$ T cells in the brain in a part of the mice **(Figure 3B, Supplementary Figure S1A)**. In contrast, on day 14, CAR T cells were scarcely detected among both $CD4^+$ and $CD8^+$ populations **(Figure 3B, Supplementary Figure S1B)**. To test whether increasing the number of injected CAR-T cells could help them remain in the brain longer, we performed an additional dose-escalation experiment. In this experiment, 4×10^6 B7-H3 CAR-T cells, which was twice the dose used in the standard treatment experiment, were injected directly into the tumor using the same coordinates and procedures described above.

Mice were euthanized 14 days after CAR-T cell injection, and CAR-T cells in the brain were evaluated using the same detection methods described above (Supplementary Figure S4). To further examine whether the disappearance of CAR-T cells from the brain was associated with their migration to peripheral lymphoid organs, we analyzed spleens harvested 14 days after intracranial CAR-T cell injection. Flow cytometric analysis was performed using CD3, CD4, CD8, and tEGFR. tEGFR-positive CAR-T cells were not detected among either $CD4^+$ or $CD8^+$ T-cell populations in the spleen (Supplementary Figure S5). These results suggest that the loss of detectable CAR-T cells in the brain was not accompanied by apparent accumulation of CAR-T cells in the spleen. At both 1 and 2 weeks after CAR T-cell administration, tumors were still very small, and only a limited number of residual tumor cells (GFP-positive cells) could be detected in either group **(Supplementary Figure S2A, Supplementary Figure S2B)**.

In addition, we also performed immunohistochemical analysis to examine where the injected CAR-T cells were located in the brain. We sacrificed the mice that had been injected with CAR-T cells on day 14 and performed immunohistochemical analysis of their brains. CAR-T cells were not detected in the whole brain (Supplementary Figure S3A), nor in the tumor or the surrounding brain tissue, including both the intratumoral and tumor margin regions (Supplementary Figure S3B), suggesting that the CAR-T cells have disappeared not only from the tumor but also from the brain by day 14.

Discussion

In this study, we evaluated the efficacy and persistence of intracranially injected B7-H3 CAR-T cells in a syngeneic mouse model of GBM using GL261 murine GBM cells expressing human B7-H3. We found that the injected CAR T cells disappeared in the brain 2 weeks after intracranial CAR-T cell injection. These results demonstrate the effect of intracranially injected CAR-T cells for a short period. Intracranially injected CAR-T cells have consistently proven effective in all mice; however, the duration of this effect is limited and the tumor eventually recurred. Our results showed the limitation of single dose CAR-T cell administration and support the repeated intracranial injection of CAR T cells, which has been performed in most clinical trials against GBM [4, 5] for their sustained effects [8, 11, 12, 14, 24]. To address whether the limited persistence of intracranially injected CAR-T cells could be overcome by increasing the initial cell dose, we performed an additional experiment using a two-fold higher dose of CAR-T cells. However, CAR-T cells were still not detected in the brain 2 weeks after injection. This result suggests that simple dose escalation may be insufficient to maintain CAR-T cells in the brain for a prolonged period. Therefore, repeated administration or biological strategies to enhance CAR-T cell persistence, rather than merely increasing the single injected dose, may be required to achieve sustained antitumor activity.

Intracranial injection of CAR-T cells enhanced the accumulation of CAR T cells at the tumor sites; however, the CAR T cells did not persist in the brain for a long time. In contrast, intravenous injection of CAR T cells may induce long-term persistence [4, 15] but trafficking into the brain and infiltrating GBM tumors is inefficient. These results suggested that a combination of intracranial and intravenous injections may function synergistically and merit further investigation. Indeed, a combination of intracranial and intravenous injections is effective against diffuse midline gliomas, as shown in a clinical trial [4].

The route of CAR-T cell administration is an important issue in the treatment of GBM. In the present study, we focused on intracranial/intratumoral administration because our primary aim was to examine whether CAR-T cells directly delivered into the tumor site can persist locally in the brain. Local delivery allows CAR-T cells to be placed directly at the intracranial tumor site and avoids the confounding effect of inefficient trafficking across the blood–brain barrier or blood–tumor barrier. In contrast, intravenous administration would evaluate additional biological processes, including systemic expansion, peripheral distribution, CNS trafficking, and tumor infiltration. Therefore, intravenous administration would address a different question from the main focus of this study. We acknowledge that intravenous administration is an important and clinically relevant route of CAR-T cell delivery. However, because B7-H3 is expressed not only in tumor cells and tumor-associated vasculature but also at low levels in some normal tissues [25-

[27] , systemic administration of B7-H3-targeted CAR-T cells may require separate evaluation of biodistribution and potential off-tumor toxicity. Thus, we selected intracranial delivery as the first step to evaluate the local antitumor effect and persistence of B7-H3 CAR-T cells in an immunocompetent syngeneic GBM model. Future studies should compare intracranial and intravenous administration and examine whether combined local and systemic delivery can improve both tumor trafficking and long-term CAR-T cell persistence.

It remains unclear how the injected CAR-T cells disappear within a short period. CAR-T cells might leave the brain for systemic circulation or glymphatic system or cerebrospinal fluid [9-14]. Previous reports suggest that intracranially injected CAR-T cells could be detected in the peripheral blood[9, 14]. Therefore, we examined the spleen 14 days after intracranial CAR-T cell injection. However, tEGFR-positive CAR-T cells were not detected in the spleen. This result suggests that migration to the spleen is unlikely to explain the disappearance of CAR-T cells from the brain. However, we did not examine blood, cerebrospinal fluid, lymph nodes, or earlier time points, and therefore we cannot exclude the possibility that CAR-T cells transiently migrated to other compartments or were rapidly lost after leaving the brain. To avoid this, CAR-T cell accumulation into GBM sites should be induced, for example, by enforced expression of chemokine or chemokine receptors in CAR-T cells such as IL15, CXCR1- or CXCR2, IL7, and IL7 Flt3L [23, 28-30]. Alternatively, CAR-T cells may be rapidly exhausted in the brain, especially

in the GBM immunosuppressive microenvironment [31-34], which also influence the persistence of CAR-T cells is influenced by their exhaustion. As we have not examined the exhaustion of the CAR-T cells, future studies are necessary. To prevent this, similar to the case of CAR-T cells against other cancers, CAR-T cells should be modified, for instance, by deletion or enforced expression of genes associated with T cell exhaustion such as c-jun overexpression and PD-1 immunocheckpoint blockade [6, 32-35]. In addition, post-hoc analyses of tumor cell proliferation (Ki67), apoptosis markers, and T-cell composition in the tumor microenvironment may help to better understand the biological effects of CAR T-cell treatment and the mechanisms of tumor control and recurrence. One limitation of this study is that we did not include a non-functional CAR-T cell as a control. In addition, since the CAR sequences are humanized, allogeneic response to human-derived sequences may reduce the persistence of the CAR T cells.

In addition, another important aspect of CAR-T cell efficacy against GBM is lymphodepletion. While some previous studies incorporated lymphodepletion prior to CAR-T cell administration [4, 7, 36], others did not [3, 5, 6, 8, 9]. There is currently no clear consensus regarding the necessity of lymphodepletion for intracranial CAR-T cell injection. Future studies will be required to determine whether lymphodepletion is required to enhance the efficacy and persistence of intracranially injected CAR-T cells.

Finally, the other aspect of this study is rechallenge of tumor cells for the cured mice, Re-

challenge is an important aspect of immunotherapy preclinical studies [37-39].As we have not examined in this study, this represents a limitation of the present study and should be addressed in future investigations.

Compliance with Ethical Standards

Disclosure of potential conflicts of interest: The authors have no relevant financial or non-financial interests to disclose.

Research involving Human Participants and/or Animals: All animal experiments were approved by the Institutional Animal Care and Use Committee of The University of Osaka Graduate School of Medicine (approval numbers: 03-071-000 and 04-028-002) and were performed following the animal use guidelines of the Animal Experiment Committee of The University of Osaka Graduate School of Medicine.

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Ha.K., and N.Ho.: Writing – original draft. No.K., Ha.K., N.Ho., K.M., Hi.K., T.T., S.I., K.N., Y.W., T.H., N.Ha., and R.H.: Writing – review & editing.

Data Availability: The data generated in this study are available upon request from the corresponding author.

Ethics approval: All animal experiments were approved by the Institutional Animal Care and Use Committee of The University of Osaka Graduate School of Medicine (approval numbers: 03-071-000 and 04-028-002) and were performed following the animal use guidelines of the Animal Experiment Committee of The University of Osaka Graduate School of Medicine.

Consent to participate: Not applicable.

Consent to publish: Not applicable.

Figure caption

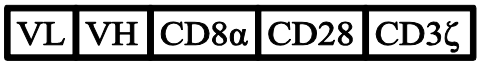
Fig. 1 T cells transduced with B7-H3 CAR produce higher amounts of cytokines upon co-culturing with B7-H3 expressing GL261 in vitro. a: CAR-T cell constructs targeting B7-H3 cells. VH, variable heavy; VL, variable light. **b**: Flow cytometry plots showing B7-H3 CAR transduction efficiency in murine T cells. SSC, side scatter. **c**: IFN-γ and IL-2 secreted by B7-H3 CAR-T cells or control CAR-T cells after co-culturing with B7-H3 expressing GL261 cells.

Mock-transduced T cells were used as controls. All experiments were performed in technical quintuplicates. IFN-γ, interferon-gamma; IL, interleukin

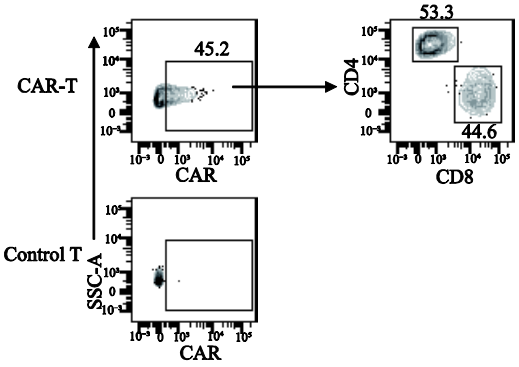
Fig. 2 T cells transduced with B7-H3 CAR show significant anti-tumor effects in vivo. a: Experimental design for in vivo CAR-T cell therapy using B7-H3 expressing GL261 cells. **b**: Bioluminescence imaging of mice after injection of B7-H3 and control CAR-T cells. **c**: Quantification of brain luminescence in mice treated with B7-H3 or CAR-T cells. **d**: Survival curves of mice treated with either B7-H3 or control CAR T cells. **P < 0.01 by log-rank test

Fig. 3 Intracranially injected B7-H3 CAR-T cells are distinctly detected in the brain 1 week later, but are scarcely detectable 2 weeks after injection. a: Truncated EGFR (tEGFR) was co-expressed with B7-H3 CAR in T cells to identify T cells transduced with the CAR **b:** Frequencies of $CD4^+$ or $CD8^+$ CAR T cells in $CD45^+$ and CD3+ hematopoietic cells in the brain (n=7 for day 7 and n=4 for day 14). All results are shown in Supplementary Fig. S1a and b.

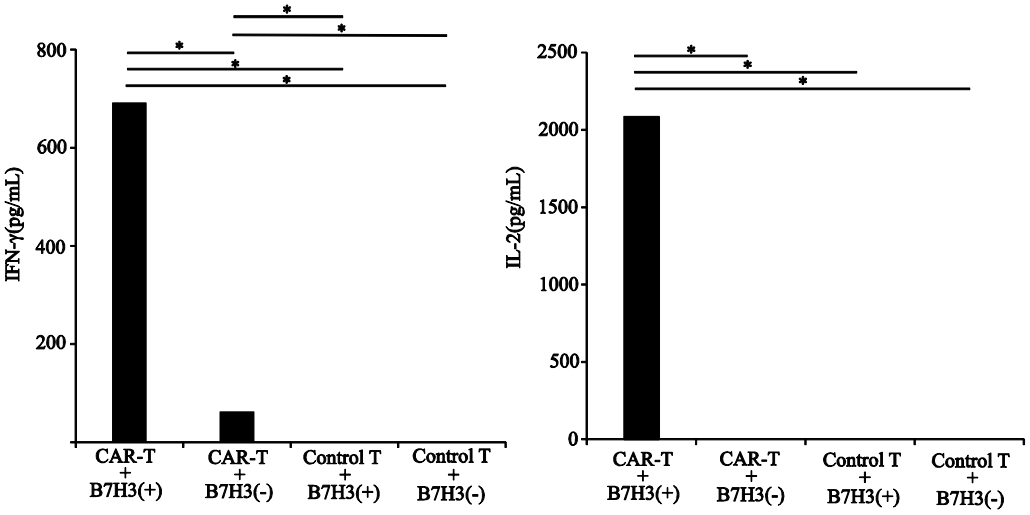
A

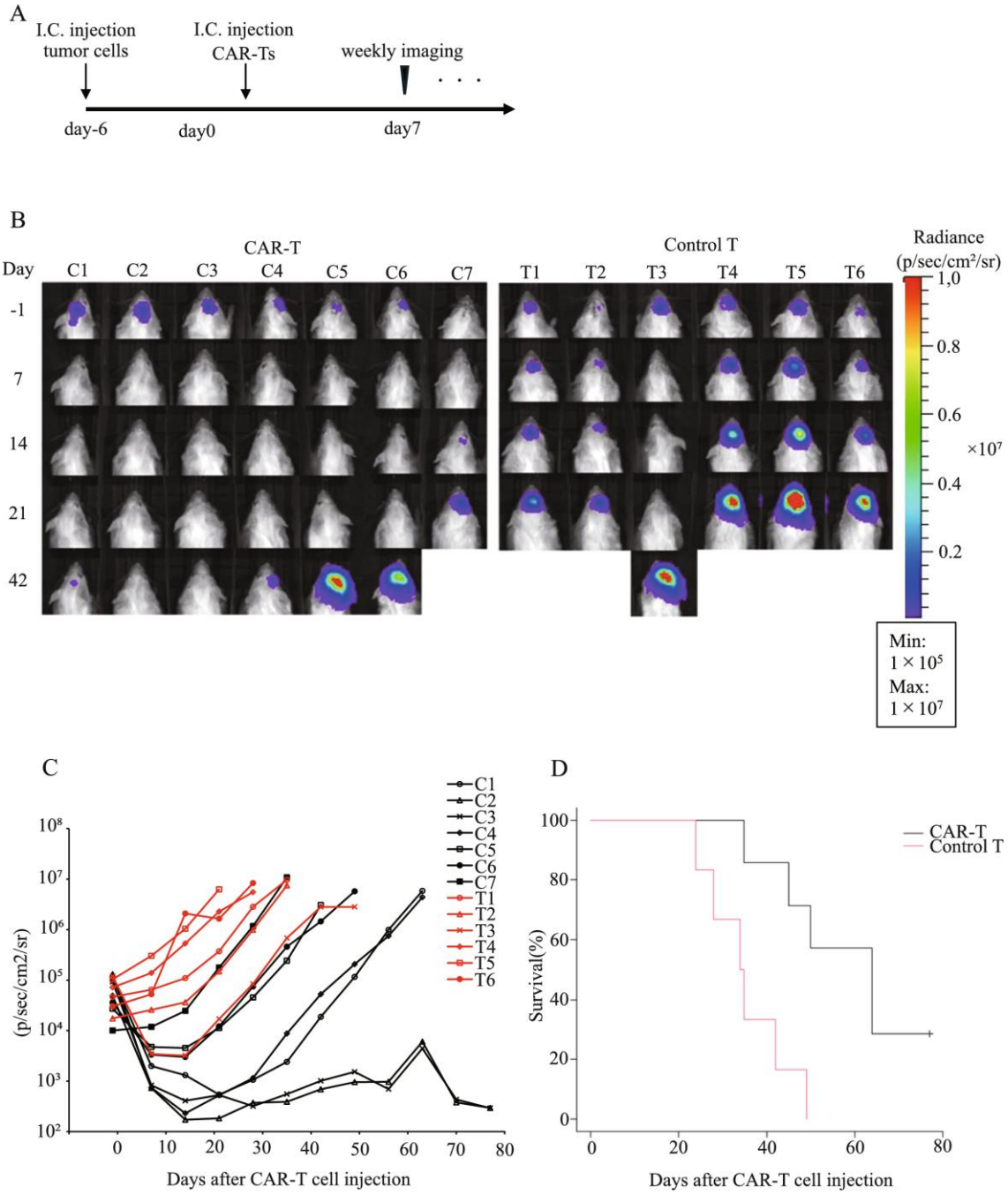

VL
VH
CD8α
CD28
CD3ζ

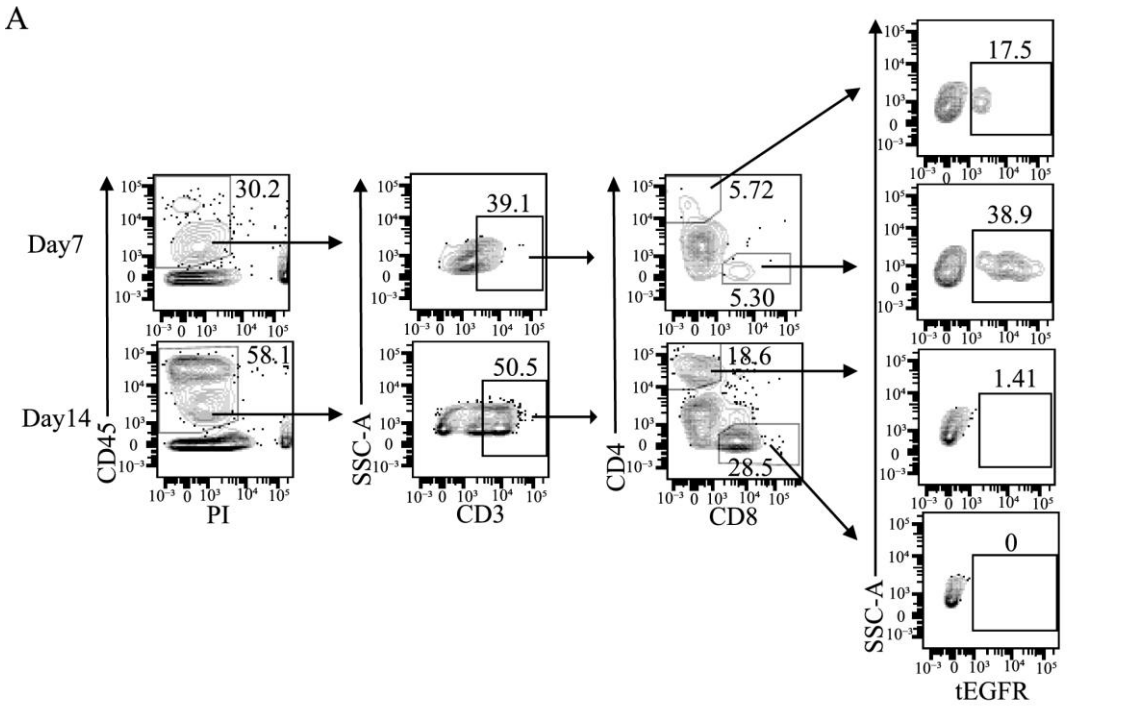
B

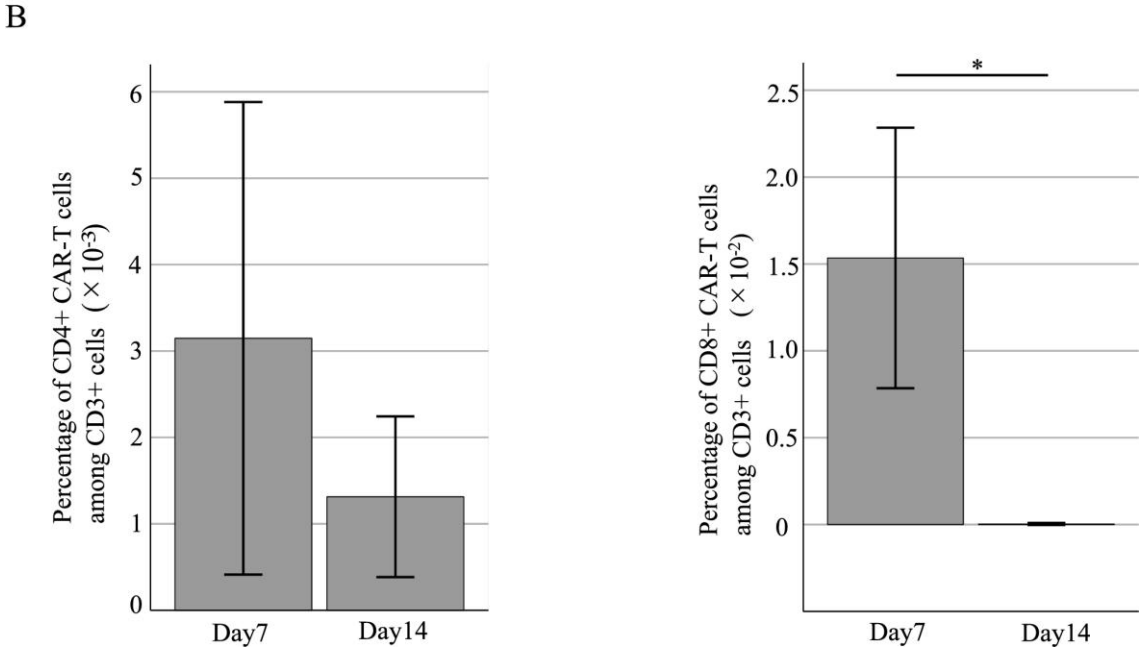

CAR-T
45.2
CAR
53.3
CD4
CD8
44.6
Control T
SSC-A
CAR

C


IFN-γ(pg/mL)
800
600
400
200
CAR-T + B7H3(+)
CAR-T + B7H3(-)
Control T + B7H3(+)
Control T + B7H3(-)
IL-2(pg/mL)
2500
2000
1500
1000
500
0
CAR-T + B7H3(+)
CAR-T + B7H3(-)
Control T + B7H3(+)
Control T + B7H3(-)


A
I.C. injection
tumor cells
I.C. injection
CAR-Ts
weekly imaging
day-6
day0
day7
B
CAR-T
Control T
Day
C1
C2
C3
C4
C5
C6
C7
T1
T2
T3
T4
T5
T6
-1
7
14
21
42
Radiance
(p/sec/cm²/sr)
1,0
0.8
0.6
×10⁷
0.4
0.2
Min:
1 × 10⁵
Max:
1 × 10⁷
C
(p/sec/cm2/sr)
Days after CAR-T cell injection
D
Survival(%)
CAR-T
Control T
Days after CAR-T cell injection


A
Day7
Day14
CD45
PI
30.2
58.1
SSC-A
CD3
39.1
50.5
CD4
CD8
5.72
5.30
18.6
28.5
SSC-A
tEGFR
17.5
38.9
1.41
0


B
Percentage of CD4+ CAR-T cells among CD3+ cells (×10-3)
Day7
Day14
Percentage of CD8+ CAR-T cells among CD3+ cells (×10-2)
*
Day7
Day14