B7-H3 specific CAR-T cells exhibit potent activity against prostate cancer

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Article

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Abstract

The high expression across multiple solid tumor, including prostate cancer and restricted expression in normal tissues makes B7-H3 an attractive target for immunotherapy. Among various types of tumor immunotherapy, chimeric antigen receptor T (CAR-T) cell therapy has shown remarkable success in hematological tumors. However, the potency of CAR-T cell therapy in solid tumors is still limited so far. Here, we examined the expression of B7-H3 in prostate cancer tissues and cells, and developed a second-generation CAR that specifically targets B7-H3 and CD28 as costimulatory receptor to explore its tumoricidal potential against prostate cancer in vitro and in vivo. The high expression of B7-H3 was detected on both the surface of PC3, DU145 and LNCaP cells and prostate cancer tissues. B7-H3 CAR-T cells efficiently controlled the growth of prostate cancer in an antigen-dependent manner in vitro and in vivo. What is more, tumor cells could induce the proliferation of CAR-T cells and the release of high levels of cytokines of IFN-γ and TNF-α in vitro. These findings elucidate that B7-H3 is a potential target for prostate cancer therapy, and support the clinical development of B7-H3 specific CAR-T cells for prostate cancer.

Introduction

The incidence of prostate cancer (PCa) is increasing year by year, and PCa has become the second leading malignancy in males and the fifth most common tumor worldwide. Radical surgery and castration are the major treatment strategies for early stage prostate cancer. However, the recurrence rate of PCa is high, and a certain number of patients will progress to the advanced castration-resistant PCa (CRPC) stage and eventually develop into metastatic castration-resistant PCa (mCRPC) [3]. Endocrine therapy is considered as palliative, which can alleviate symptoms but cannot cure the disease. Chemotherapy alone has limited efficacy and is hindered by drug resistance, while radiotherapy often shrinks PCa tumor with obvious complications. Following the FDA approval of sipuleucel-T for prostate treatment, some studies evaluated the role of immunotherapy drugs in prostate cancer, but found no significant effect [4–5]. Novel therapeutic strategies are in urgent need to provide durable disease control and to improve the survival of patients with PCa.

Cancer immunotherapy has emerged as an effective regimen alone or in combination with other treatments, such as surgery, radiotherapy, chemotherapy and targeted therapy. It activates the host immune cells, especially T cells, to specifically target tumor cells. Among cancer immunotherapy, chimeric antigen receptor (CAR) T cell therapy has overcome cancer immune tolerance and made significant breakthroughs in liquid cancer treatment. CAR is an artificial T cell receptor consisted of tumor antigen-binding domain (such as single-chain fragment variable scFv) fused with T cell costimulatory and activating motifs [9]. Hence, CAR-modified T cell (CAR-T) could specifically recognize and kill tumor cells without antigen-presenting process and major histocompatibility complex (MHC)-restriction. CAR-T cell therapy was initially used in hematological malignancies and has shown high remission rates and persistence in chronic lymphocytic leukemia (CLL), acute lymphocytic leukemia (ALL), and refractory B-
cell lymphoma \cite{10-15}. It also demonstrated promising result in early clinical trials for other hematological malignancies including multiple myeloma \cite{16}. Clinical trials of CAR-T cell therapy for solid tumors such as breast, liver, ovarian, and lung cancer are increasing \cite{17}. The target antigen of CAR-T cell therapy should be overexpressed on the surface of cancer cells in most patients, while no expression or low expression in normal tissues, thus CAR-T cells can exert cancer-specific immune response without damaging normal tissue and organs \cite{18-19}.

B7-H3, also called CD276, a type I transmembrane protein, is highly expressed in various solid tumors such as prostate cancer, renal cell carcinoma, non-small cell lung cancer, and breast cancer \cite{20}, while it is expressed at low levels in normal human tissues. It contributes to tumor immune evasion \cite{21} and metastasis \cite{22}, and is associated with disease progression \cite{23}, recurrence, poor prognosis \cite{24} and drug resistance. Roth et al performed immunohistochemical analysis of tissue samples from PCa patients and found that B7-H3 was expressed in all tissues and its expression is associated with tumor aggressiveness, metastasis, and disease recurrence. Zang et al showed that high expression of B7-H3 in PCa tissue is associated with tumor metastasis, postoperative recurrence and higher mortality. Hence, B7-H3 is a potential therapeutic target for prostate cancer. To date, multiple clinical trials have shown that monoclonal, bispecific and drug-conjugated B7-H3 antibodies (MGA271 and 8H9) are safe and effective for the treatment of advanced malignant tumors \cite{25-29}. A number of therapies targeting B7-H3 are already in preclinical or clinical trials stages \cite{30}. B7-H3 CAR-T cells have been shown to be effective in solid tumors, such as neuroblastoma, ovarian cancer, pancreatic ductal adenocarcinoma and melanoma \cite{31-32}. Furthermore, it has been reported that B7-H3 CAR-T cells significantly repress tumor growth in syngeneic tumor models and multiple preclinical studies without apparent toxicity \cite{33}. Therefore, CAR-T targeting B7-H3 might be a promising therapeutic strategy for prostate cancer.

In this study, we firstly constructed a second generation CAR targeting B7-H3 with the humanized scFv from 8H9. cells and verified B7-H3 CAR-T cells killed B7-H3 positive PCa cells in an antigen-dependent manner \textit{in vitro}. In addition, infusion of B7-H3 CAR-T cells also significantly inhibited the growth of DU145 xenograft tumors in NCG mice. Such finding suggests that B7-H3 targeted CAR-T therapy might be a novel immunotherapeutic strategy for PCa.

## Results

### Overexpression of B7-H3 in prostate cancer cell lines and tumor tissues

To assess whether B7-H3 could be a target for PCa, we examined the expression of this molecule in PCa tissues and cell lines. Immunohistochemistry of tumor tissue from three clinical prostate cancer patients revealed high expression of B7-H3 (Figure 1A). The result of flow cytometry (FCM) showed that B7-H3 was highly expressed on the surface of prostate cancer cell lines PC3, DU145 and LNCaP (Figure 1B). These results indicate that B7-H3 is a potential PCa target for the development of novel therapeutic strategies.
Construction of CAR-T cells targeting B7-H3

After confirming that B7-H3 is highly expressed in prostate cancer, we synthesized the B7-H3 scFv sequence based on 8H9 clone, and constructed the second-generation B7-H3 CAR containing CD8α transmembrane region, CD28 intracellular costimulatory domain and CD3ζ intracellular signaling domain (Figure 2A). B7-H3 CAR-T cells were constructed by infecting CD3/CD28-activated T cells with a retroviral vector encoding B7-H3 CAR, and the activated T cells were used as control T cells. The positive rate of CAR expression on the surface of CAR-T cells reached 85% (Figure 2B, C). On the sixth day after transfection, CAR-T cells expanded approximately 60 fold, which was comparable to control T cells, indicating that the expression of CAR did not affect the proliferation of T cells (Figure 2D).

B7-H3 redirected CAR-T cells efficiently kill PCa cells

We tested the function of B7-H3 specific T cells in vitro using FCM and RTCA techniques. Three prostate cancer cells were co-incubated with control T and B7-H3 CAR-T cells at effector-to-target ratio (E:T) of 1:1, respectively. RTCA was used to record the cell index in real time to reflect the adherence and killing of tumor cells. After 4 days of co-culture, the percentage of surviving CAR-T cells and tumor cells was analyzed by flow cytometry to calculate the killing rate. The results of RTCA showed that, tumor cells alone as blank group, control T cells had no obvious effect on three B7-H3 positive tumor cells. Compared with blank and control T groups, B7-H3 CAR-T cells induced almost complete elimination of tumor cells (Figure 3A, B, C). FCM results demonstrated that B7-H3 CAR-T cell group had less residual tumor cells at higher target ratio, that is, CAR-T cells had stronger killing effects on PC3, DU145 and LNCaP cells. The killing effect was dose-dependent and enhanced with the increase of effector-to-target ratio (Figure 3D, E, F). The above results suggest that B7-H3 CAR-T cells have significant anti-tumor effect on B7-H3 positive PCa cells.

Control T and CAR-T cells were stained with CFSE, then incubated with PC3 or DU145 cells at E:T of 1:1. After 48 h, the CFSE fluorescence intensity of T cells was detected by flow cytometry, and the fluorescence intensity decreased with the increase of T cell proliferation. As shown in Figure 3G and 3H, the fluorescence intensity of control T cells co-cultured with the two tumor cells was not different from that of T cells alone, while the CFSE intensity of B7-H3 CAR-T cells was significantly weaker after co-culture, demonstrating that tumor cells enhanced the proliferation of CAR-T cells. The above shows that B7-H3 CAR-T cells could eradicate tumor cells, and tumor cells stimulate CAR-T cells to proliferate efficiently. CAR-T cells activate, proliferate, and release cytokines IFN-γ, TNF-α, Granzyme A, and Granzyme B in the presence of target antigen. Hence, we collected T cells co-cultured with tumor cells at E:T of 1:5 for 48 hours, then detected the release of IFN-γ and TNF-α from CAR-T cells by flow cytometry. The detection of the cytokines illustrated PC3, DU145 and LNCaP cells expressing B7-H3 significantly increased the release of IFN-γ (Fig 3I) and TNF-α (Fig 3J) from CAR-T cells compared with control T cells.

B7-H3 CAR-T cells do not kill B7-H3 negative PCa cells
In order to clarify the specificity of B7-H3 CAR-T cells, we used CRISPR-Cas9 technology to knock out B7-H3 in PC3 and DU145 cells, and constructed PC3 (B7H3-) and DU145 (B7H3-) cells by puromycin selection and flow sorting. Flow cytometry was used to detect the expression of B7-H3 on cell clones (Figure 4A, B), and western blot was used to verify the successful construct of PC3(B7H3-) and DU145(B7H3-) cells (Figure 4C, D). The effect of B7-H3 CAR-T cells on B7-H3 negative cells was detected by RTCA and flow cytometry. RTCA curve showed that control T cells did not kill PC3(B7H3-) and DU145(B7H3-) cells, and the curve of B7-H3 CAR-T group was similar to blank group and control T group, suggesting that B7-H3 directed CAR-T cells had no cytotoxicity against B7-H3 negative tumor cells (Figure 4E, F). The results of FCM were consistent with RTCA (Figure 4G, H).

As shown in Figure 4I and 4J, the CFSE intensity of B7-H3 CAR-T cells co-incubated with PC3(B7H3-) or DU145(B7H3-) cells had no discernible difference from that of CAR-T cells alone, but higher than co-incubation with PC3/DU145 cells, denoting that B7-H3 negative PC3 and DU145 cells had no effect on the proliferation of B7-H3 CAR-T cells. Cytokine assays revealed that PC3(B7H3-) and DU145(B7H3-) cells did not promote the release of IFN-γ (Figure 4K) and TNF-α (Figure 4L) from B7-H3 CAR-T cells. The above results imply that B7-H3 CAR-T cells specifically target and kill B7-H3 positive tumor cells and release cytokines.

**B7-H3 CAR-T cells inhibit the growth of prostate cancer in vivo**

Since B7-H3 specific CAR-T cells could effectively eliminate B7-H3 positive PCa cells in vitro, we evaluated anti-tumor activity of B7-H3 CAR-T cells in vivo. We infused immunodeficient NCG mice with DU145 or DU145(B7H3-) cells to construct subcutaneous xenograft models of prostate cancer (Figure 5A). On day 29 and 65, 5×10^6 control T or B7-H3 CAR-T cells were infused via tail vein. After treatment, blood was collected from the tail vein every week to detect the content of T cells and CAR-T cells in peripheral blood to evaluate the survival of T cells in mice. As show in Figure 5B, tumors in PBS group and control T group of DU145 mice continued to grow, while B7-H3 CAR-T cells significantly inhibited the tumor growth (p < 0.05). Tumors in the B7-H3 CAR-T group of DU145(B7H3-) mice were slightly smaller than PBS and control T groups, but not statistically significant (Figure 5C). Treatment with control T and B7-H3 CAR-T cells in both models did not lead to weight loss in mice (Figure 5D, E), demonstrating that B7-H3 CAR-T cells had no obvious toxic or side effects on mice.

As for the content of T cells in the peripheral blood of DU145 mice, there were fewer T cells in control group than in B7-H3 CAR-T group (p < 0.05), while T cells in both groups of DU145(B7H3-) mice were lower. The contents of T cells and CAR-T cells in the peripheral blood of CAR-T group of DU145 mice were higher than those of CAR-T group of DU145(B7H3-) mice (p < 0.01) (Figure 5F, G). At the end of experiment, mouse spleen was taken and ground to detect the ratio of T cells (Figure 5H) and CAR-T cells (Figure 5I). There was no difference in the proportion of spleen T cells between control T group and B7-H3 CAR-T group of the two mice, and the ratio of T cells and CAR-T cells in the spleen of DU145(B7H3-) mice was obvious lower than that of DU145 mice. The tumors of mice were ground to measure the content of T cells and CAR-T cells, and excised tumor tissues for CD3 immunohistochemical staining to evaluate the
infiltration of T cells. The numbers of T cells in the tumors of control T group and B7-H3 CAR-T group in the two mouse models were comparable, and the numbers of T cells and CAR-T cells in the tumors of B7-H3 knockout mice were less than that of the DU145 mice (p < 0.05) (Figure 5J, K). There were obvious T cell infiltration in the tumor tissues of control T group and B7-H3 CAR-T group (Figure 5L).

Overall, the above results verified that B7-H3 specific CAR-T cells inhibited the tumor growth of B7-H3 positive DU145 mice, and effectively expanded and infiltrated in mice, but had no obvious side effects.

**Discussion**

In this study, we constructed a CAR targeting B7-H3 based on 8H9 clone. We used CRISPR-Cas9 technology to knock out B7-H3 in PC3 and DU145 cells, then verified that B7-H3 CAR-T cells specifically recognized B7-H3 positive target cells and produced cytokines IFN-γ and TNF-α to induce tumor cells lysis in vitro. In vivo, DU145 tumor-bearing mice showed better response to B7-H3 specific CAR-T cell treatment without obvious side effects.

The high heterogeneity of antigens limits the efficacy of CAR-T cells in solid tumor \[^{34}\]. B7-H3 has limited expression in normal tissues and is widely expressed in tumor cells and tumor vasculature. In some malignancies, overexpression of B7-H3 is associated with fewer tumor-infiltrating lymphocytes, cancer severity and poor prognosis, such as prostate cancer \[^{35}\], clear cell renal cancer, lung cancer, and ovarian cancer. Difference of B7-H3 between healthy and malignant tissues makes it an attractive target for cancer immunotherapy. 8H9 antibody has been used to treat a variety of tumors, ensuring the efficacy and safety of the treatment when applied to CAR-T cells targeting B7-H3 \[^{36-37}\]. Researchers found that B7-H3 antibody-drug conjugate (ADC) eradicates established tumors and metastases, improves overall survival, and has been used in a broad range of anticancer treatments with favorable safety profile in phase I clinical trials.

This study demonstrates that B7-H3 CAR-T cell therapy is a promising treatment strategy for PCa, while there are some issues to be further investigated. On the one hand, clinical trials have not yet been conducted, and the clinical effects and toxic side effects in humans are still uncertain. Therefore, the safety and efficacy of B7-H3 CAR-T cells in PCa patients need to be explored in the future. In addition, B7-H3 plays a role in cancer migration, invasion and angiogenesis, so it can be further investigated whether it can be used as an anti-metastatic marker for prostate cancer.

In this study, we validated the antitumor effect of B7-H3 redirected CAR-T cells against PCa in vitro and in vivo. Collectively, our findings provide a new target for the treatment of prostate cancer, and B7-H3 CAR-T cell is a potential treatment for PCa patients.

**Material And Methods**

**Clinical samples and cell lines**
Clinical samples of prostate tissues were obtained from patients diagnosed with prostate cancer in the Affiliated Hospital of Xuzhou Medical University (Approved No. XYFY2021-KL325-01). Three PCa cell lines (PC3, DU145, LNCaP) and 293T were purchased from the Cell Bank of the Chinese Academy of Sciences. All media contained 10% serum (fetal bovine serum FBS or Hyclone) and 1% penicillin and streptomycin. Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood by density-gradient centrifugation using Ficoll reagent. 1% recombinant human IL-7 (rhIL-7) and rhIL-15 were added to the medium of T cells. Cells were cultured in a sterile incubator at 37°C with 5% CO2. The study was approved by the Ethics Committee of the Affiliated Hospital of Xuzhou Medical University, and informed consent was obtained from all patients (XYFY2016-JS033-01).

**Reagents**

The Activator of human T, CD3 monoclonal antiboby (OKT3) and CD28 monoclonal antiboby (OKT28), was purchased from Life Technologies. Recombinant human IL-7 and IL-15 were purchased from PrimeGene BioTech, China. RetroNectin was purchased from Takara Bio Inc (T100A). The following antibodys used for flow cytometry (FCM) were purchased from BioLegend: anti-human PE B7-H3 (331606), APC CD3 (317318), PE-Cy7 TNF-α (502930), FITC IFN-γ (502506) and PE-immunoglobulin G Fc (410708)). Fetal bovine serum, medium, and penicillin / streptomycin were purchased from Gibco. 5(6)-Carboxuorescein diacetate succinimidyl ester (CFSE) was purchased from Thermo Fisher Scientific (C34554). Brefeldin A (BFA) was purchased from Biogems. Cytofix/Cytoperm Kit was purchased from BD Pharmingen. B7-H3 (14058), GAPDH antibody (649202) and peroxidase-coupled goat anti-rabbit IgG secondary antibody (SA00001-2) for Western Blot were purchased from CST, BioLegend and Proteintech, respectively.

**Flow Cytometry**

The expression of B7-H3 on the surface of prostate cancer cells was detected with anti-B7H3 antibody. For the analysis of CAR expression, control T cells or CAR-T cells were incubated with B7-H3 protein (SinoBiological, 11188-H02H), then stained with IgG-Fc, followed by anti-CD3 antibody. The proliferation of T cells were evaluated by CFSE staining. To measure cytokines of CAR-T cells, B7-H3 CAR-T cells were co-incubated with tumor cells at an effector-to-target (E:T) ratio of 1:5 for 24 h. For measurement of intracellular cytokines, T cells were fixed and permeabilized, then stained with IFN-γ (Biolegend, DCN.70) and TNF-α (Biolegend, DCN.70) antibodies. B7-H3 knockout tumor cells were sorted by flow cytometry (BD FACS Aria). All samples were detected using BD FACS Canto II and Data were processed by FlowJo software.

**Generation of B7-H3 specific CAR-T cells**

The scFv sequence based on 8H9 antibody was synthesized by the company, and then introduced into a plasmid vector containing CD8α transmembrane region, CD28 and CD3ζ intracellular signal domains to construct B7-H3 CAR. The B7-H3 CAR plasmid and two packaging plasmid were co-transfected into 293T cells. The virus supernatants were collected at 48h and 72h after transfection, then centrifuged at 2000
rpm for 10 minutes to remove cell debris and stored at -80°C for later use. PBMC cells from whole blood were isolated using Ficoll reagent and cultured in L500 medium supplemented with 10% serum, 1% penicillin/streptomycin, 1‰ rhIL-7 and rhIL-15. PBMC cells were stimulated with 1 μg/ml CD3/28 dynabeads in 24-well plate for 48 hours. The 24-well low-adsorption plate coated with RetroNectin was used to infect T cells with virus solution, and then replaced with L500 growth medium after centrifugation at 30°C 1500g for 2 hours. T cells were expanded and cultured according to the density of 0.7-1×10⁶ cells/ml, and cells were collected 48 h after infection to detect the CAR positive rate of T cells by flow cytometry. Activated T cells without infection served as control cells.

**Cytotoxicity assay**

Cytotoxicity of CAR-T cells was assessed with Real-Time Cell Analysis (RTCA) instrument or flow cytometry. In the first step, 50 μl L500 complete medium was added to each well of the E-plate to detect the background baseline value. In the second step, 1.5×10⁴ tumor cells were added to each well, then placed plate on the instrument to continuously monitor the adherence and proliferation of tumor cells through impedance values. The second step was terminated at an appropriate time, then effector cells were added to the target cells at a certain effect-to-target ratio, so that the activity of CAR-T cells can be directly reflected by cell index. Flow cytometry was used to detect the ratio of T cells to tumor cells at the beginning and end of co-culture to calculate the killing rate of CAR-T cells. Wells with only tumor cells served as control.

**Cell proliferation assay**

Effector cells were stained with 1.5 μM CFSE dye for 10 minutes at room temperature in the dark, then stopped with an equal volume of serum at 37°C, and finally washed with 2% serum in PBS. Flow cytometry was used to check whether the staining was successful. Target cells and effector cells were co-cultured at E:T of 1:5, and a control well without target cells was set at the same time. After 48 hours, T cells were collected and the CFSE fluorescence intensity was measured by flow cytometry to evaluate the proliferation of T cells. CFSE fluorescence intensity decreased with T cell proliferation. Data were analyzed using FlowJo software.

**Cytokine production assay**

Tumor cells and effector cells (control T or CAR-T cells) were plated in a 24-well plate at E:T of 1:5, then BFA was used to block the extracellular release of cytokines after 24 h. After 6 h of blocking, T cells were harvested for CAR staining, fixation and membrane permeabilization, IFN-γ and TNF-α antibody staining, and finally detected by flow cytometry.

**CRISPR-mediated B7-H3 knockout**

The gRNA sequence targeting B7-H3 (5’-CACCGACCCAGTGGTGGCACTGGT-3’) derives from the article Oncogene 2019 01;38(1). The gRNA was introduced into laboratory's Lenti CRISPR v2 plasmid, then
transfected into 293T cells, and finally the viral supernatant was collected to infect PC3 and DU145 tumor cells. After 48 hours of infection, monoclones of cells were sorted into 96-well plates, then cells were expanded and tested for knockout of B7-H3. Three monoclones were mixed to construct PC3 (B7H3-) and DU145 (B7H3-) cells.

**Western blot**

Prostate cancer cells were lysed with protein lysing buffer to extract total cell proteins. The protein extracts were separated by 10% SDS-PAGE, transferred to PVDF membrane, and incubated with antibodies against B7-H3 and GAPDH. Horseradish peroxidase-conjugated goat anti-rabbit IgG was used as the secondary antibody, and the protein content was displayed with enhanced chemiluminescence liquid (ECL).

**Xenografted mouse model**

All animal treatment procedures were approved and performed in strict accordance with the regulations of the animal care and use committee of Xuzhou Medical University. 4-week-old immunodeficient NCG male mice were purchased from Nanjing Jicui Yaokang and housed in specific pathogen-free (SPF) animal facility of the Experimental Animal Center of Xuzhou Medical University. 4×10^6 DU145/DU145(B7H3-) tumor cells were resuspended in 150µl PBS and subcutaneously inoculated into the right lower limb of mice to construct prostate cancer model. The body weight and tumor size of mice were measured every 4 days, and tumor volume was calculated using the formula length × width² × 0.5. To assess the effect of B7-H3 CAR-T cells, mice were randomly divided into three groups when tumor volume reached 100-150 mm³, and PBS, 5×10⁶ B7-H3 CAR-T cells or control T cells were infused via tail vein on day 29 and 65 after tumor injection. Blood was collected weekly through tail vein to detect the proliferation of T cells in peripheral blood during the treatment. Mice were sacrificed at the end of the experiment, and the spleen and tumor tissues were grounded to detect the content of T cells and CAR-T cells. At the same time, the infiltration of T cells in tumor tissue was assessed by immunohistochemistry.

**Statistical analysis**

All data were repeated three times, and the experimental data were statistically analyzed with Graphad Prism 7.0 software. The data were expressed as mean ± standard error (SEM). Unpaired Student t test was used to compare two independent samples, and ANOVA was used to compare significant differences among multiple groups. P < 0.05 was considered statistically significant.

**Declarations**

**Availability of Data and Materials**

All data involved in this study are available in the main text and we will provide the original data to support the findings upon reasonable request. Materials can be available through agreement with
corresponding authors.

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**Author contributions**

JZ, PM, and GW designed the research. SL, LJM and MZ provide samples. MMZ, HTW, and HW collected the data. SL, MMZ, and MW analyzed and interpreted the results. SL and MMZ wrote the manuscript. All authors contributed to the article and approved the submitted version.

**Competing interests**

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

**Ethical approval**

The study was approved by the Ethics Committee of the Affiliated Hospital of Xuzhou Medical University, and informed consent was obtained from all patients.

**References**


Figures

Figure 1

A

Sample 1

Sample 2

Sample 3

4x

40x

B

PC3

DU145

LNCaP

PE-B7H3

count

0 10^3 10^4 10^5

0 100 200 300 400

0 10^3 10^4 10^5
Expression of B7-H3 protein in prostate cancer tissues and cells. A. The expression of B7-H3 protein in tissue samples of prostate cancer patients was detected by immunohistochemical analysis. Scale bar, 4× 500 μm, 40× 500 μm. B. Flow cytometry was used to detect the expression of B7-H3 on the surface of prostate cancer cells.

Figure 2

A

B

C

D

Construction of B7-H3 CAR-T cells. A. Structural schema of CAR, LTR: long terminal repeat, scFv: single-chain Fragment variable. B. The expression of IgG-Fc on the surface of T cells was detected by flow...
cytometry to show the transfection efficiency of CAR. C. Statistical graph of the CAR positive rate of T cells. D. Proliferation curves of control T and B7-H3 CAR-T cells. Paired data were analyzed using Student’s t-test.

Figure 3

A. PC3
- Tumor only
- Control T
- B7-H3 CAR-T

B. DU145

C. LNCaP

D. Control T
- B7-H3 CAR-T

PC3
- Effector:Tumor

E. DU145

F. LNCaP

G. CAR-T+PC3 48h
- CAR-T 48h
- CAR-T 0h

H. CAR-T+DU145 48h
- CAR-T 48h
- CAR-T 0h

I. % of IFN-α+ CAR-T cell

J. % of TNF-α+ CAR-T cell
Cytotoxicity of B7-H3 CAR-T cells against prostate cancer cells. A-C. Cytotoxicity analysis of B7-H3 CAR-T cells against B7-H3 positive target cells by real-time cell assay. D-F. Flow cytometry detected the final proportion of T cells co-cultured with PC3 (Figure D), DU145 (Figure E) or LNCaP (Figure F), and calculated the percentage of killing. G, H. Effect of tumor cells on the proliferation of B7-H3 CAR-T cells by FCM. After co-incubating CFSE-stained T cells with PC3 (Figure G) or DU145 (Figure H), flow cytometry was used to detect the fluorescence intensity of control T and B7-H3 CAR-T cells to reflect the proliferation of T cells. I, J. Effect of tumor cells on the cytokine release of B7-H3 CAR-T cells by FCM. T cells were subjected to membrane permeation, fixation, and antibody staining to detect the IFN-γ (Figure I) and TNF-α (Figure J) levels of control T and B7-H3 CAR-T cells under the stimulation of prostate cancer cells. ** $P < 0.05$, *** $P < 0.001$. 
Cytotoxicity of B7-H3 CAR-T cells against B7-H3 negative tumor cells. **A, B.** The expression of B7-H3 protein on the surface of PC3(B7H3-) (Figure A) and DU145(B7H3-) (Figure B) monoclonal cells was detected by flow cytometry. **C, D.** Western blot was used to confirm the expression of B7-H3 in PC3, PC3(B7H3-), DU145 and DU145(B7H3-) cells. **E, F.** RTCA was used to verify the killing of control T and B7-H3 CAR-T cells to B7-H3 negative PC3(B7H3-) (Figure E) and DU145(B7H3-) (Figure F), and the right figure
is the corresponding statistics. **G, H.** The killing of PC3(B7H3-) (Figure G) or DU145(B7H3-) (Figure H) by B7-H3 CAR-T and control T cells was assessed by FCM. **I, J.** CFSE staining was used to detect the proliferation of control T and CAR-T cells co-incubated with PC3(B7H3-) (Figure I) or DU145(B7H3-) (Figure J). **K, L.** The release of IFN-γ (Figure K) and TNF-α (Figure L) from T cells under the stimulation of B7-H3 negative tumor cells. **P < 0.05.

**Figure 5**
Antitumor effect of B7-H3 CAR-T cells in xenografted mice. A. Treatment protocol for subcutaneous xenografts tumors, DU145 and DU145 (B7H3-), in NCG mice. B, C. Tumor volume curves of DU145 mice and DU145(B7H3-) mice treated with PBS, control T cells and B7-H3 CAR-T cells (n=6). D, E. Body weight curves of the two mouse models. F, G. The content of T cells (Figure F) and CAR-T cells (Figure G) in the peripheral blood of mice. H, I. The percentage of T cells (Figure H) and CAR-T cells (Figure I) in the spleen. J, K. The numbers of T cells (Figure J) and CAR-T cells (Figure K) in tumor tissues. L. Immunohistochemical analysis of T cell infiltration in tumor tissue, Scale bar, 20× 50 μm. **P < 0.01, *P < 0.05 by ANOVA for multiple comparisons of the three treatment group.