Development of T Cells Redirected to Glypican-3 for the Treatment of Hepatocellular Carcinoma

Running title: GPC3-targeted CAR T Cells for HCC treatment

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Translational Relevance

There are limited strategies for the treatment of hepatocellular carcinomas (HCCs). A majority of HCC cells express Glypican-3 (GPC3), which has restricted expression in normal tissues. In this study, we engineered T cells with a first and a third generation (composed of costimulatory signaling domains from CD28 and CD137) chimeric antigen receptor (CAR) targeting GPC3. Our study indicates that the third generation GPC3-targeted CAR T cells can efficiently destroy GPC3-positive human HCC cells in vitro and in vivo. Our preclinical study suggests that adoptive transfer of T cells expressing a CAR targeting GPC3 presents a promising therapeutic strategy to treat HCC. Future clinical trials on this novel strategy are warranted.
Abstract

**Purpose:** The aim of our study is to elucidate whether T cells expressing GPC3-targeted chimeric antigen receptor (CAR) can efficiently eliminate GPC3-positive HCC cells and their potential in the treatment of HCC.

**Experimental Design:** T cells expressing a first generation and third generation GPC3-targeted CAR were prepared using lentiviral vector transduction. The *in vitro* and *in vivo* cytotoxic activities of the genetically engineered CAR T cells were evaluated against various HCC cell lines.

**Results:** GPC3-targeted CAR T cells could efficiently kill GPC3-positive HCC cells but not GPC3-negative cells *in vitro*. These cytotoxic activities appeared to be positively correlated with GPC3 expression levels in the target cells. Additionally, T cells expressing the third generation GPC3-targeted CAR could eradicate HCC xenografts with high level of GPC3 expression and efficiently suppress the growth of HCC xenografts with low GPC3 expression level *in vivo*. The survival of the mice bearing established orthotopic Huh-7 xenografts was significantly prolonged by the treatment with the third generation GPC3-targeted CAR T cells.

**Conclusions:** GPC3-targeted CAR T cells could potently eliminate GPC3-positive HCC cells, thereby providing a promising therapeutic intervention for GPC3-positive HCC.
Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer and the third most common cause of cancer mortality worldwide (1). Currently, surgery is the most effective treatment for HCC. However, tumor recurrence after a curative liver resection is very high, and the 5-year survival rate remains at only 10% (2). Moreover, because the majority of patients with HCC are diagnosed at a late disease stage, potentially curative therapies, including chemotherapy, chemoembolization, ablation and proton beam therapy, are frequently ineffective. Sorafenib (Nexavar), the first clinically approved targeted drug therapy for HCC, could only extend the overall survival by 2-3 months (3). Thus, it remains an urgent need for effective, life-prolonging strategies in the management of HCC patients.

Immunotherapy based on T cells modified with a chimeric antigen receptor (CAR) has been demonstrated as a promising strategy for cancer treatment (4). CAR T cells can specifically recognize tumor-associated antigen and eliminate tumor cells in a non-major histocompatibility complex-restricted manner. Several pilot clinical trials using CAR T cells have recently been reported with promising clinical outcomes (5).

GPC3 is a member of the glypican family of heparan sulfate (HS) proteoglycans that are attached to the cell surface by a glycosylphosphatidylinositol (GPI) anchor. GPC3 plays an important role in cellular growth, differentiation and migration (6). Multiple studies have demonstrated that GPC3 is an attractive liver-cancer-specific target because it is highly expressed in HCC but limited expressed in normal tissues (7). Currently, GPC3-targeted immunotherapeutic strategies utilizing antibodies or a
peptide-vaccine have been explored for HCC treatment (8-12). The clinical data of the monoclonal antibody GC33 indicated that it was well tolerated in advanced HCC, suggesting that GPC3 is a relatively safe target. However, according to experimental knockdown results, GPC3 is not a lethal gene to HCC cells (13). Moreover, the naked antibody could not completely eliminate tumors in mouse models, and no partial or complete response was observed in the fifteen patients evaluated during the phase I clinical trial of GC33, suggesting that the therapeutic effects of a naked antibody may not be potent enough for curative treatment of HCC (14). In the phase I clinical trial of a GPC3-derived peptide vaccine for HCC patients, the median overall survival appeared to positively correlate with the GPC3-specific CTL frequencies, suggesting that GPC3-targeted T cells could be potential agents for HCC treatment (12). However, GPC3-based, CAR-engineered T cells have never been reported. Therefore, in this study, we developed GPC3-specific CAR T cells and explored their potential for HCC treatment.
Materials and Methods

Construction of lentiviral vectors encoding CARs

The sequence encoding the anti-GPC3 scFv antibody in the VL-VH orientation (Supplementary Fig. S1), based on the sequence of GC33, which recognizes the C-terminal region of GPC3 (8), was obtained by splicing PCR and the overlap extension technique. As shown in Figure 1A, the αGPC3-Z CAR (first generation) comprising the scFv GPC3 linked to the human CD8α hinge and transmembrane domain (nucleotides 412-609, GenBank NM 001768.6) was fused to the intracellular signaling domain derived from only the CD3ξ molecule (nucleotides 154-492, GenBank NM 198253.2). The αGPC3-28BBZ CAR (third generation) comprising the scFv GPC3 linked in-frame to the hinge domain of the CD8α molecule (nucleotides 412-546, GenBank NM 001768.6) was fused to the transmembrane region of the human CD28 molecule (nucleotides 457-537, GenBank NM 006139.3), and the intracellular signaling domains (nucleotides 538-660, GenBank NM 006139.3), CD137 (nucleotides 640-765, GenBank NM 001561.5), and CD3ξ molecules (nucleotides 154-492, GenBank NM 198253.2) in tandem. One CAR (αGPC3-DZ) carrying the truncated CD3ξ (nucleotides 154-189, GenBank NM 198253.2), and another CAR (2D3-28BBZ) containing the irrelevant scFv 2D3 derived from a hybridoma against the intracellular domain of EGFRvA (15) were set as controls. All CAR constructs were overlapped with eGFP through a ribosomal skipping sequence (F2A) derived from the foot and mouth disease (FMD) virus (16). Finally, all the products contained an MluI site at the 5’ end and a SalI site...
at the 3’ end and were ligated into the third generation non-self-inactivating EF-1α promoter-based lentiviral expression vector pWPT-eGFP (17). The primers are listed in Supplementary Table S1.

**Cell lines**

Human HCC cell lines (HepG2, Hep3B, PLC/PRF/5 and SK-HEP-1) and 293T were obtained from the American Type Culture Collection (ATCC, Manassas, VA). Huh-7 cell line was obtained from the RIKEN Cell Bank (Tsukuba, Japan). These cell lines were tested and authenticated by DNA profiling for polymorphic short-tandem repeat (STR) markers. For bioluminescence assays, Huh-7 cells with enforced expression of firefly luciferase (fLuc+) were established. 293T and HCC cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FBS (Gibco, UK). All cells were routinely tested for mycoplasma contamination.

**Lentivirus production**

Recombinant lentiviral particles were produced by a calcium phosphate transfection system (17). Lentiviral particles were concentrated 30-fold by ultracentrifugation (Beckman Optima™ XL-100 K, Beckman, Germany) for 2 h at 28,000 rpm.

**Isolation, activation, transduction and expansion of human T cells**

Peripheral blood mononuclear cells (PBMCs) derived from human donors were
provided by the Shanghai Blood Center. Primary human CD4⁺ and CD8⁺ T cells were isolated from PBMCs by the negative selection using RosetteSep kits (Stem Cells Technology, Vancouver BC, Canada). CD4⁺ and CD8⁺ T cells mixed at a 1 : 1 ratio were stimulated for 24 h with anti-CD3/anti-CD28 antibodies immobilized on tosyl-activated paramagnetic beads (Invitrogen, Carlsbad, CA) at a cell : bead ratio of 1 : 1 in accordance with the manufacturer’s instructions. T cells were then transduced with the lentiviral vector at a multiplicity of infection (MOI) of = 8 unit/cell. The transduced T cells were cultured at a concentration of 5 × 10⁵ cells/ml in the presence of recombinant human IL-2 (300 U/ml) (Shanghai Huaxin High Biotech, Shanghai, China) every other day. Genetically modified T cells were used for functional assays when the lymphocyte volume and proliferation showed a decrease.

**Western blot analysis**

The cell lysate was denatured and electrophoresed by SDS-PAGE. The samples were then transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA) and immunoblotted with the targeted antibodies.

**Cytotoxicity assays in vitro**

The specific cytotoxicity of CAR-modified T cells towards various HCC cells at the different effector : target ratios of 3 : 1, 1 : 1 and 1 : 3 was measured after coculturing for 18 hours by lactate dehydrogenase (LDH) assay using the CytoTox 96® non-radioactive cytotoxicity kit (Promega, Madison, WI) according to the
manufacturer’s instructions.

Cytokine release assays

The IFN-γ and IL-2 cytokines secreted by the varying genetically modified T cells were measured using an ELISA kit (MultiSciences Biotechnology, Hangzhou, China).

Xenograft models of human hepatocellular carcinoma

Six- to eight-week-old NOD/SCID mice were housed and treated under specific pathogen-free conditions at the Experimental Animal Center of Shanghai Jiaotong University School of Medicine. All animal experiments were carried out according to the protocols approved by the Shanghai Medical Experimental Animal Care Commission. For the established subcutaneous (s.c.) Huh-7 models, mice were inoculated s.c. with $2 \times 10^6$ Huh-7 cells on the right flank on day 0. When the tumor burden was approximately 200-300 mm$^3$, mice were randomly allocated into four groups (n = 6) and assigned to receive one of the following injections: (i) genetically modified $\alpha$GPC3-28BBZ CAR T cells dissolved in sterile saline ($\alpha$GPC3-28BBZ); (ii) genetically modified 2D3-28BBZ CAR T cells in sterile saline (2D3-28BBZ); (iii) genetically modified mock T cells in sterile saline (Mock); (iv) Saline only without T cells. On day 13, To deplete of host lymphocyte compartments using cyclophosphamide and enhance the tumor treatment efficacy of the administered T cells (18), mice were injected intraperitoneally (i.p.) with 200 mg/kg of cyclophosphamide. Then $8 \times 10^6$ $\alpha$GPC3-28BBZ CAR T cells were intravenously (i.v.)
injected on day 14. In established s.c. PLC/PRF/5 models, mice were inoculated with $5 \times 10^6$ PLC/PRF/5 cells in the right flank on day 0. On day 21, when the tumor burden was about 100-150 mm$^3$, the mice were received cyclophosphamide (200 mg/kg i.p.), two doses of $8 \times 10^6 \alpha$GPC3-28BBZ CAR T cells were i.v. injected on day 22 and 30. Tumor dimensions were measured with calipers, and tumor volumes were calculated using the formula $V = \frac{1}{2} (\text{length} \times \text{width}^2)$. Mice were euthanized when the mean tumor burden in the control mice reached 1500-2000 mm$^3$. Xenografts were fixed with formalin, embedded in paraffin, and sections were cut and processed for immunohistochemistry staining. For the established orthotopic HCC model, mice were inoculated with $2 \times 10^6$ Huh-7 (fLuc+) cells in the liver on day 0. On day 13, mice were grouped ($n = 5$) and received the injection of cyclophosphamide (200 mg/kg i.p.) followed by the treatments with two doses of i.v. injections of $5 \times 10^6$ genetically modified T cells on day 14 and 21. The mice were imaged every week following T cell administration to evaluate whether $\alpha$GPC3-28BBZ CAR T cells could suppress tumor growth or prolong the survival of the mice bearing orthotopic HCC xenografts. The transduction efficiencies of genetically modified T cells used in experimental assays were about 50%.

**Bioluminescence imaging**

Isoflurane-anesthetized animals were imaged using the IVIS system (IVIS, Xenogen, Alameda, CA) followed by the intraperitoneally (i.p.) injection of 150 mg/kg D-luciferin (Promega, Madison, WI). The data were quantified using Living
Image software (Caliper Life Sciences, Hopkinton, MA).

**Immunohistochemistry**

The HLiv-HCC150CS-01 tissue microarray (Outdo Biotech, Shanghai, China) and sections of the normal kidney and gastric glands were immunostained using an anti-GPC3 antibody (mAb 1G12, BioMosaics Inc, Burlington, VT). To assess the persistence of the administrated human T cells in xenografts, the sections of formalin-fixed, paraffin-embedded tumor tissues were immunostained using an anti-CD3 antibody (Thermo Scientific RM-9107-S0).

**Statistical analysis**

Statistical analysis was performed by 2-way repeated measures ANOVA with Bonferroni post-tests for the tumor burden (tumor volume, tumor weight and photon counts). Differences in the absolute number of various transferred T cells were evaluated by Student's *t*-test. The overall survival statistics were calculated using the log-rank test (19). GraphPad Prism 5.0 was used for the statistical calculations. *P* < 0.05 (*), *P* < 0.01 (**) and *P* < 0.001(***) was considered statistically significant.

Additional information was described in Supplementary Methods.
Results

Generation of CAR-GPC3 T cells by lentiviral vector transduction

As shown in Figure 1A, the lentiviral expression vectors encoding the GPC3-targeted CARs including αGPC3-Z, αGPC3-2BBBZ or negative control CARs including αGPC3-DZ and 2D3-2BBBZ efficiently transduced human T cells to co-express the CAR protein and eGFP using the “self-cleaving” F2A peptide, respectively. The genetically modified T cells began to expand after activation. On day 7, the expression of the different CARs in the transduced T cells was demonstrated through eGFP expression. The transduction efficiencies were about 55% (Fig. 1B). According to viable cell counting assays (trypan blue exclusion), the αCD3/αCD28-coated beads could induce 200-300 fold expansions of CAR-modified T cells on day 18. In the presence of OKT3 (100 ng/ml) and recombinant human IL-2 (300 U/ml), the expansion could be further increased by 10-20 fold (Fig. 1C) after subsequent stimulation for 7 days with cells of γ-irradiated K562-based artificial antigen-presenting cells (aAPC) (referred to as aK562-64/86) which express the membrane-bound extra-domains of CD64 and CD86 (Supplementary Fig. S2). To determine the antigen-specific expansion, the well-known GPC3-positive Huh-7 cells and GPC3-negative SK-HEP-1 cells were used for specific stimulations on day 18. The results indicated that T cells expressing αGPC3-2BBBZ were expanded by the stimulation of GPC3-positive Huh-7 cells but not by GPC3-negative SK-HEP-1 cells. Additionally, both GPC3⁺ and GPC3⁻ HCC cell lines failed to elicit the expansion of mock or 2D3-2BBBZ transduced T cells (Fig. 1D).
**Cytokines produced by GPC3-targeted CAR T cells**

Surface expression of GPC3 on five HCC cell lines was determined. The results of FACS and western blot (Fig. 2A) confirmed that SK-HEP-1 had no GPC3 expression, and the other four HCC cell lines had varying levels of GPC3 expression. In the cytokine production assay, greater amounts of IL-2 (Fig. 2B) and IFN-γ (Fig. 2C) were produced by αGPC3-28BBZ CAR T cells than αGPC3-Z CAR T cells or the control T cells including mock, αGPC3-DZ or 2D3-28BBZ transduced T cells, when cocultured with GPC3-positive cells. However, almost no induction of IL-2 or IFN-γ was observed when any of the engineered T cells were cocultured with SK-HEP-1 cells (Fig. 2B, C). Intriguingly, there was a positive correlation between IFN-γ secretion in GPC3-redirected CAR T cells transfected with either αGPC3-28BBZ or αGPC3-Z and the MFI of GPC3 expression on the target cells (Fig. 2D).

**In vitro cytotoxicity of T cells redirected to GPC3 on HCC cells**

To determine whether T cells targeting GPC3 could specifically recognize and kill GPC3-positive HCC cells, cytotoxicity assays were performed by incubating the genetically modified T cells with each of the five HCC cell lines. The results indicated that both αGPC3-28BBZ and αGPC3-Z CAR T cells could efficiently lyse the four GPC3-positive HCC cell lines, but not the GPC3-negative SK-HEP-1 cell line (Fig. 3A), while the control effector cells (mock, αGPC3-DZ or 2D3-28BBZ) could not initiate specific lysis on those HCC cells.
It was reported that two types of soluble GPC3 (sGPC3) exist in the blood serum of patients with HCC (20, 21). One is the N-terminal region of GPC3 (GPC3N, residues: S25-R358); the other is the full-length GPC3 without GPI (GPC3ΔGPI, residues: Q25-S560). To elucidate whether sGPC3 can prevent the activation and functional activity of the GPC3-targeted CAR T cells, the cytotoxic activities of the αGPC3-28BBZ-modified T cells towards Huh-7 cells in the presence or absence of three types of recombinant sGPC3 were determined. The results indicated that the GC33 binding peptide fusion protein and GPC3ΔGPI, but not GPC3N, could inhibit the cytotoxic activities of the αGPC3-28BBZ-modified T cells. However, this suppression was mild, as the highest inhibition ratio was approximately 10% even at a concentration of 1000 ng/ml GPC3ΔGPI (Fig. 3B).

**CAR T cells redirected to GPC3 suppress the tumorigenesis of subcutaneous Huh-7 xenografts**

NOD/SCID mice were coinjected s.c. with the Huh-7 tumor cells and transduced T cells at an E : T ratio of 1 : 1 on the right flanks, with an additional group receiving the αGPC3-28BBZ T cells at an E : T ratio of 1 : 2. The results indicated that two out of the six mice were tumor-free in the group that received αGPC3-Z CAR T cells, while all mice were tumor-free in the group that received αGPC3-28BBZ CAR T cells, even at an E : T ratio of 1 : 2 (Supplementary Fig. S3A). In contrast, tumor outgrowth was observed in all mice in the control groups (Supplementary Fig. S3B). The results indicated the αGPC3-28BBZ CAR T cells displayed a significantly stronger lytic
activity than the αGPC3-Z CAR T cells did.

**αGPC3-28BBZ CAR T cells suppressed the growth of established subcutaneous GPC3-positive HCC xenografts**

To further explore the antitumor activities of αGPC3-28BBZ CAR T cells, NOD/SCID mice bearing established s.c. Huh-7 xenografts were employed. The potent antitumor effect was observed in the mice treated with αGPC3-28BBZ CAR T cells, while the other genetically modified T cells did not suppress tumor growth (Fig. 4A). At the experimental endpoint (day 28), three of the six mice treated with the αGPC3-28BBZ CAR T cells were tumor-free while all mice in the control groups carried large tumors (Supplementary Fig. S4A). The antitumor effect of the αGPC3-28BBZ CAR T cells was very significant compared with the control groups, respectively (P < 0.001) (Fig.4A, Supplementary Fig. S4B). These results suggested that GPC3-targeted CAR T cells could specifically eliminate Huh-7 cells *in vivo*.

To elucidate whether the *in vivo* cancer cell eliminating ability of αGPC3-28BBZ CAR T cells is dependent on the expression level of GPC3, mice bearing PLC/PRF/5 xenografts were also treated with the genetically modified T cells. Compared with the control groups, the αGPC3-28BBZ CAR T cells could significantly suppress the growth of PLC/PRF/5 tumors (Fig. 4B). At the end of the study (day 47), the mice bearing PLC/PRF/5 tumors treated with αGPC3-28BBZ CAR T cells still carried residual tumors (Supplementary Fig. S4C). The tumors treated with αGPC3-28BBZ CAR T cells were significantly smaller in weight than those in the control groups.
In two models, \( \alpha \)GPC3-28BBZ vs saline, \( P = 0.0332 \); \( \alpha \)GPC3-28BBZ vs mock, \( P = 0.021 \); \( \alpha \)GPC3-28BBZ vs 2D3-28BBZ, \( P = 0.0367 \); Supplementary Fig. S4D). The results of these two models implicated that the cytotoxic activities of GPC3-targeted CAR T cells were dependent on the GPC3 expression level on the target cells.

Previous studies have indicated that the persistence of transferred T cells in vivo is highly correlated with tumor regression (22, 23). Therefore, we also detected the numbers of human T cells in the peripheral blood of mice bearing s.c. established Huh-7 or PLC/PRF/5 xenografts 1 week after T cell infusion. The results indicated that CD4\(^+\) and CD8\(^+\) T cell numbers were highest in the group treated with \( \alpha \)GPC3-28BBZ CAR T cells in both groups (\( p < 0.01 \)) Fig. 4C, D). Additionally, the persisting T cell number of the PLC/PRF/5 group was less than that of the Huh-7 group (CD4\(^+\) T cells: 123.5 ± 16 vs 148 ± 20; CD8\(^+\) T cells: 246.5 ± 26 vs 374.5 ± 33).

The persistence of human T cells was further certified by immunostaining of the sections of Huh-7 tumors treated with \( \alpha \)GPC3-28BBZ CAR T cells. The results revealed that human CD3\(^+\) T cells had accumulated in residual tumors 2 weeks after i.v. T cell administration (Fig. 5), while fewer T cells could be detected in the sections of tumors treated with 2D3-28BBZ or mock transduced T cells. There was no specific staining in the sections treated with saline alone.

**Human GPC3-targeted CAR T cells potently suppressed the growth of established orthotopic Huh-7 xenografts**

To better evaluate the antitumor activities of GPC3-targeted CAR T cells, mice
orthotopically transplanted with Huh-7 (fLuc⁺) cells were employed. After one week of treatment, the group with αGPC3-28BBZ CAR T cells had the lowest tumor burden among the T cell-transferred groups (Fig. 6A). In the third week after the first dose of T cell administration, the mice in the three control groups had swollen abdomens, while the mice of the αGPC3-28BBZ CAR T cell treated group had the normal abdomen (Supplementary Fig. S5). At the end of this study, 3 out of 5 of the mice in the group receiving the αGPC3-28BBZ CAR T cells were liver tumor-free, while all mice in the control groups carried large hepatomas (data not shown). One week after the last infusion, the total number of human CD4⁺ and CD8⁺ T cells and CAR-positive T cells from mice treated with αGPC3-28BBZ CAR T cells was significantly higher than that of mice treated with 2D3-28BBZ CAR T cells or mock T cells (Fig. 6B), while no significant difference was observed between the 2D3-28BBZ and mock groups (P = 0.197). The persistence of CAR⁺ T cells were consistent with the total T cells (αGPC3-28BBZ vs mock, P < 0.01; αGPC3-28BBZ vs 2D3-28BBZ, P < 0.01; 2D3-28BBZ vs mock, P = 0.22; Fig. 6C). These results suggested that tumor antigen recognition drove the survival of the infused T cells in vivo. Previous studies indicated that the persistence of the CD137 signaling domain could upregulate Bcl-XL expression and enhance the survival of T cells (24). We also observed increased expression of Bcl-XL in αGPC3-28BBZ CAR T cells driven by the GPC3 antigen in vitro (Supplementary Fig. S6), suggesting that the increased number of CAR⁺ T cells among the αGPC3-28BBZ CAR T cells might be ascribed to Bcl-XL up-regulation. More importantly, all mice treated with the GPC3-specific
CAR T cells survived for longer than 60 days while the median survival durations of the saline, mock and 2D3-28BBZ-treated mice were 33 days, 34 days and 39 days, respectively (Fig. 6D).
Discussion

In this study, we developed GPC3-targeted CAR T cells with or without the costimulatory signaling domains of CD28 and CD137. Both types of GPC3-targeted CAR T cells could specifically destroy the HCC cells in an antigen-dependent manner. Similar to previous reports (25), the αGPC3-28BBZ CAR T cells that had the costimulatory signaling domains displayed a significantly stronger cytotoxic activity against the GPC3+ HCC cells than the αGPC3-Z CAR T cells did, suggesting that the intracellular CD28 and CD137 costimulatory domains contributed to the increased cytotoxic activities.

Our data on established Huh-7 subcutaneous and orthotopic xenografts indicated that αGPC3-28BBZ CAR T cells could eradicate liver cancer cells possessing a high level of GPC3 expression. Compared with Huh-7 cells, PLC/PRF/5 cells, which had a significantly lower level of GPC3 expression, displayed less sensitivity to GPC3-targeted CAR T cells both in vitro and in vivo. Additionally, there is a positive relationship between IFN-γ secretion from GPC3-targeted CAR T cells and the GPC3 expression level in HCC cells. Together, these data suggested that GPC3 expression level on the cell surface of HCC cell lines might be used as an indicator of the antitumor efficacy of GPC3-targeted CAR T cells.

Our previous study revealed that the levels of sGPC3 were $99.94 \pm 267.20$ ng/ml with a median level of 15.11 ng/ml (26). Our in vitro data in this study indicated that even a high concentration (approximately 1000 ng/ml) of GPC3ΔGPI could only mildly inhibit the cytotoxic activity of GPC3-targeted CAR T cells against
GPC3-positive HCC cells. We also observed that the GPC3ΔGPI (1000 ng/ml) had no significant effect on IFN-γ and IL-2 secreted by the GPC3-targeted CAR T cells as well as the expansion of GPC3-redirec ted T cells (data not shown). Therefore, the persistence of sGPC3 in most of the HCC patients might not significantly inhibit the clinical efficacy of GPC3-targeted CAR T cells. However, the actual effect of the sGPC3 on the antitumor effect of CAR T cells in vivo needs further study.

A major concern regarding CAR T cells, especially when the costimulatory signaling domain of CD28 or CD137 is included, is the on-target off-tumor effect. Previously, one breast cancer patient died after the treatment with CAR T cells redirected to Erbb2 due to the expression of this receptor in lung tissues (27). Therefore, the specificity of the cancer-associated antigen targeted by the CAR T cells is vitally important. Although several studies have indicated that GPC3 is absent in normal tissues, a study by Daniel Baumhoer revealed that while most normal tissues stained negatively for GPC3, gastric glands (3/7 [43%]), kidney tubules (9/17 [53%]), and testicular germ cells (2/16 [13%]) stained positively (7). Therefore, we examined the GPC3 expression in the kidney and gastric glands. Fortunately, our study revealed that there are no obvious GPC3 expression in normal kidney tissues (10/10) and gastric glands (10/10) (Supplementary Fig. S7). The contradictory results may be ascribed to the different samples used. Another possibility is the false-positivity of immunostaining. Anyway, considering the individual difference, we thought it should be cautious when the GPC3-redirec ted CAR T cells were applied in clinical trial.

Several strategies such as the incorporation of suicide switches (28, 29), a “tumor
sensing” approach by combining two types of antigen recognition with balanced signaling (30) or iCARs technology (31) might be used to minimize the potential off-target toxicity of the CAR T cells.

As we know, one of the major challenges for cancer treatment lies in its heterogeneity. Several testis-antigens have been targeted by T cell-based immunotherapy. However, most of the testis antigens are heterogeneously expressed in the tumor tissue (32). In the tumor tissue microarray of 75 primary HCC patients, the different levels of GPC3 expression were evaluated by two experienced pathologists using a 4-point scale according to the staining intensity (Supplementary Fig. S8A). Similar to previous reports (7, 33), our data indicated that GPC3 was expressed in 70.7% (53 out of 75) of HCC patients (Supplementary Fig. S8B). In GPC3-positive patients, 77% (41 out of 53) of patients carried high levels of GPC3 expression (score ≥ 2). Importantly, unlike testis-antigen, in patients carrying a high level of GPC3 expression, GPC3 appeared to be homogenously expressed in HCC tissues with a mean rate of GPC3-positive hepatoma cells equal to 88% (Supplementary Fig. S8C). This will be an important factor in the long-term clinical efficacy of GPC3-directed CAR T cells.

Taken together, the results of this study constitute the first report concerning CAR T cells redirected to GPC3 and the first report about HCC treatment using CAR T cells. With their potent cancer cell-eliminating capacity, GPC3-targeted CAR T cells may be a promising therapeutic option for HCC treatment.
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Figure legends:

Figure 1. Characterization of GPC3-specific CAR T cells. (A) Schematic representation of a bicistronic lentiviral vector expressing CARs along with eGFP using the F2A ribosomal skipping sequence is shown. (B) GPC3-specific CARs expression on human T cells transduced with lentiviral particles were analyzed using Flow cytometry by detection of GFP autofluorescence. Transduction efficiency was shown. (C) In vitro expansion of T cells following activation with one cycle stimulation of αCD3/αCD28 coated magnetic beads on day 0 and transduction with the indicated CARs on day 1. Genetically modified T cells were cultured in vitro for up to 2 weeks. On day 18, T cells were re-stimulated by aK562-64/86 with co-expression of the extracellular domain of CD64 and CD86, in the presence of OKT3 (100 ng/ml) and recombinant human IL-2 (300 U/ml). (D) On day 18, the general or antigen-specific re-proliferation of αGPC3-28BBZ, and the negative control mock and 2D3-28BBZ transduced T cells was quantified.

Figure 2. IL-2 and IFN-γ produced by GPC3-targeted CAR T cells co-cultured with various HCC cell lines. (A) Surface GPC3 expression (black line) on various human HCC cell lines detected by flow cytometry and isotype antibody control (filled red histograms). Moreover, GPC3 expression in various HCC cell lines was analyzed by western blot. IL-2 (B) and IFN-γ (C) were secreted by the indicated genetically modified T cells co-cultured with various HCC cell lines for 24h. Results are representative of triplicates. (D) Correlation between the IFN-γ secretion from
GPC3-targeted T cells and the MFI of GPC3 expression on the five HCC cell lines.

Figure 3. *In vitro* cytotoxic activities of GPC3-targeted CAR T cells. (A) Primary human T cells transduced with the indicated lentiviral vectors were co-incubated with the five HCC cell lines at the varying effector : target ratios for 18h, respectively. Cell lysis was determined by a standard non-radioactive cytotoxic assay. Each data reflects the mean ± SEM of triplicates. (B) Cytotoxicity of αGPC3-28BBZ CAR T cells on Huh-7 cells in the absence or presence of mammalian cell-expressed GPC3ΔGPI and GPC3N, E.coli-expressed GST-fused GC33-binding peptide or BSA at the effector : target ratio of 3:1 for 18h.

Figure 4. *In vivo* antitumor activities of αGPC3-28BBZ CAR T cells on established s.c. HCC tumor xenografts. (A) Growth curve of Huh-7 xenografts treated with the indicated T cells or saline. At the endpoint, the residual tumors treated with αGPC3-28BBZ CAR T cells was significantly smaller than those in the control groups, respectively (**P < 0.001). (B) Growth curve of PLC/PRF/5 xenografts treated with the indicated T cells or saline. On day 47, the tumor in αGPC3-28BBZ group was significantly smaller than that in each control group (* P < 0.05). The time point of T cells infusion or saline in figure (A) and (B) was shown with arrow. (C) and (D) The quantities of circulating human CD4⁺ and CD8⁺ T cells from mice bearing Huh-7 xenografts or PLC/PRF/5 treated with the indicated genetically modified T cells. Mean cell concentration (cells/µL) ±SEM for mice in genetically modified T cells
treatment group and $P$ values are shown.

Figure 5. αGPC3-28BBZ CAR T cells could located in Huh-7 tumors. Tumors were collected from mice bearing Huh-7 s.c. xenografts treated with αGPC3-28BBZ CAR T cells, 2D3-28BBZ, Mock T cells or saline. Formalin-fixed, paraffin-embedded tumor sections were consecutively cut and stained for human CD3 expression (brown). (The images were taken with the microscope (BX41, Olympus, PA) and camera (DP70) under $\times$ 200 magnifications. Scale bar was 200 μm).

Figure 6. αGPC3-28BBZ CAR T cells potently suppressed the established orthotopic Huh-7 xenografts in vivo. (A) NOD/SCID mice bearing Huh-7 (fLuc+) orthotopic tumor were intravenous injected with $5 \times 10^6$ genetically-modified T cells on day 14 and 21, respectively. Mice were imaged weekly. Tumor growth was assessed by total bioluminescence signals. The growth of tumors treated with αGPC3-28BBZ CAR T cells was potently suppressed when compared with the control groups. (B) The quantitative analysis of human CD4$^+$ and CD8$^+$ T cells by TruCount tubes. (C) GFP-positive peripheral blood cells were also counted. Mean cell concentration (cells/μL) ±SEM for all evaluable mice in each treatment group are shown. (D) The overall survival of mice treated with the indicated T cells or saline.
**Figure 1**

(A) Schematic representation of the constructs used in the study. The diagram shows the structure of different constructs, including \( \alpha \text{GPC3-DZ} \), \( \alpha \text{GPC3-Z} \), \( \alpha \text{GPC3-28BBZ} \), and a negative control. The constructs are labeled with their corresponding signal peptides and domain structures.

(B) Flow cytometry histograms showing the percentage of GFP-positive cells for each construct: \( \alpha \text{GC3-DZ} 56\% \), \( \alpha \text{GC3-Z} 55\% \), \( \alpha \text{GC3-28BBZ} 57\% \), and \( 2D3-28BBZ 58\% \).

(C) Graphs depicting fold expansion of different cell lines over time following transduction. The constructs include \( \alpha \text{K562-64/86} \), \( \alpha \text{GPC3-DZ} \), \( \alpha \text{GPC3-Z} \), and \( \alpha \text{GPC3-28BBZ} \) compared to untreated controls.

(D) Additional graphs showing fold expansion for different cell lines, including \( \alpha \text{K562-64/86} \), \( \text{Huh-7} \), \( \text{SK-HEP-1} \), and untreated controls. The graph for \( 2D3-28BBZ \) also includes fold expansion data.
Figure 5

Anti-human CD3ε Ab

Isotype control Ab

daGPC3-28BBZ

2D3-28BBZ

Mock

Saline
Figure 6

A

Total bioluminescence signal (Photons/s)

Weeks post-tumor inoculation

- Saline
- Mock
- 2D3-28BBZ
- αGPC3-28BBZ

B

CD4⁺ T cell
CD8⁺ T cell

P < 0.01
P = 0.0156

Peripheral blood T cell count (cells/µL)

Mock 2D3-28BBZ αGPC3-28BBZ

C

Peripheral blood GFP⁺ T cell count (cells/µL)

Mock 2D3-28BBZ αGPC3-28BBZ

D

% Overall survival

Mock 2D3-28BBZ αGPC3-28BBZ

Days post-inoculation

Infusion Week 1 Week 2 Week 3

Saline

Mock

2D3-8BBZ

αGPC3-28BBZ
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