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ORIGINAL ARTICLE

EGFRvIII-CAR-T Cells with PD-1 Knockout Have Improved Anti-Glioma Activity

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Abstract

Glioblastoma multiforme (GBM) is the most malignant form of the brain tumors. EGFR variant III (EGFRvIII) is expressed in about 30% of GBM specimens, but not expressed in normal brain tissues. Therefore, EGFRvIII protein offers an ideal CAR-T therapeutic target for EGFRvIII-positive GBM patients. PD-L1 is expressed in a variety of cancer cells, including GBM. Tumor-associated PD-L1 can bind to PD-1 on T cells and promote apoptosis of T cells, thus suppressing the anti-cancer immune response. In our current studies, PD-1^{WT} EGFRvIII-CAR-T cells and PD-1^{KD} EGFRvIII-CAR-T cells were generated. Cytokine production and lytic activity of these two CAR-T cells against to PD-$L1^{WT}$ EGFRvIII^{+} U373 cells or PD-$L1^{KO}$ EGFRvIII^{+} U373 cells were evaluated. The results showed that PD-1^{KD} EGFRvIII-CAR-T cells and PD-1^{WT} EGFRvIII-CAR-T cells showed same levels of interferon-γ (IFN-γ) and interleukin-2 (IL-2) production as well as cytolytic activity against PD-$L1^{KO}$ EGFRvIII^{+} U373 cells; however, PD-1^{KD} EGFRvIII-CAR-T cells exhibited higher levels of IFN-γ and IL-2 production as well as cytolytic activity against PD-$L1^{+}$ EGFRvIII^{+} U373 cells than that of PD-1^{WT} EGFRvIII-CAR-T cells. PD-1^{KD} EGFRvIII-CAR-T cells also exhibited higher anti-glioma activity and longer survival in mice in vivo than that of PD-1^{WT} EGFRvIII-CAR-T cells. Taken together, our findings indicate that PD-1 knockout enhances lytic activity of EGFRvIII-CAR-T cells against PD-$L1^{+}$ EGFRvIII^{+} GBM cells. These might provide a new insight into strategy of GBM CAR-T cell therapy.

Keywords Glioblastoma multiform · EGFRvIII · CAR-T · PD-1 · PD-L1

Introduction

Glioblastoma multiforme (GBM) is the most malignant form of the brain tumors in adults. The different genetic and epigenetic abnormalities are involved in GBM formation. Although various therapeutic approaches including surgery, radiotherapy and chemotherapy have been applied in clinic, the prognosis of GBM patients remains still poor, with median survival being on average little over a year [1–3].

Chimeric antigen receptors (CARs) on CD8^{+} cytotoxic T lymphocytes (CTL) directly recognize cell surface antigens independent of MHC, therefore avoids tumor escape caused by MHC downregulation. The majority of GBM exhibits a frequent amplification of epidermal growth factor receptor (EGFR), increasing GBM proliferation, invasion, and therapeutic resistance. EGFR variant III (EGFRvIII), as a subset of EGFR alteration, accounts for about 30% of GBM specimens [4, 5]. EGFRvIII mutation represents an ideal CAR-T therapeutic target because it is expressed in GBM cells rather than normal brain cells [6, 7]. However, limited anti-glioma activity of CAR-T cells might be caused by the immunosuppressive glioma environment [8, 9].

The mechanisms of cancer immunosuppression involve many factors including programmed death protein 1 (PD-1) and programmed death-ligand 1 (PD-L1) [10–12]. PD-L1, also called B7-H1, belongs to the B7 family members [13]. PD-L1 is expressed on cell membrane of a variety of cancer

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cells, such as lung cancer, gastric cancer, breast cancer and GBM [14–17]. Tumor-associated PD-L1 binds to PD-1 on CTL and promotes apoptosis of CTL, thus suppressing the anti-cancer immune response. Since inhibition of PD-1 expression can promote survival of antigen-specific CAR-T cells, CAR-T cells with PD-1 blockage therapy might provide effective strategy for GBM therapy [18].

In our present studies, EGFRvIII-CAR-T cells and PD-1^{KD} EGFRvIII-CAR-T cells were generated, respectively. Next, cytokine production and lytic activity of these two CAR-T cells against to PD-$L1^{+}$ $EGFRvIII^{+}$ U373 cells or PD-$L1^{KO}$ $EGFRvIII^{+}$ U373 cells in vitro were compared with each other. Additionally, the anti-glioma activity and longer survival of PD-1^{KD} EGFRvIII-CAR-T cells and EGFRvIII-CAR-T cells were also compared in mice in vivo.

Material and Methods

Cell Culture

The human glioma cell line of U373 was obtained from Cell Bank of Chinese Academy of Sciences (Shanghai, China). Cells were maintained in DMEM containing 10% fetal bovine serum (FBS) in a humidified chamber at 37 °C with 5%CO2.

Generation of PD-$L1^{WT}$ $EGFRvIII^{+}$ U373 Cells and PD-$L1^{KO}$ $EGFRvIII^{+}$ U373 Cells

Lentivirus vector encoding EGFRvIII was constructed by GenePharma (Shanghai, China). Cultured U373 cells were infected with EGFRvIII expression lentivirus to generate stably transformed cell line of $EGFRvIII^{+}$ U373 cells. PD1 knockout (KO) $EGFRvIII^{+}$ U373 cell line (PD-$L1^{KO}$ $EGFRvIII^{+}$ U373) was established by CRISPR/Cas9.

Generation of PD-$L1^{WT}$ EGFRvIII-CAR-T Cells and PD-1^{KD} EGFRvIII-CAR-T Cells

Lentiviral vector encoding a third generation (G3) anti-EGFRvIII CAR (MR1-CD8TM-CD28-OX40-CD3ζ) was created according to published studies.[19] Instant transfection of lentivirus into human T cells was performed, and GFP-positive cells were selected after 4 days. These GFP-positive T cells were transfected with PD-1 siRNA for 24 h, and PD-1 expression was identified by flow cytometry and Western blot.

Flow Cytometry

Cells were stained with PE-labeled anti-human PD-L1 (329,706, Biolegend), PE-labeled anti-human PD-1 (329,906, Biolegend) or EGFRvIII (64,952, Cell Signaling Technology) at 4 °C for 30 min. Cells were washed with PBS for two times, and then the expression of PD-L1, PD-1 and EGFRvIII on cells was detected by flow cytometry on BD FACSCalibur (BD Biosciences, San Jose, CA, USA).

Western Blot Analysis

After protein extraction with RIPA lysis buffer, equal amounts of protein were subjected to 4–20% ExpressPlus™ PAGE Gel (Genscript, Nanjing, China) at the quantity of 30 μg/lane. Primary antibodies against PD-L1 (ab210931, Abcam), PD-1 (ab140950, Abcam) as well as HRP-conjugated anti-rabbit IgG (7074, CST) were used to detect the levels of PD-L1 and PD-1 proteins. The bands on PVDF membranes were visualized through ECL detection system, and density analysis was performed with Quantity One (Bio-Rad, Hercules, CA, USA).

Elisa

The secretion levels of interferon-γ (IFN-γ) and interleukin-2 (IL-2) in the supernatant were detected by commercial ELISA kits from R&D Systems (Minneapolis, MN, USA) according to the instructions.

BCECF Release Experiment

Cytotoxic activity of CAR-T cells was measured by BCECF leakage assay [19]. In brief, U373 cells were seeded in a 96-well plate, and cultured for 24 h, and then treated with different inhibitors for 9 h. Next, U373 cells were incubated with 5 μM BCECF-AM for 30 min at 37 °C. After washing with PBS three times, the BCECF-labeled U373 cells were cultured with CAR-T cells for 12 h at 37 °C, then released BCECF in the supernatants was measured by a Synergy HTX Multi-Mode Microplate Reader (BioTek, Winooski, VT, USA) with excitation and emission wavelengths of 485 nm and 538 nm, respectively. The percentage of BCECF release (U373 cell damage) = $[(A - B)/(C - B)] \times 100\%$, where A represents the experimental BCECF release of U373 cells incubated with CAR-T cells, B represents the spontaneous release BCECF release of U373 cells without CAR-T cells, and C represents the maximum BCECF release of U373 cells caused by 0.1% Triton X-100.

Animal Study

Glioma xenograft mouse model was induced with EGFRvIII+ U373 cells. Cultured EGFRvIII+ U373 cells were injected stereotactically into the brains of BALB/c nude mice, followed by PBS control, PD-1WT EGFRvIII-CAR-T cells and PD-1KD EGFRvIII-CAR-T cells on day 10 after U373 cells injection. Then, tumor size and survival time of mice were detected. Intracranial tumor growth was quantified by Bioluminescence imaging (BLI) using an IVIS SPECTRUM

200 system (Perkin Elmer) at day 1, 10, and 28. Animals received 150 mg/kg D-luciferin by intraperitoneal (i.p.) injection and 15 min after the luciferin administration were anesthetized by gas anesthesia (2.5% isoflurane) and placed into black paper in the IVIS Imaging System box to be imaged.

Statistical Analysis

Data are presented as means ± SD. T-Test or One-way ANOVA followed by Bonferroni post-hoc test was used to determine significant differences among groups. $P < 0.05$ was considered significant.

Results

Generation of PD-L1^{WT} EGFRvIII^{+} U373 Cells and PD-L1^{KO} EGFRvIII^{+} U373 Cells

Cultured U373 cells were infected with EGFRvIII expression lentivirus to generate EGFRvIII^{+} U373 cells, and PD-L1 knockout was performed by CRISPR/Cas9. The expression of EGFRvIII and PD-L1 were identified by flow cytometry (Fig. 1a-b) and Western blot (Fig. 1c-d). The results showed that PD-L1WT EGFRvIII+ U373 cells and PD-L1KO EGFRvIII+ U373 cells were established successfully.

Generation of PD-1^{WT} EGFRvIII-CAR-T Cells and PD-1^{KD} EGFRvIII-CAR-T Cells

To generate EGFRvIII-CAR-T cells, we designed lentiviral vector encoding anti-EGFRvIII CAR (MR1-CD8TM-CD28-OX40-CD3ζ) was created. Instant transfection of CD8^{+} T cells with this lentiviral vector was performed, and GFP-positive cells were selected after 4 days by flow cytometry. To generate PD-1^{KD} EGFRvIII-CAR-T cells, these GFP-positive cells were transfected with PD-1 siRNA for 24 h, and PD-1 expression was identified by flow cytometry (Fig. 2a-b) and Western blot (Fig. 2c-d).

PD-1 Knockdown Enhances Cytokine Production of EGFRvIII-CAR-T Cells in Response to PD-L1^{+} EGFRvIII^{+} GBM Cells

After generation of PD-1^{WT} EGFRvIII-CAR-T cells and PD-1^{KD} EGFRvIII-CAR-T cells, we tested their effector function in vitro. PD-1^{WT} EGFRvIII-CAR-T cells or PD-1^{KD} EGFRvIII-CAR-T cells were incubated with PD-L1^{WT} EGFRvIII^{+} U373 cells or PD-L1^{KO} EGFRvIII^{+} U373 cells at a ratio of 5:1 effector-to-target (E:T) for 24 h. Supernatant was collected from the co-culture system, and the concentrations of IFN-γ and IL-2 were determined by ELISA. The results showed that PD-1^{KD} EGFRvIII-CAR-T cells produced

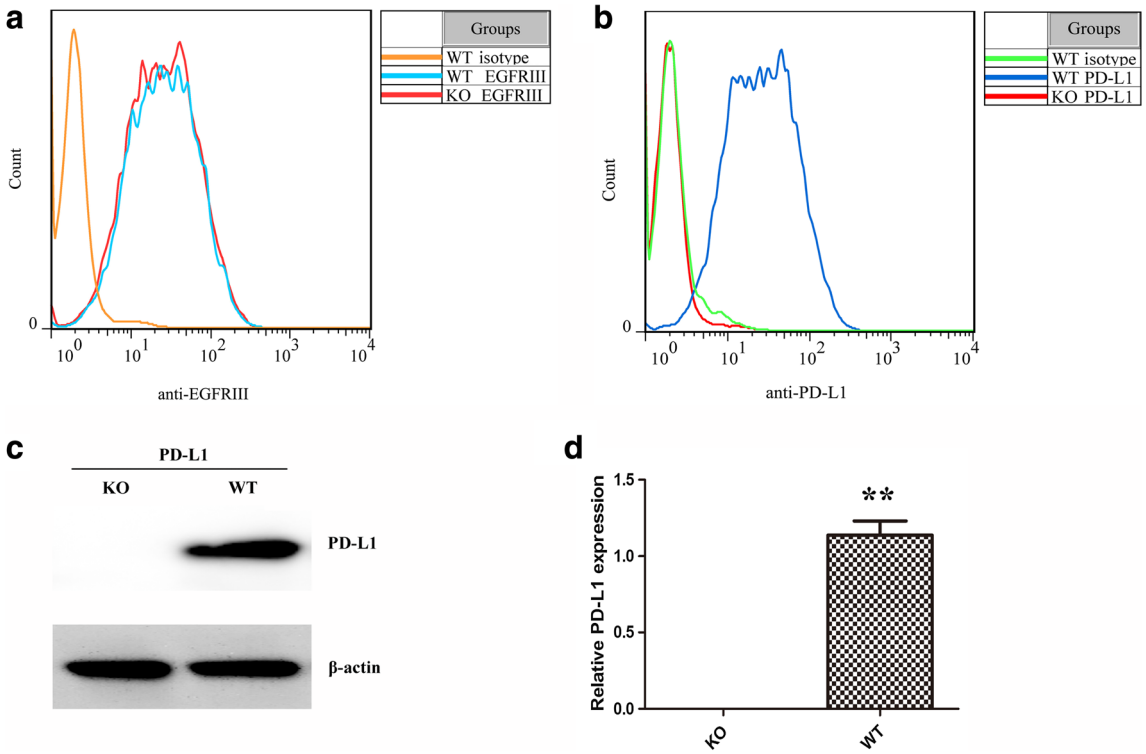

Fig. 1 The expression of EGFRvIII and PD-L1 in PD-L1^{WT} EGFRvIII^{+} U373 cells and PD-L1^{KO} EGFRvIII^{+} U373 cells. The expression of EGFRvIII and PD-L1 in PD-L1^{WT} EGFRvIII^{+} U373 cells and PD-L1^{KO} EGFRvIII^{+} U373 cells were identified by flow cytometry (**a** and **b**) and Western blot (**c** and **d**)

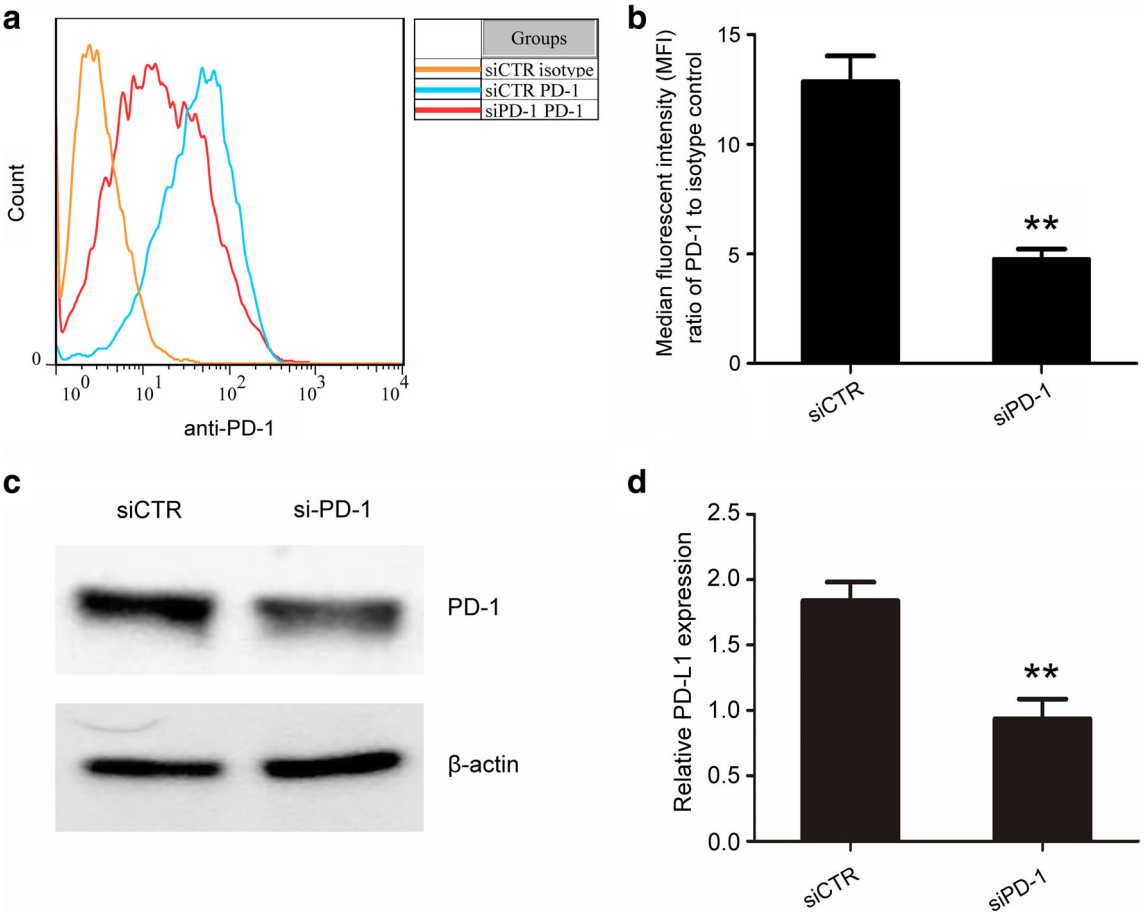

Fig. 2 PD-1 expression in PD-1^{KD} EGFRvIII-CAR-T cells. EGFRvIII-CAR-T cells were transfected with siPD-1 or siCTR for 24 h, and then the expression of PD-1 was detected by flow cytometry (**a** and **b**) and Western blot (**c** and **d**)

same amount of IFN-γ and IL-2 as PD-1^{WT} EGFRvIII-CAR-T cells when they were incubated with PD-L1^{KO} EGFRvIII^{+} U373 cells (Fig. 3). We also observed that PD-1^{KD} EGFRvIII-CAR-T cells produced higher levels IFN-γ and IL-2 than that of PD-1^{WT} EGFRvIII-CAR-T cells after incubation with PD-L1^{+} EGFRvIII^{+} U373 cells (Fig. 3), suggesting that PD-1 knockout enhanced cytokine production of EGFRvIII-CAR-T cells in response to PD-L1^{+} EGFRvIII^{+} U373 cells.

PD-1 Knockdown Enhances Lytic Activity of EGFRvIII-CAR-T Cells against PD-L1^{+} EGFRvIII^{+} GBM Cells In Vitro

We confirmed the lytic activity of PD-1^{WT} EGFRvIII-CAR-T cells and PD-1^{KD} EGFRvIII-CAR-T cells on the PD-L1^{+} EGFRvIII^{+} U373 cells or PD-L1^{KO} EGFRvIII^{+} U373 cells in the co-culture at a 5:1 effector-to-target (E:T) ratio for

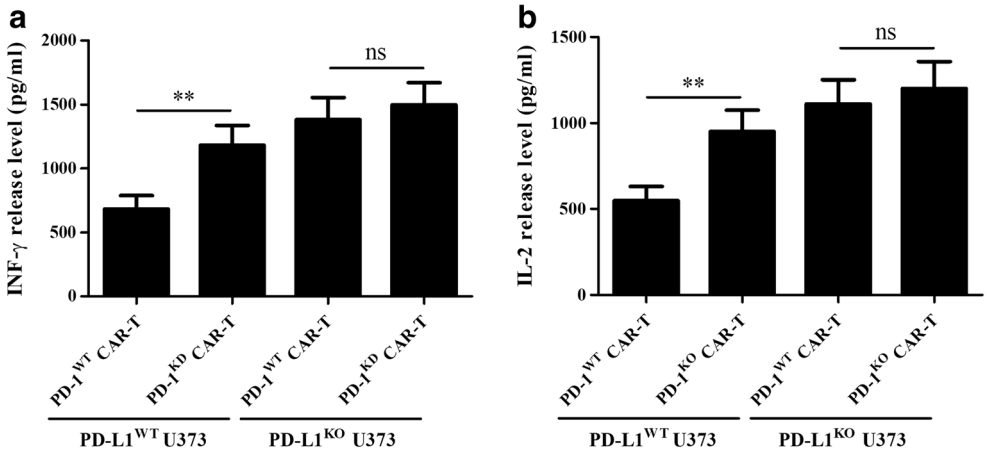

Fig. 3 PD-1 knockdown enhances the IFN-γ and IL-2 production of EGFRvIII-CAR-T cells in response to PD-L1^{+} EGFRvIII^{+} GBM cells. The amount of IFN-γ (**a**) and IL-2 (**b**) release was detected by ELISA when PD-1^{WT} EGFRvIII-CAR-T cells or PD-1^{KD} EGFRvIII-CAR-T cells were incubated with PD-L1^{WT} EGFRvIII^{+} U373 cells or PD-L1^{KO} EGFRvIII^{+} U373 cells

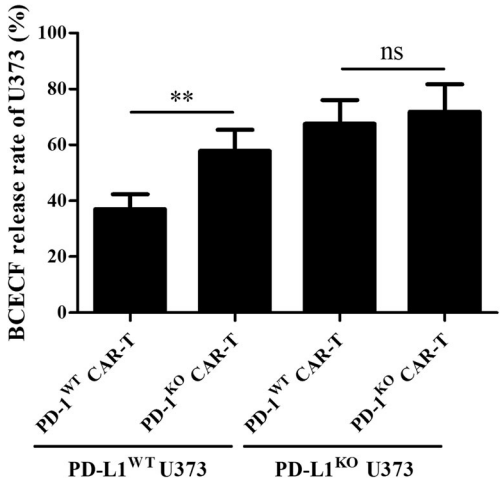

Fig. 4 PD-1 knockdown enhances lytic activity of EGFRvIII-CAR-T cells against PD-L1^{+} EGFRvIII^{+} GBM cells in vitro. PD-1^{WT} EGFRvIII-CAR-T cells or PD-1^{KD} EGFRvIII-CAR-T cells were incubated with PD-L1^{WT} EGFRvIII^{+} U373 cells or PD-L1^{KO} EGFRvIII^{+} U373 cells. Then, BCECF release from U373 cells was examined and BCECF release rates in different groups were expressed

24 h. BCECF release assay showed that PD-1^{KD} EGFRvIII-CAR-T cells and PD-1^{WT} EGFRvIII-CAR-T cells exhibited same levels of cytolytic activity against PD-L1^{KO} EGFRvIII^{+} U373 cells; however, PD-1^{KD} EGFRvIII-CAR-T cells exhibited higher levels of cytolytic activity than that of PD-1^{WT} EGFRvIII-CAR-T cells against PD-L1^{+} EGFRvIII^{+} U373 cells (Fig. 4). Namely, PD-1 knockout enhanced lytic activity of EGFRvIII-CAR-T cells against PD-L1^{+} EGFRvIII^{+} U373 cells rather than PD-L1^{KO} EGFRvIII^{+} U373 cells.

EGFRvIII-CAR-T Cells with PD-1 Knockout Have Improved Anti-Glioma Activity In Vivo

We compared anti-glioma activity of PD-1^{WT} EGFRvIII-CAR-T cells and PD-1^{KD} EGFRvIII-CAR-T cells in the glioma xenograft mouse model of GFP^{+} U373 cells. Cultured GFP^{+} U373 cells were injected stereotactically into the brains of BALB/c nude mice, followed by PBS control, PD-1^{WT} EGFRvIII-CAR-T cells and PD-1^{KD} EGFRvIII-CAR-T cells on day 10. Mice treated with PBS showed continuous tumor growth. PD-1^{WT} EGFRvIII-CAR-T cells had significant anti-tumor activity in comparison with the PBS injection (Fig. 5a). Notably, PD-1^{KD} EGFRvIII-CAR-T cell treatment exhibited stronger anti-tumor activity than that of PD-1^{WT} EGFRvIII-CAR-T cell treatment (Fig. 5a). Additionally, PD-1^{KD} EGFRvIII-CAR-T cell-treated mice exhibited longer survival than that of PD-1^{WT} EGFRvIII-CAR-T cell-treated mice (Fig. 5b).

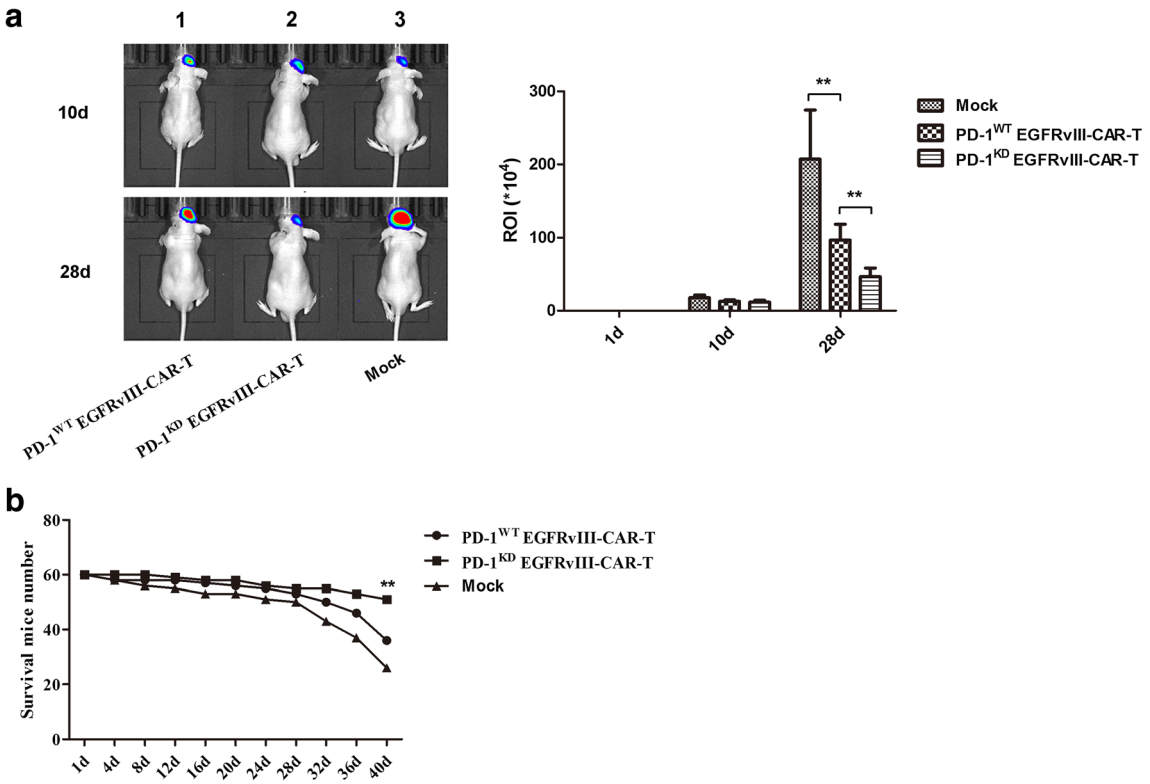

Fig. 5 The improved anti-glioma activity of EGFRvIII-CAR-T cells with PD-1 knockout in mice. Cultured U373-eGFP cells were injected stereotactically into the brains of BALB/c nude mice, followed by PBS control, PD-1^{WT} EGFRvIII-CAR-T cells and PD-1^{KD} EGFRvIII-CAR-T cells on day 10. Then, tumor size (**a**) and survival time of mice (**b**) were detected

Discussion

GBM is one of the most lethal adult cancers, as the majority of patients die within 15 months after diagnosis although the combined treatment of surgery, radiation and chemotherapy with temozolomide (TMZ) [2]. Adoptive anti-CD19 CAR-T cell therapy has produced promising results in the treatment of advanced B cell malignancies [20, 21]. However, the treatment of solid tumors including GBM with CAR-T cells is difficult, partially caused by the immunosuppressive effects of tumor microenvironments on infiltrated T cells [22].

The tumor-specific variant of the EGFR, namely EGFRvIII, is exclusively expressed on the tumor cell surface of up to 30% of GBM patients and other neoplasms but not expressed on normal tissues [23]. Therefore, EGFRvIII represents an ideal therapeutic target for CAR-T cell therapies against GBM [24, 25]. PD-L1 expression occurs in a variety of cancer cells including GBM cells [14–16, 26]. Acceptably, tumor-associated PD-L1 binds to PD-1 on CTL and promotes apoptosis of CTL, thus suppressing the anti-cancer immune response. It has been reported that glycogen synthase kinase 3 inhibition decreases PD-1 expression, promotes survival of CAR-T cells [18]. Therefore, we conceived that EGFRvIII-CAR-T cells with PD-1 blockage might exhibit stronger effect on PD-L1^{+} EGFRvIII^{+} GBM.

In our current studies, PD-1^{WT} EGFRvIII-CAR-T cells and PD-1^{KD} EGFRvIII-CAR-T cells were generated. Cytokine production and lytic activity of these two CAR-T cells against to PD-L1^{WT} EGFRvIII^{+} U373 cells or PD-L1^{KO} EGFRvIII^{+} U373 cells were evaluated. The results showed that PD-1^{KD} EGFRvIII-CAR-T cells and PD-1^{WT} EGFRvIII-CAR-T cells showed same levels of cytokine production and cytolytic activity against PD-L1^{KO} EGFRvIII^{+} U373 cells; however, PD-1^{KD} EGFRvIII-CAR-T cells exhibited higher levels of cytolytic activity against PD-L1^{WT} EGFRvIII^{+} U373 cells than that of PD-1^{WT} EGFRvIII-CAR-T cells. In vivo studies showed that PD-1^{KD} EGFRvIII-CAR-T cells also exhibited higher anti-glioma activity and longer survival in mice than that of PD-1^{WT} EGFRvIII-CAR-T cells.

Collectively, our studies indicate that EGFRvIII-CAR-T cells with PD-1 blockage might provide effective method for PD-L1^{+} EGFRvIII^{+} GBM therapy.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that there is no conflict of interest.

Ethical Approval All procedures performed in this study were in accordance with the ethical standards of the Affiliated Kunshan Hospital of Jiangsu University Ethics Committee for Scientific Research (No. 201901276) and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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