Nucleofection with Plasmid DNA for CRISPR/Cas9-Mediated Inactivation of Programmed Cell Death Protein 1 in CD133-Specific CAR T Cells

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CRISPR/Cas9-mediated programmed cell death protein 1 (*PD-1*) disruption in chimeric antigen receptor (CAR) T cells could be an appealing choice to improve the therapeutic efficacy of CAR T cells in an immunosuppressive tumor microenvironment. In most of the reported cases, Cas9 was delivered into T cells by way of electroporation with RNA or protein. However, transient expression of Cas9 by transfection with a plasmid encoding its gene is apparently simpler, as it avoids the steps of *in vitro* transcription of DNA or protein production. This study tried nucleofection into human primary T cells of plasmids encoding both CRISPR/Cas9 for disrupting the *PD-1* gene and the *piggyBac* transposon system for expressing CD133-specific CAR in one reaction. Based on drug selection, CD133-specific CAR T cells were obtained in which, on average, 91.5% of the *PD-1* gene sites were disrupted, but almost no Cas9 gene expression was found in the final engineered CAR T cells. The PD-1-deficient CD133-specific CAR T cells showed similar levels of cytokine secretion and improved proliferation and cytotoxicity *in vitro*, and enhanced inhibition of tumor growth in an orthotopic mouse model of glioma, compared to conventional CD133-CAR T cells. The described method could be useful for the production of PD-1-deficient CAR T cells for cancer immunotherapy.

Keywords: chimeric antigen receptor, immunotherapy, CRISPR/Cas9, transposon, PD-1, CD133

INTRODUCTION

CHIMERIC ANTIGEN RECEPTORS (CARs), which are composed of an antigen recognition unit (normally a single-chain variable fragment from a tumor-specific antibody), the hinge and transmembrane chain, and one to three intracellular signaling domains (derived from the T-cell receptor [TCR] complex and co-stimulatory molecules such as CD28 and 4-1BB), have been designed to modify T cells to target tumor cells.¹ When encountering the targeted tumor cells, the engineered CAR T cells can be activated and show strong antitumor activity. CAR T cells have shown very encouraging results in the treatment of hematological malignancies such as leukemia and lymphoma,² and two CD19-specific

CAR T-cell therapies, Kymriah and Yescarta, were recently approved for the treatment of acute lymphoblastic leukemia (ALL) and non-Hodgkin's lymphoma, respectively, in the United States. Although several reports have provided evidence that CAR T cells also show activity against solid tumors, less treatment benefits were found in patients with solid tumors.²⁻⁴ One important reason is that the tumor microenvironment is immunosuppressive, leading to the inhibition of CAR T-cell function. Checkpoint receptors, including programmed cell death protein 1 (PD-1), cytotoxic T lymphocyte-associated antigen 4 (CTLA-4), T-cell membrane protein 3 (TIM-3), lymphocyte activation gene 3 (LAG-3), and so on, were reported to be involved.⁵ PD-1 upregulation

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was found on tumor-infiltrating T cells,⁶ and its ligand PD-L1 was known to be constitutively expressed or upregulated on tumor cells in response to interferons.^{7,8} The recent success of anti-PD1 and anti-PD-L1 antibodies in the treatment of solid tumors clearly demonstrated that the PD-1/PD-L1 pathway is crucial for the immunotherapy of solid tumors.⁹ *PD-1* gene disruption seems to be a promising strategy to counteract immunosuppressive effects of the solid tumor environment on CAR T cells. Several groups have shown the feasibility of using the CRISPR/Cas9 genome-editing tool to knock out the *PD-1* gene in CAR T cells.^{10–12}

Genome editing has so far been reported mainly for CD19-specific CAR T cells. However, CAR T cells targeting solid tumors might benefit even more from genome editing, with the aim of counteracting the inhibition caused by components of an immunosuppressive microenvironment.

CD133 is a well-known marker for cancer stem cells in many solid tumor types, including glioma, lung, colon, and ovarian cancer.¹³ CD133-specific CAR T cells or natural killer (NK) cells have already been reported to kill patient-derived glioma stem cells or ovarian cancer stem cells.^{14,15} A 4.5-month-long partial response from CD133-specific CAR T-cell treatment has been achieved in a patient with advanced cholangiocarcinoma.¹⁶ *PD-1* gene targeting by CRISPR/Cas9 might help to improve CD133-specific CAR T cells.

The CRISPR/Cas9 system requires two essential components: the nuclease Cas9 and a chimeric single-guide RNA (sgRNA). With the guidance of the sgRNA, Cas9 binds to the target site and produces double-strand breaks, which are repaired by non-homologous end joining, causing unexpected gene mutations.¹⁷ In previous studies aiming at *PD-1* disruption in CAR T cells, Cas9-encoding mRNAs or Cas9 proteins were delivered into T cells by electroporation.^{10–12} However, for clinical use, the synthesis of RNA or protein would increase the number of manufacturing steps compared to the use of traditional gene delivery vehicles such as plasmid DNA. In addition, RNA or protein delivery has the disadvantage that the delivered elements are not stably integrated into the genome of the recipient cell. This means that for generating genome-edited CAR T cells, the T cells have to be modified at least twice (e.g., by using a combination of electroporation and virus transduction). Therefore, a simplified and efficient gene delivery method would be highly desirable for this purpose. Successful delivery of all elements into T cells using plasmids would be an attractive strategy, facilitating manufacture and reducing the modification steps.¹⁸

This study developed a simplified protocol for generating PD-1-deficient CD133-specific CAR T cells. Plasmids (not RNAs or proteins) encoding all elements for both genome editing (CRISPR/Cas9 system) and CAR gene delivery and expression (*piggyBac* transposon system) were nucleofected together into primary T cells. The efficiency of gene modification was evaluated, and the functionality of the PD-1-deficient CD133-specific CAR T cells was analyzed subsequently *in vitro* and *in vivo*.

MATERIALS AND METHODS

Plasmids

The plasmids used in this study have been described previously. The CD133-CAR *piggyBac* transposon vector and the Super *piggyBac* transposase plasmid for expression of the CD133-specific CAR were prepared according to Zhu *et al.*¹⁴ pST1374-Cas9-GFP and pGL3-U6-hPD-1-sgRNA(1+2) for PD-1 disruption in T cells were prepared following the protocols of Su *et al.*¹⁹

Primary cells and cell lines

Frozen peripheral blood mononuclear cells (PBMCs) from healthy donors were purchased from ALLCELLS (PB005F). The tumor cell lines used in this study were generated based on a previous report with modifications.²⁰ Briefly, the glioma cell line U251 was purchased from the Cell Bank of the Chinese Academy of Sciences. Based on this cell line, a CD133-overexpressing line (U251 CD133-OE) was generated by nucleofection with a *piggyBac* transposon vector encoding the human CD133 protein and the transposase plasmid, followed by drug selection. The stable transfectant was nucleofected again with a *piggyBac* transposon vector encoding firefly luciferase and the transposase plasmid, followed by drug selection to obtain a luciferase-expressing tumor cell line (U251 CD133-OE luc).

Gene editing and expansion of T cells

PBMCs were thawed and rested for 1.5–2 h. Then, 2×10^7 cells were re-suspended in 100 μ L of buffer from the Human T cell Nucleofector[®] kit (Lonza; VPA-1002) and used for one nucleofection reaction; 5 μ g of pST1374-Cas9-GFP, 5 μ g of pGL3-U6-hPD-1-sgRNA(1+2), 5 μ g of CD133-CAR *piggyBac* transposon vector, and 5 μ g of Super *piggyBac* transposase plasmid were added. For preparation of the conventional CAR T cells, only the *piggyBac* transposon and transposase were used. Electroporation was performed with a Nucleofector[™] II/2b device (Lonza) using the U-014 program. After

nucleofection, the cells were immediately transferred into 3 mL of prewarmed T-cell medium in a 12-well plate.

To stimulate the T cells, on day 0, Dynabeads™ Human T-Expander CD3/CD28 (ThermoFisher Scientific; 11141D) were added at a ratio of three beads per cell, and the cell culture was supplemented with 300 IU/mL of interleukin (IL)-2. On day 14, allogeneic PBMCs from five donors were irradiated with 40 Gy using an X-ray biological irradiator (Rad Source Technologies, Inc.; RS2000PRO) and added to the T-cell culture at a ratio of 10:1. In addition, the cell culture was supplemented with 50 ng/mL of anti-CD3 antibody (Miltenyi; 130-093-387) and 300 IU/mL of IL-2 for the second round of stimulation and expansion. Two days after each stimulation, the T cells were washed and further cultured in T-cell medium (RPMI 1640 + 10% fetal bovine serum, penicillin–streptomycin, and GlutaMAX) supplemented with 300 IU/mL of IL-2 for expansion. The medium was refreshed every 2–3 days. Seven days after the first stimulation, the beads were removed, and CD133-CAR-expressing T cells were selected with 1 µg/mL of puromycin. Transfected T cells were analyzed on day 27 (13 days after the second stimulation). Non-transfected T cells were generated as described above but without nucleofection and drug selection.

Flow cytometric analysis

Cells were washed with FACS buffer (phosphate-buffered saline [PBS] containing 0.5% bovine serum albumin and 2 mM of ethylenediaminetetraacetic acid) and stained in the same buffer. Antibodies were used at the concentrations according to the manufacturers' instructions. The cells were analyzed by flow cytometry on a CytoFLEX instrument (Beckman Coulter). The data were analyzed by CytExpert (Beckman Coulter) or FlowJo (Tree Star).

To determine CD133-CAR-positive cells after transfection, cells were collected 13 days after the second stimulation, washed and stained with mouse anti-c-Myc antibody (clone 9B11; Cell Signaling; 2276S), followed by a secondary rabbit anti-mouse immunoglobulin G F(ab')₂ antibody (Jackson ImmunoTech). CAR T-cell phenotype characterization was performed using anti-CD95 (BD; 562616), anti-CD45RO (BD; 555493), and anti-CD62L (BD; 559772).

To detect the expression of PD-L1 and CD133 on tumor cells, the cells were incubated with human FcR blocking reagent (Miltenyi; 130-059-901) for 10 min at 4°C. Then, the following antibodies were used: PE mouse anti-human CD274 (BD; 557924), CD133/1 (AC133)-PE (Miltenyi; 130-098-826). The

corresponding isotype control antibodies were used as controls.

PD-1 disruption in T cells was analyzed based on the expression of PD-1 on the cell surface: 10⁶ conventional CD133-CAR or PD-1-deficient CD133-CAR T cells were co-cultured with 10⁵ irradiated (70 Gy) U251-CD133 OE cells in 24-well plates in 2 mL of T-cell medium per well with 100 IU/mL of IL-2. Three days later, PE mouse anti-human CD279 (BD, 560795) and APC-H7 mouse anti-human CD3 (BD, 560176) were used to detect the PD-1 expression on CD3-positive T cells.

To determine the expression of Cas9 protein in T cells, indirect evidence was collected by flow cytometry based on green fluorescent protein (GFP) signals 1 day and 8 days after nucleofection. GFP was co-expressed with Cas9 as a fusion protein.

To determine the number of human T cells in mouse blood, red blood cell lysis buffer (Beyotime; C3702) was added for removing the red cells. The human T cells were stained with the following antibodies: PE-Cy™ 7 mouse anti-human CD45 (BD; 557748) and APC anti-human CD3 (4A Biotech; FHA003-050). Finally, the cells were fixed with 4% formalin solution, washed extensively using PBS, and analyzed by flow cytometry.

Efficiency of PD-1 gene disruption

PD-1 gene disruption was assessed, as previously described.¹⁹

Cytotoxicity assay

U251 CD133-OE luc cells (10⁴) were seeded into 96-well plates. Conventional or PD-1-deficient CD133-specific CAR T cells were added according to different effector-to-target cell ratios. The cells were cultured in 200 µL of T-cell medium without cytokines for 24 h. All cells were collected and transferred to a 96-well white microplate (PerkinElmer); 0.75 mg/mL of D-luciferin K⁺ salt (PerkinElmer; 122799) was added, and the signals were read immediately using an EnSpire Multimode plate reader (PerkinElmer). The percentage of target cell death was calculated according to the following formula: Lysis (%) = (1 – signal_{co-culture well} / signal_{tumor alone well}) × 100.

Cytokine secretion assay

Conventional or PD-1-deficient CD133-specific CAR T cells (2 × 10⁵) were co-cultured with 10⁵ U251 CD133-OE cells at an effector-to-target cell ratio of 2:1 in a 96-well plate in 200 µL of T-cell medium without cytokines for 24 h. The supernatant was collected, and the concentrations of cytokines in the

medium including interferon (IFN)- γ , IL-2, tumor necrosis factor (TNF)- α , and granulocyte-macrophage colony-stimulating factor (GM-CSF) were measured with the AlphaLISA kits from PerkinElmer (IL-2, AL221C; IFN- γ , AL217C; TNF- α , AL208C; and GM-CSF, AL216C) according to the manufacturer's instructions.

T-cell proliferation assay

T cells were labeled with 0.5 μ M of carboxy-fluorescein diacetate succinimidyl ester (CFSE; eBioscience; 65-0850-84) following the manufacturer's protocol. Target cells were irradiated with 70 Gy using an X-ray irradiator (Rad Source Technologies, Inc.; RS2000PRO). CFSE-labeled T cells (10^6) were co-cultured in a 24-well plate with 5×10^5 irradiated target cells in 1 mL of T-cell medium supplemented with 300 IU/mL of IL-2 per well. At the indicated time points, T-cell proliferation was analyzed by flow cytometry.

Antitumor function *in vivo*

All animal experiments in this study were approved by the Shanghai Administrative Committee for Laboratory Animals. The orthotopic mouse glioma model used has been described previously.¹⁴ For intracranial tumor implantation, 2×10^5 luciferase-expressing U251 CD133-OE luc cells were injected into the brain of 6- to 8-week-old female NPG mice (NOD-Prkdc^{scid} Il2rg^{null}; Beijing Vitalstar). To monitor tumor growth, the bioluminescence imaging (BLI) signals were measured using an IVIS spectrum imaging system (PerkinElmer) twice per week. For these measurements, the mice were anesthetized and received 150 mg/kg of D-luciferin K⁺ salt (PerkinElmer; 122799) intraperitoneally. On days 7, 11, and 15 after tumor cell injection, 2×10^6 PD-1-deficient or conventional CD133-CAR T cells or non-transfected T cells were injected via the original injection hole. BLI signals were continually monitored thereafter. Animals were regularly checked for health conditions and sacrificed if they had lost 20% of their body weight or showed signs of neurological deficits. The survival curve was generated using Prism.

Bio-safety assay

Female NOG (NOD.Cg-Prkdc^{scid} Il2rg^{tm1Sug}/JicCr1) immunodeficient mice (Beijing Vital River) at 4–6 weeks of age were inoculated with 5×10^6 conventional or PD-1-deficient CD133-specific CAR T cells in 200 μ L of PBS solution by intravenous injection. After inoculation, the animals were checked daily for mobility, food and water consumption, body weight gain/loss, ruffled fur, and any other abnormal effects.

Death and any other observed clinical signs were recorded. At days 7 and 30 post inoculation, peripheral blood samples of all mice were collected, and the remaining human T cells were measured by flow cytometry. At study termination, the mice were sacrificed and the liver, spleen, lung, and bones were harvested, fixed in 4% formalin solution, and further analyzed histopathologically by hematoxylin and eosin staining.

Statistical analysis

All the calculations were performed using GraphPad Prism v6.0 (GraphPad Software, Inc.). *p*-Values of <0.05 was considered statistically significant.

RESULTS

Development of PD-1-deficient CD133-specific CAR T cells

To generate PD-1-deficient CD133-specific CAR T cells, a protocol was developed based on two key points: (1) all the exogenous genes were delivered into T cells by nucleofection with plasmids; and (2) the cells with *PD-1* disruption were enriched according to their CAR expression by puromycin selection. The enrichment of PD-1-deficient CD133-specific CAR T cells had been designed according to the following two hypotheses: (1) multiple plasmids could enter one cell simultaneously; and (2) stable transposon-mediated integration of exogenous DNA could be assumed to occur less frequently than CRISPR/Cas9-mediated genome editing. The important elements in the plasmids encoding Cas9 and CAR are shown in Fig. 1A. GFP and c-Myc tag have been included in the constructs for the detection of Cas9 and CAR, respectively, and the puromycin resistance gene was co-expressed with the CAR through a T2A peptide for the selection of CAR-positive cells, as shown in Fig. 1A. The process is summarized in Fig. 1B. Briefly, PBMCs were isolated from blood. The four plasmids (coding for Cas9, the sgRNA, the transposase, and the transposon) were nucleofected into human T cells in one reaction. One day later, transfected T cells were activated by anti-CD3/CD28 beads and expanded in complete medium supplemented with IL-2. One week after activation, puromycin was added to enrich CAR-expressing T cells. On day 14, the T cells were activated again with anti-CD3 antibody and feeder cells, and IL-2 was supplemented to support expansion for another 13 days.

To determine whether the expansion folds of cells could be adequate for future clinical usage, a proliferation assay of cells from three individual

Figure 1. Generation of programmed cell death protein 1 (PD-1)-deficient CD133-specific chimeric antigen receptor (CAR) T cells. **(A)** Diagram of the transposon and the Cas9 expression cassettes. CD133-specific CAR contains the single-chain variable fragment with a c-Myc detection tag, cytoplasmic costimulatory domains from CD28 and 4-1BB, and a cytolytic CD3ζ domain. A puromycin resistance gene is separated from the CAR with a T2A ribosomal skip sequence for selecting CAR-expressing cells. **(B)** The plasmids encoding both the required components of the CRISPR/Cas9 and the *piggyBac* transposon system were nucleofected together into T cells on day -1. Anti-CD3/anti-CD28 beads were used to stimulate the T cells 1 day later. Engineered T cells were enriched by puromycin selection from day 7 onwards. On day 14, feeder cells from irradiated allogeneic peripheral blood mononuclear cells were used for the second round of expansion. Color images are available online.

donors was performed, and the cell numbers were recorded every 2–3 days. The proliferation rate varied depending on different donors (Fig. 2A). The average fold change of total cell number was 1.00 for the early stage (days 0–7 in the first round of expansion), 1.99 for the 7 days of puromycin selection, and 39.35 for the second round of expansion with puromycin. In total, an average 80-fold increase of cell number was achieved after two rounds of expansion. In addition, a big cell loss before stimulation was observed, as nucleofection was toxic to T cells, and part of cells in PBMC even died without nucleofection (Fig. 2B).

To assess the expression of the CD133-specific CAR, T cells were analyzed by flow cytometry for expression of the c-Myc tag included in the CAR construct. As shown in Fig. 2C, CAR-positive T cells were enriched by puromycin selection significantly, and a majority of the drug-selected cells expressed the CD133-specific CAR after the second round of expansion. In addition, the transposition efficiency of transposon system we used has been evaluated according to the following formula: percentage of c-Myc positive cells/transient transfection efficiency (shown in Fig. 2D).

To determine whether the genome-edited CAR T cells express the non-human Cas9 protein, the expression of Cas9 was determined by flow cytom-

etry by detecting the GFP co-expressed with Cas9. One day after nucleofection, the transfection efficiency for Cas9 was about 39.7% (Fig. 2E). However, 7 days later, Cas9 expression was no longer detected by flow cytometry. Therefore, a Cas9-induced immune response can probably be avoided when genome-edited CAR T cells are therapeutically used in patients.

To confirm the differentiation state of the products, CD95, CD45RO, and CD62L on the cell surface were checked by antibody staining. All the CAR T cells were CD95 positive, and more than half of them expressed CD62L and CD45RO, a surface phenotype typical of central memory T cells,²¹ corresponding to an intermediate state of T-cell differentiation (Fig. 3), which has also been reported in other studies when using allogeneic PBMCs as feeder cells for the T-cell expansion.^{22,23}

PD-1 knockout efficiency on both the DNA and protein levels

To determine the efficiency of CRISPR/Cas9-mediated genome editing in CD133-specific CAR T cells, first the PD-1 knockout efficiency was analyzed on the genome level. Genomic DNA was extracted from CD133-specific CAR T cells, and the *PD-1* gene was amplified by PCR. The PCR products were incubated with the T7EN1 enzyme. As

Figure 2. Proliferation and exogenous gene expression of PD-1-deficient CD133-specific CAR T cells. **(A)** Expansion folds of T cells were determined for different stages. **(B)** Survival cell proportion 1 day after nucleofection compared to the number before nucleofection. **(C)** CAR expression on the enriched T cells was determined according to flow-cytometric detection of the c-Myc tag. T cells were stained with mouse anti-c-Myc antibody followed by a PE-labeled rabbit anti-mouse secondary antibody. Data shown are representative of several independent experiments. **(D)** Transposition efficiency from three independent experiments was calculated according to the following formula: percentage of c-Myc positive cells/transient transfection efficiency. **(E)** Cas9 expression was determined according to the fused green fluorescent protein. The expression was transiently visible on day 0 but already absent on day 7. Color images are available online.

shown in Fig. 4A, the edited *PD-1* genes were recognized by T7EN1 and cut to produce more than one smaller fragment. In addition, the PCR products were cloned and sequenced. As shown in Fig. 4B, insertions and deletions were found in 89.5–95% of the *PD-1* gene sites, summarized for three individual donors.

To stimulate PD-1 expression on the cell surface, conventional CD133-specific CAR T cells or PD-1-deficient CD133-specific CAR T cells were co-cultured with irradiated U251 CD133-OE cells, and PD-1 expression was thereafter measured by flow cytometry. As shown in Fig. 4C, after 3 days of co-incubation, PD-1 was upregulated on 49.2% of

Figure 3. T-cell differentiation marker expression after two rounds of expansion. CAR T cells were stained with anti-CD95 (A), anti-CD62L and anti-CD45RO (B), or matched isotype control antibodies for setting gates. More than half of the cells in both groups had a central memory phenotype (CD45RO⁺ CD62L⁺). (C) Summary data for four donors ($M \pm SD$). Color images are available online.

the conventional CD133-specific CAR T cells. In contrast, only 4.0% of the cells were positive for PD-1 in the genome-edited CAR T-cell group.

Effector functions of PD-1-deficient CD133-specific CAR T cells

First, the expression levels of both the CAR target and PD-L1 on the target cells were checked by flow cytometry. U251 CD133-OE cells were found to express both CD133 and PD-L1 highly on their surface (Fig. 5A).

To determine the cytolytic capacity of the PD-1-deficient CD133-specific CAR T cells, firefly luciferase-expressing target cells (CD133-overexpressing luc⁺ U251 glioma cells) were co-cultured with CAR T cells for 24 h. Target cell death and viability were analyzed based on the luciferase signals. As shown in Fig. 5B, significantly stronger lytic capacity was found for the PD-1-deficient CD133-specific CAR T cells when

the effector-to-target cell ratios were 8:1, 4:1, or 2:1 compared to conventional CAR T cells.

To analyze cytokine secretion, the T cells were stimulated with the target cells for 24 h. IL-2, IFN- γ , TNF- α , and GM-CSF released into the medium were measured thereafter by AlphaLISA assay. As shown in Fig. 5C, the PD-1-deficient CD133-specific CAR T cells secreted similar amounts of all four cytokines when compared with conventional CAR T cells.

To explore the expansion ability of PD-1-deficient CD133-specific CAR T cells, conventional or PD-1-deficient CD133-specific CAR T cells were co-cultured with irradiated PD-L1⁺ CD133⁺ target cells. As shown in Fig. 5D, on days 3 and 4, there was no significant difference in the proliferation rates between conventional and PD-1-deficient CD133-specific CAR T cells. However, on day 5, a proportion of the PD-1-deficient CD133-specific CAR T cells showed a higher proliferation rate than the conventional CD133-specific CAR T cells, as assessed by CFSE dilution.

Figure 4. *PD-1* knockout efficiency in three individual donors. **(A)** The efficiency of *PD-1* gene disruption was tested in three individual donors: #319, #429, and #522. All samples showed high rates of insertions and deletions (indels) compared to the controls. **(B)** The most common form of indels was whole-fragment deletion, with rates of 14/20, 14/19, and 15/20. **(C)** *PD-1* disruption was confirmed by assessing PD-1 expression after co-culture with U251 CD133-OE cells, which caused PD-1 upregulation on conventional (CAR alone) but not on the PD-1-deficient CAR T cells (PD1 KO CAR). Color images are available online.

In vivo antitumor activity

To determine whether the PD-1-deficient CD133-specific CAR T cells enhanced antitumor activity *in vivo*, an orthotopic glioma model was used in immunodeficient mice, as described previously. CD133-overexpressing U251 glioma cells were orthotopically injected into the brains of immunodeficient mice;

7 days later, non-transfected T cells, conventional CAR T cells, or PD-1-deficient CAR T cells were injected into the tumor sites, followed by two additional rounds of treatment in the next 2 weeks. Both the mice treated with conventional CD133-CAR T cells and those treated with PD-1-deficient CD133-CAR T cells showed prolonged survival.

Figure 5. Conventional and PD-1-deficient CD133-specific CAR T cells had comparable antitumor effects *in vitro*. **(A)** Flow cytometric analysis showing the expression of both CD133 and PD-L1 on the target cells (U251 CD133-OE) used in this study. **(B)** Cytotoxic assays. Upon co-culture with the U251 CD133-OE target cells at effector-to-target cell ratios of 2:1, 4:1, and 8:1, PD-1-deficient CAR T cells showed a higher antitumor effect than conventional CAR T cells. * $p < 0.05$ by multiple t -test. **(C)** Cytokine secretion by CAR T cells upon contact with target cells. Different types of CAR T cells were co-cultured with target cells for 24 h. Thereafter, culture supernatants were collected and analyzed for IFN- γ , IL-2, TNF- α , and GM-CSF by AlphaLISA. PD-1-deficient CAR T cells produced similar amounts of cytokines upon stimulation with U251 CD133-OE cells compared to conventional CAR T cells. **(D)** Comparison of the CAR T-cell proliferation rates induced by contact with target cells. CAR T cells were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE) and then co-cultured with the irradiated target cells for 5 days. The extent of T-cell proliferation was reflected by the loss of incorporated CFSE. On day 5, a proportion of cells in the cultures of PD-1-deficient CD133-specific CAR T cells showed a higher proliferation rate. The experiments were confirmed in at least three individual donors. In **(B)** and **(C)**, data are shown as $M \pm SD$ from triplicate experiments. Color images are available online.

However, the mice treated with PD-1-deficient CD133-CAR T cells had a significantly longer survival time compared to those treated with the conventional CAR T cells (Fig. 6A). Tumor sizes were also assessed based on BLI signals. Using this assay, it was found that both the conven-

tional and the PD-1-deficient CD133-CAR T cells inhibited tumor growth. However, on day 28, the mice treated with PD-1-deficient CD133-CAR T cells showed lower luciferase signals compared to those treated with conventional CAR T cells (Fig. 6B).

Figure 6. PD-1-deficient CAR T cells showed therapeutic efficacy against established, orthotopic glioma xenografts in immunodeficient mice. Firefly luciferase-expressing U251 CD133-OE cells (2×10^5) were injected into the forebrains of immunodeficient mice. On days 7, 11, and 15 of tumor growth, 2×10^6 CAR T cells or non-transfected T cells were injected via the same coordinates. Mice with $>20\%$ weight loss were euthanized. **(A)** Kaplan-Meier analysis revealed a significantly longer survival for mice treated with PD-1-deficient CAR T cells compared to those treated with conventional CAR T cells ($p = 0.0132$ by log-rank [Mantel-Cox] test). **(B)** Bioluminescence images taken before (day 2) and after (days 9 and 28) treatment with non-transfected control T cells, conventional CAR T cells, or PD-1-deficient CAR T cells. NT, non-transfected control T cells. Color images are available online.

Safety in immunodeficient mice

To assess the safety of the PD-1-deficient CD133-specific CAR T cells *in vivo*, potential uncontrolled proliferation, signs of graft-versus-host disease (GvHD), and other potential signs of acute toxicity were investigated upon intravenous injection of conventional or PD-1-deficient CD133-specific CAR T cells into immunodeficient mice. CAR T-cell numbers in the blood were detected by flow cytometry (Fig. 7A). Both conventional and PD-1-deficient CAR T cells were detectable (mean 0.5% and 0.67%, respectively) 1 week after injection, but had almost disappeared (mean 0.02% and 0.04%, respectively) by day 28 (Fig. 7B), demonstrating that there was no excessive proliferation or lymphoma formation due to the deletion of PD-1. Likewise, no GvHD signs or deaths were found. All the mice were euthanatized 28 days after the start of treatment, and the vital organs were collected and subjected to pathology analysis in which no toxicity-related abnormalities were detected (Supplementary Table S1). These findings together with the efficacy data presented above warrant further exploration of PD-1-deficient CAR T cells in clinical trials.

DISCUSSION

This study developed a protocol in which plasmids encoding components of both the CRISPR/Cas9 and the *piggyBac* transposon/transposase system required for the production of genome-edited CAR T cells were nucleofected together into T cells. The CRISPR/Cas9-edited cells were selected based on stable transposon integration indicated by CAR expression. Using this protocol, CD133-specific CAR T cells with *PD-1* gene knock-down were generated successfully and showed similar or enhanced *in vitro* functions and improved tumor inhibition *in vivo* compared to conventional CAR T cells. The study presents a simple but efficient method for CRISPR/Cas9-mediated genome editing in CAR T cells, and a new type of CAR T cells (*i.e.*, PD-1-deficient CD133-specific CAR T cells), which might be therapeutically useful in treating a wide range of tumor types containing CD133+ cancer stem cells.

In previous studies, *in vitro* transcribed Cas9 mRNA or Cas9 protein was delivered into T cells to generate PD-1-deficient CD19-specific CAR T cells based on the combination with virus transduction.^{10–12} However, compared to RNA, protein, and virus, plasmid DNA is easier to manufacture at lower cost, especially in Good Manufacturing Practice (GMP) grade. Using DNA as gene vehicle has additional advantages. First, multiple plasmids can be electroporated simultaneously into one cell, and plasmids have sufficiently high capacity to carry multiple elements. Thus, the system will possibly enable editing of multiple genes in CAR T cells, thus generating so-called universal CAR T cells, or CAR T cells in which multiple inhibitory checkpoints are silenced. Second, transient, plasmid-mediated expression of the *Cas9* gene does not pose the problem of stable *Cas9* gene integration, which may cause immunogenicity when used in humans, as the Cas9 protein is not of human origin. Lastly, in the present protocol, transduction using virus is avoided by employing the *piggyBac* transposon system. Besides, if a viral system is chosen for delivery of the CAR gene, additional steps for transfection of CRISPR/Cas9 system need to be performed. In contrast, the current protocol does not need too many additional steps, except mixing the plasmid-encoding CRISPR/Cas9 system together with the encoding transposon system for nucleofection. Thus, the protocol may provide an alternative method for genome-edited CAR T-cell production.

However, DNA electroporation is quite toxic for T cells, causing substantial cell death at the beginning of the manufacturing process. This, combined

Figure 7. PD-1-deficient CAR T cells did not show uncontrolled proliferation in immunodeficient mice. **(A)** Conventional CAR (CAR alone) or PD-1-deficient CAR (PD1 KO CAR) T cells were intravenously injected into immunodeficient mice. The number of human T cells (human CD45+ CD3+) in mouse blood was measured by flow cytometry on days 7 and 28. **(B)** The frequency of human CAR T cells from both groups significantly decreased from day 7 to day 28. * $p < 0.05$ and ** $p < 0.01$ by multiple t -tests. Color images are available online.

with the relatively low transgene efficiency of transposon transfection compared to retrovirus transduction, renders the whole manufacture process longer. Some groups working on the generation of clinical-grade CAR T cells using the transposon system have tried to optimize the expansion time (e.g., by using PBMCs or artificial antigen-presenting

cells for enrichment or mini-circle plasmids for gene delivery).^{18,24,25} Currently, improved electroporation devices are available, and in the future, it may be possible to reduce the toxic effects on human T cells caused by electroporation (e.g., by optimizing the electroporation conditions). Currently, the puromycin resistance gene and antibiotic selection are used

for enrichment of CAR-positive T cells. For further development of CAR T cells in the clinical setting, CAR T cells may be enriched by sorting based on CAR expression, using GMP-grade equipment (e.g., Clin-iMACS Prodigy from Miltenyi). To obtain sufficient cells for adoptive transfer, the expansion rate should be judged. According to a previous clinical report, two batches of IL13R α 2-targeting CAR T-cells were manufactured for the treatment of a patient with recurrent multifocal glioblastoma, and regression of all intracranial and spinal tumors was observed.³ For the 16 infusions, a total of 9.4×10^7 and 5.0×10^7 cells have been taken from the two batches of products. Considering the current protocol, to obtain 1.0×10^8 PD-1 KO CAR T cells, 1.0×10^7 PBMCs of starting materials will be required, which should not be difficult in most cases, especially when a leukapheresis device is applied to the patient instead of drawing blood directly. It has been reported that an average of 6.7×10^9 PBMCs could be acquired from one leukapheresis product.²⁶

In principle, *PD-1* disruption will give CD133-specific CAR T cells the capacity to resist PD-1/PD-L1-induced immunosuppression. The present data show that *PD-1* disruption did improve the *in vitro* cytotoxicity and the *in vivo* antitumor activity in immunodeficient mice. In addition, the study found no uncontrolled proliferation of PD-1-deficient CAR T cells or evident toxicity induced by them in immunodeficient mice. These data support the evaluation of PD-1-deficient CD133-specific CAR T cells for safety and clinical efficacy within clinical trials. Moreover, the data more generally advocate the evaluation of genome-edited CAR T cells such as PD-1-deficient CAR T cells for clinical use in the therapy of cancer patients.

How PD-1-deficient CD133-specific CAR T cells contribute to better antitumor activity *in vivo* is an important question and requires further studies. The authors and other groups have analyzed the *in vitro* functions of genome-edited T cells, including cytotoxicity, cytokine secretion, and proliferation.^{11,12} Liu *et al.* found significant improvement in cytokine secretion for PD-1 knockout CD19-CAR T cells.¹¹ Rupp *et al.* observed enhanced cytotoxicity of PD-1 knockout CD19-CAR T cells.¹² In this study, only enhanced cytotoxicity and proliferation were found for PD-1-deficient CD133-specific CAR T cells; cytokine secretion was similar to that of conventional CAR T cells. The clear molecular and cellular effects caused by *PD-1* gene disruption in CAR T cells need further exploration in the future. In addition, CARs with different intracellular signaling domains may have different abilities to resist immunosuppression.

Cherkassky *et al.* showed that a 4-1BB CAR worked better in the immunosuppressive tumor microenvironment than a CD28 CAR.²⁷ In this study, a third-generation CAR was gene-edited that includes both CD28 and 4-1BB signaling domains. How different types of CAR-associated signaling domains are coordinated with the PD-1 signaling pathway will also require future studies.

Independent of CAR transduction or transfection, Su *et al.* observed enhanced cytokine secretion and cytotoxicity following the disruption of *PD-1* in the primary T cells of patients.¹⁹ In contrast, a study on zinc finger nuclease-mediated gene editing of *PD-1* in tumor-infiltrating lymphocytes (TILs) reported a significantly increased polyfunctional cytokine profile (TNF- α , GM-CSF, and IFN- γ) but no enhanced cytotoxicity in the *PD-1* genome-edited TILs.²⁸

In this study, simple models were used, including co-culture of CAR T cells with tumor cells *in vitro* and treatment of tumor-bearing immunodeficient mice with human CAR T cells, as used before.^{14,20,29} Although a CD133 highly expressed U251 cell line cannot be a very suitable model to represent the patient situation, it was used to judge the CAR T cells' capability of killing tumors on a quantified level. Furthermore, though the tumor cells used expressed high levels of PD-L1, it is not clear whether the PD-1/PD-L1 pathway is the main negative regulator of CAR T cells in the models. Other immunosuppressive elements that play roles in the regulation of T-cell-mediated immune responses are not considered in the models. In the future, humanized mice, which rebuild the human immune system, may be used to study the impact of genome-edited CAR T cells.

In conclusion, a simple protocol is provided for CRISPR/Cas9-mediated genome editing in CAR T cells based on electroporation of DNA. The PD-1-deficient CD133-specific CAR T cells showed efficient antitumor function. Further research on the clinical performance and the mechanism of action of this new type of CAR T cells in the treatment of solid tumors is warranted.

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AUTHOR DISCLOSURE

No competing financial interests exist.

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