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Research article

PD-1 blocking strategy for enhancing the anti-tumor effect of CAR T cells targeted CD105

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ABSTRACT

Purpos: CD105 has become a promising target of immunotherapy development for highly specific expression on the neovascular surface of most types of tumor cells. In previous studies, we constructed a CAR T cell (CD105 CAR T cell) and observed significant antitumor activity. In this study, we optimized the structure of CD105 CAR to increase PD-1 antibody secretion function (CD105 × PD-1 CAR T cells).
Methods: we tested whether Increased PD-1 antibody secretion with CAR T cells targeted CD105 could promote in vitro proliferation, proinflammatory cytokine production and cytotoxicity,or not. For the in vivo experiments, we constructed a subcutaneously transplanted tumor model and placed it in NOD/SCID mice to verify the anti-tumor effect of this therapy.
Results: Our data showed that the PD-1 antibody secreted by CD105 × PD-1 CAR T cells could specifically bind to the PD-1 receptor of T cells then blocked the PD-1/PD-L-1 signaling pathway, thus enhancing the activation and proliferation of CAR T cells. After incubation of CD105 × PD-1 CAR T cells with HepG2 as a hepatocellular carcinoma cell line expressing CD105, the results showed that CD105 × PD-1 CAR T cells increased the expression levels of CD69 and CD62L, enhanced the proliferation capacity of CAR T cells, and secreted more IL-2, TNF-α and IFN-γ than CD105 CAR T cells.
Conclusion: These data showed that CD105 × PD-1 CAR T cells was specifically killing tumor cells in vitro and in vivo. Our findings may therefore provide a promising new strategy for the improvement of CAR T therapy for solid tumors.

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1. Introduction

CAR T cell therapy modifies T cells by genetic engineering technology so that antigen receptor fragments for specific targets are expressed on the cell surfaces, and antigen signals are transmitted into the cells through hinge region and transmembrane region, thus realizing targeted activation of T cells and specific attack on the targets [1,2]. This therapy is independent of MHC, with activated T cells releasing cytokines to kill target cells, and some molecules on the surface of the effector cells changing correspondingly, by which relevant memory function is obtained [3]. Current CAR T therapies have modified the CAR components to better the effect on solid tumors [4,5].

Tumor cell neovascularization is an important source of blood nutrient supply of tumor, and one of the most important channels for metastasis as well. CD105, also known as endoglin, is highly expressed in tumor cells and vascular endothelial cells around or within tumors (including HCC),as an important marker of tumor neovascularization, and in recent years, anti-tumor therapy targeting CD105 has become a research hotspot [6,7,8,9,10]. Now there are studies that show that CD105 promotes the invasion and metastases of liver cancer cells by increasing VEGF expression [10,11]. There is convincing evidence supporting that CD105 plays a key role in neovascularization and it is the best molecular target. In our previous studies [12], the CD105 CAR T cells constructed with CD105 as the target showed an excellent function against tumor neovascularization, and thus, the optimized CAR structure has been verified, indicating that this therapy is promising for treatment of solid tumors. By now, several clinical trials have verified the potential of CAR T combined with PD-1 checkpoint inhibitor for relevant applications [13,14]. Immune checkpoints has long been considered to play a key role in the initiation and preservation of tumor immune escape, which provide a potent rationale for the treatment of patients with HCC [15]. However, theoretically, such a combination method is unable to maintain the optimal plasma concentrations, and it is not conducive to the local aggregation of PD-1 antibody in tumors as well. Several studies have proved that using a soluble scFv is a better method to enhance the activity and cytotoxicity of CAR T cells, which is an important research direction at present [16,17].

In this study, what was constructed is CD105 × PD-1 CAR cell secreting anti-PD-1 ScFv (with His-tag), which retained the original capability for targeted killing of hepatocellular carcinoma cells of CD105 CAR T. With the constructing PD-1 ScFv on CAR gene, CD105 × PD-1 CAR cell gained the ability to synthesize and secrete PD-1 ScFv after activation by intracellular expression of self-cleaving P2A peptide after cleaving. The increased concentration of PD-1 antibodies in tumor microenvironment is more conducive to the replication and activation of CAR T cells, and thus, this may enhance the antitumor activity of CAR T cells. The main purpose of this study was to investigate the difference in antitumor function between two construction strategies, i.e., CD105 CAR and CD105 × PD-1 CAR.

2. Materials and methods

2.1. Materials

Female NOD/SCID mice, aged 4–6 weeks, were purchased from Beijing Vital River Lab of Animal Technology (Beijing, China) and raised in an SPF environment. all animal experiments comply with the ARRIVE guidelines and were performed in accordance with the National Institutes of Health guide for the care and use of Laboratory animals. Hepatocarcinoma cell line (SMMC7721, HepG2 and MHCC97H) was purchased from ATCC (China) and preserved in this laboratory, culture conditions: Dulbecco modified Eagle medium (DMEM) (Invitrogen, USA) and 10% fetal bovine serum (FBS) (HyClone, USA). PBMC (peripheral blood mononuclear cell) was isolated from Peripheral blood of normal volunteers by gradient density centrifugation method. Primary PBMC, cultured in RPMI-1640 supplemented with 10% human serum, 5% L-glutamine-penicillin-streptomycin solution (Sigma, U.S.A) and 200 IU/mL of IL-2 (Peprotech). These cells were stored in an incubator containing 5% carbon dioxide at 37 °C using 25 cm^2 cell culture flasks(Corning, U.S.A). Vector pCDH-CMV-MCS-EF1-CopGFP-T2A-puro was purchased from Japan SBI, DH5αcompetent escherichia coli cells were purchased from TransGen Biotech, Beijing, the plasmid mini preparation kit, Gel extraction kit and PCR kit from Tiagen Biochemical Technology Co., Ltd., and T4 DNA ligase and Taq polymerase were TaKaRa products. Restriction endonucleases *Xba*I and *Nhe*I were purchased from NEB Company, DNALadder and Lipofectamine 2000 from Thermo company, and the endotoxin-free plasmid mini preparation kit from OMEGA Company.

2.2. CAR T cells construction

The PD-1 scFv was derived from Nivolumab, whose sequence was obtained from imgt(http://www.imgt.org/). It was designed and synthesized into CD105 × PD-1 CAR, including signal sequence, CD105 Nb(nanobody) sequence, GS linker, CD28 transmembrane domain, 4-1BB costimulatory domain, CD3ζ chain, P2A sequence, PD-1 ScFv sequence, IRES, and GFP. The CD105 × PD-1 CAR was cloned into pCDH-CMV-MCS-EF1-CopGFP-T2A-puro vector and transformed into competent cells to extract the CAR plasmid. After packaging lentivirus, CAR transduction was performed, and the transduction efficiency of CAR and secretion of PD-1 antibody were detected by flow cytometry and SDS-PAGE. Written informed consent was obtained from all volunteer. This study has been approved by the local ethics committee of Hainan Medical University.

2.3. Flow cytometry

Groups of CAR T cells were cultured in vitro with HepG2 cells treated by mitamycin in a 1:1 ratio for 12 h. Then, the CAR T cells were performed CD69(BD, Cat555431), CD62L (BD, Cat555543), TIM-3(ABCAM ab210543)flow cytometric antibody staining. The expression of these surface molecules was detected byflow cytometry, and the cell proliferation was detected by flow cytometry after

PKH26(Sigma-Aldrich) staining.

2.4. ELISA and ELISPOT

After 16 h of incubation with HepG2, two groups of CAR T cells were detected for the level of IL-2 and TNF-α in the supernatant by ELISA Kits (BD) according to the manufacturer's protocols.

The density of IFN-γ secreting cells was detected by ELISPOT method. In short, CD105 × PD-1 CAR T cells and control cells (3×10^5 cells/well) were incubated overnight at 37 °C with 10^5 irradiated HepG2 cells on a 96-well plate in triplicate. After washing, captured IFN-γ was reacted with biotinylated anti–IFN–γ in a single well overnight at 4 °C. Subsequently, IFN-γ specific immunocomplex was detected with Streptavidin-AP and visualized with substrate solution (BCIP/NBT). The number of spots formed in a single well was analyzed with CTL ImmunoSpot S6 Ultimate-V Analyzer.

2.5. Proliferation and killing assay

T cell proliferation was detected in vitro by PKH26 staining. Groups of CAR T cells (1×10^6 cells/tube) was performed 5 min of labelling with PKH26 (Sigma-Aldrich) at 37 °C. The same number of HepG2 cells (100 Gy) were irradiated for 120 h, and then, incubated with groups of CAR T cells. The suspended CAR T cells were collected for determining the percentage of proliferated T cells by flow cytometry. In the cytotoxicity test, each group of CAR T cells was incubated with pkh26-labeled SMMC7721, HepG2 and MHCC97H cells in a 3:1, 1:1, or 1:3 ratio for 16 h. After washing, the adherent target cells were collected for determining the percentage of $PKH26^+PI^+$ cells in each group by flow cytometry using Propidium iodide stain (PI, Sigma).

2.6. Xenograft experiments in mice

NOD/SCID mice aged 6–8 weeks were inoculated subcutaneously with1×10^6 HepG2 cells. After 14 days, when the average tumor size reached 100–120 mm^3, 5×10^6 adoptive cell therapy by i.v. was carried out for the mice. The tumor growth was monitored twice a week, and the tumor size was measured with a caliper and calculated by the following formula: $d1 \times (d2)^2 \times 0.52$, where d1 represented the longest diameter, and d2, the shortest diameter perpendicular to d1.The mice were euthanized when they had significant weight loss, tumor ulceration, or tumor size greater than 1500 mm^3.

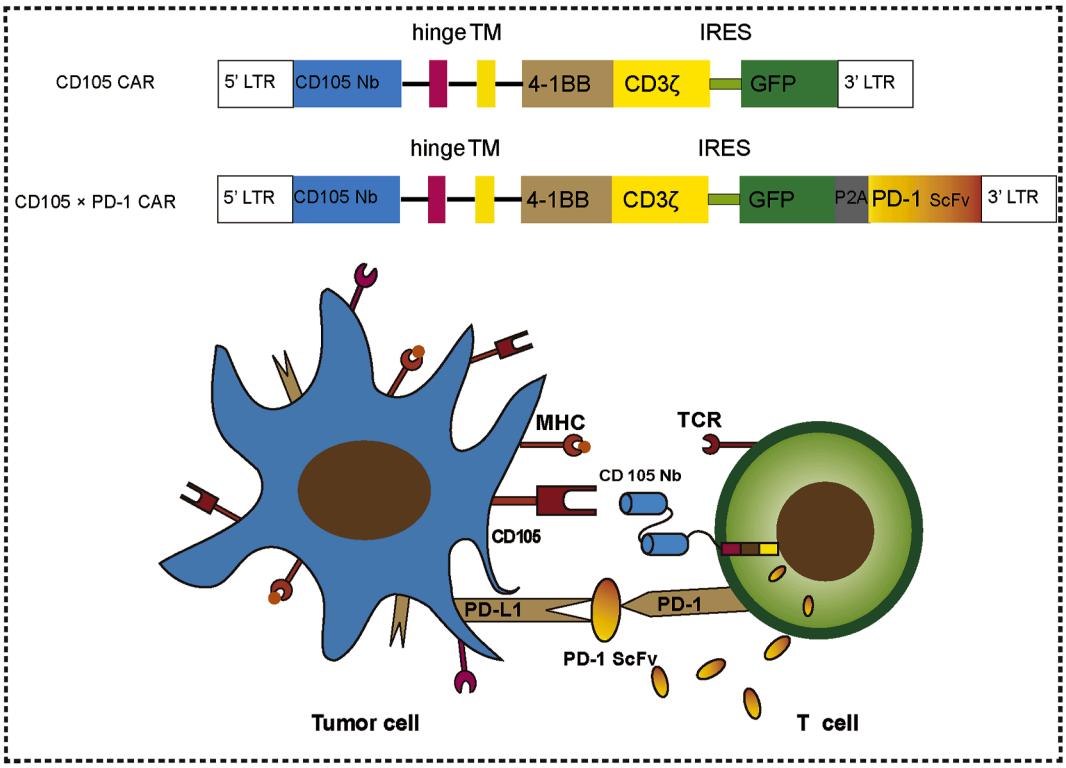

Fig. 1. The schematic of the CAR construct.

2.7. Immunohistochemistry

After dissection, the tumors of mice were fixed in 10% neutral formalin, and sectioned (4 μm) after paraffin embedding. CD34 is a specific marker of tumor blood vessels, in order to investigate the microvascular densities of xenograft tumor after treatment, the

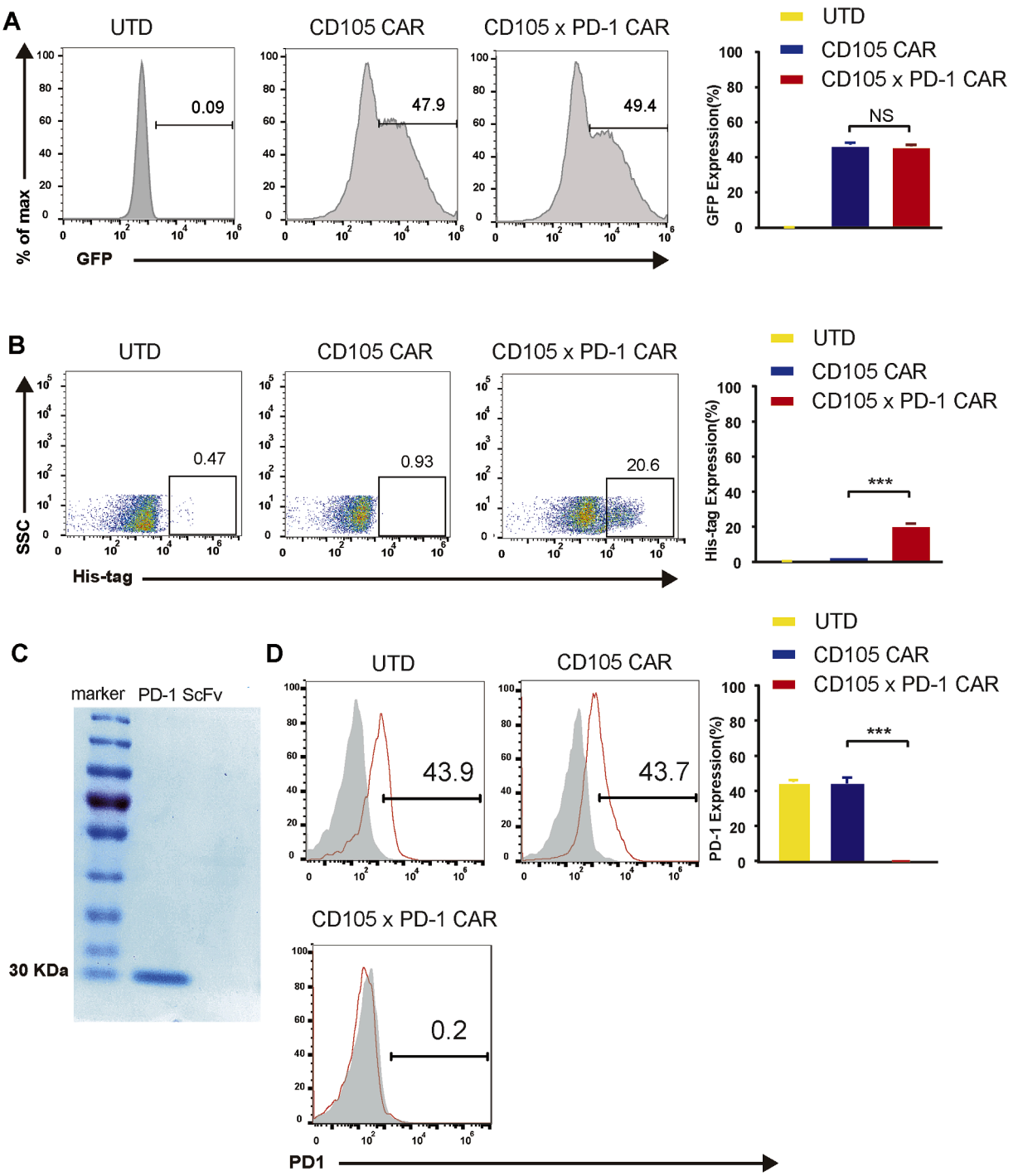

Fig. 2. Generation of CD105 × PD-1 CAR T cells. (A)Flow cytometry analysis of GFP expression of CAR T cells. $n = 3$, *NS* represents not significant. (B)Flow cytometry analysis of His-tag expression of T cells after incubating the CAR T cell culture supernatant. n = 3, NS represents not significant. (C) Analyzed the PD-1 scFv was secreted in the supernatant with SDS-PAGE.(D)Flow cytometry analysis of PD-1 expression of T cells after incubating the CAR T cell culture supernatant. $n = 3$, *NS* represents not significant.

tumor tissue paraffin sections were immuno-stained using anti-human primary antibodies (CD34) (ABCAM ab81289) according to the manufacturer's instruction.

Apoptotic cells in the tumors were detected (TUNEL) with the In Situ Cell Death Detection Kit (FITC, Roche, Switzerland) according

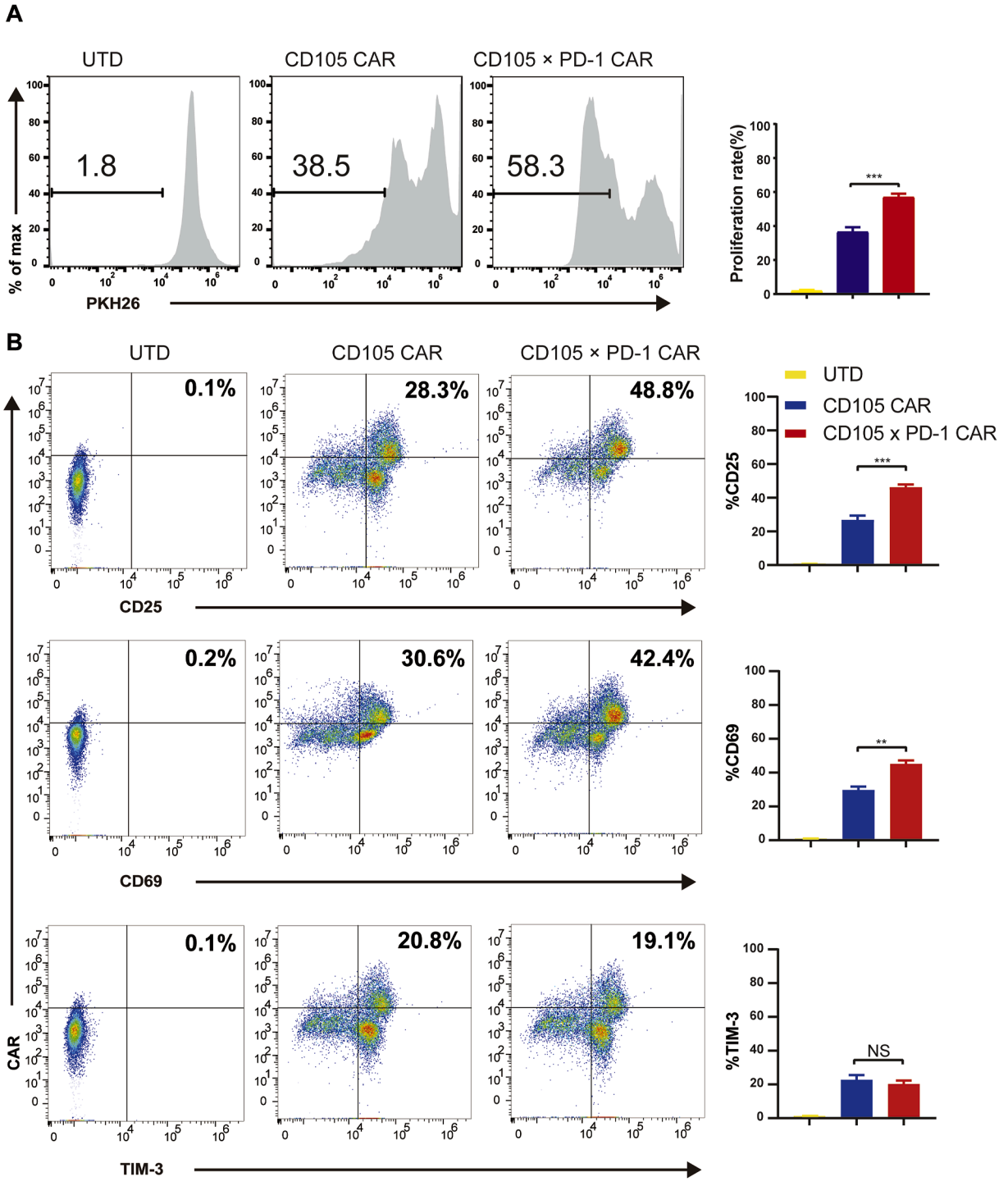

Fig. 3. Emergence of activation and memory of the CD105 × PD-1 CAR T cells in vitro with CD105 antigen-dependent. (A)Flow cytometry analysis of proliferation frequency in PKH26-labeled CAR T cells co-cultured with HepG2 $n = 3$, $^{**}P < 0.01$. (B) FACS analysis on activation markers (CD69), memory marker(CD62L) and depletion marker (TIM-3) expression percentage of CAR T cells after being co-cultured with CAFs for 12 h. *NS* was shown to be insignificant. $n = 3$, $^{**}P < 0.01$.

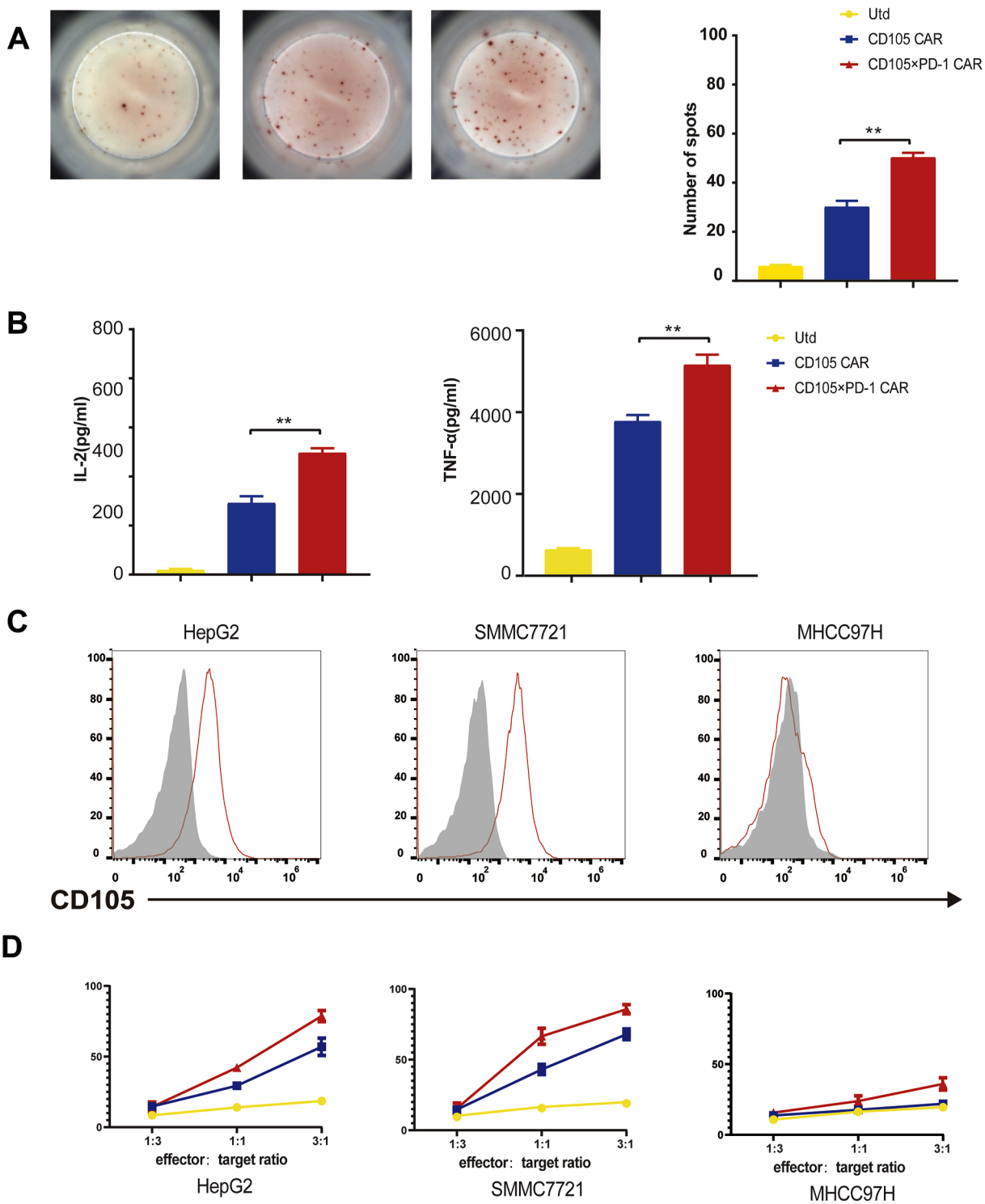

Fig. 4. CD105 × PD-1 CAR T cells specifically eliminate CD105^{+} target cells. (A) LISPOT analysis of the frequency of IFN-γ-secreted CD105 × PD-1 CAR T cells. $n = 3$, $^{**}P < 0.01$.(B) CD105 × PD-1 CAR T cells produced higher levels of pro-inflammatory cytokines viz IL-2 and TNF-α. $n = 3$, $^{*}P < 0.05$, $^{**}P < 0.01$.(C) The CD105 expression of target cells in each group.(D)Specific lysis of the CAR T cells detected by FACS.

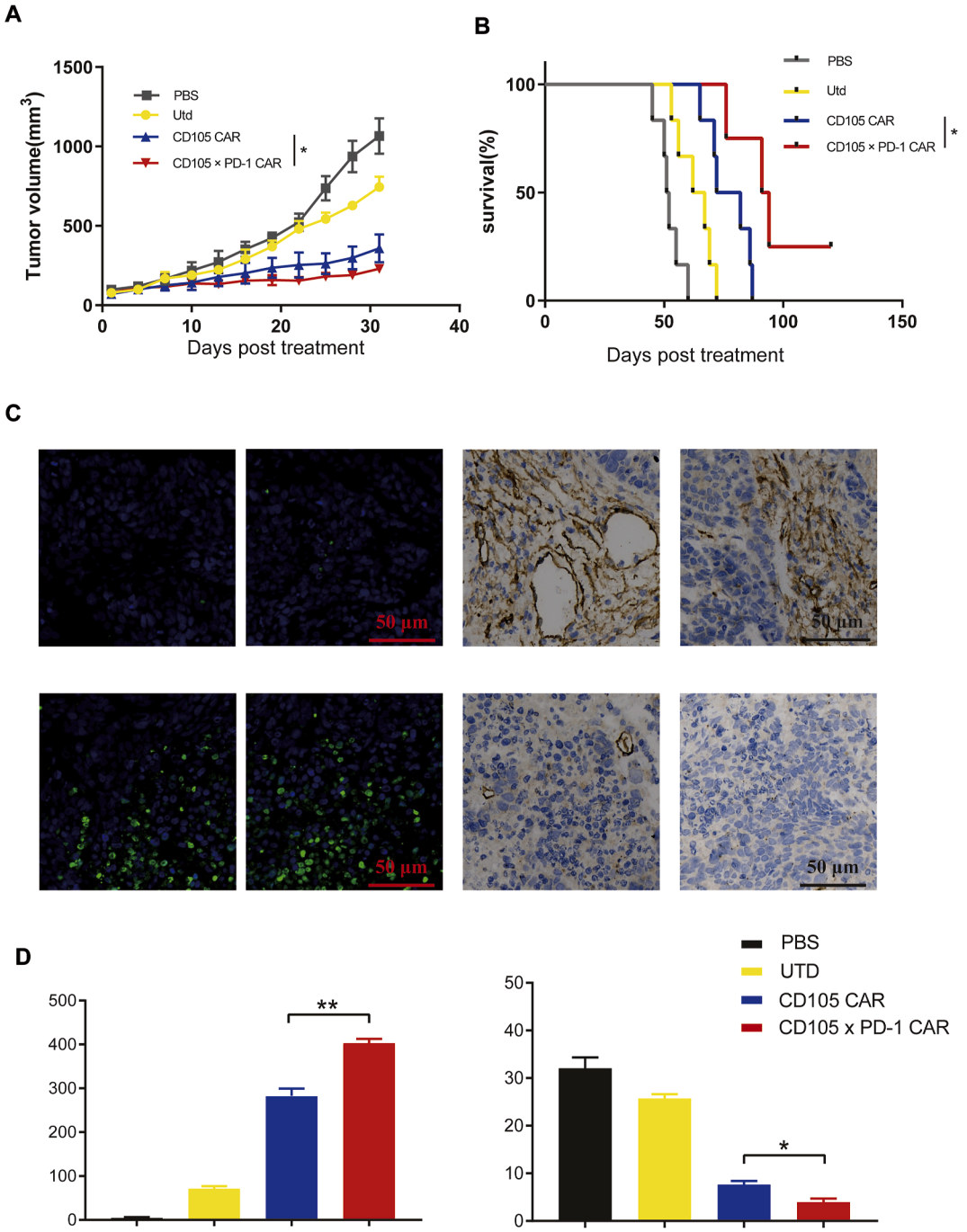

Fig. 5. In vivo antitumor activities of CD105 × PD-1 CAR T cells in the established subcutaneous human tumor xenografts (A) Measurement of tumor volume in mice of each group on day 33. $n = 5$, $^*P < 0.05$. (B) Kaplan-Meier survival curve of each group. n = 5. (C) The date showed that the number of apoptosis cells in CD105 × PD-1 CAR group was more than that in the other treatment control groups (200 ×). Green: apoptotic cells; red: nucleus. The number of apoptosis cells in each section was taken from 5 sections for statistical analysis. $^*P < 0.05$ represented that the difference was statistically significant. (D) The date showed that the number of blood vessels in the tumor tissue of the CD105 × PD-1 CAR group was less than that in the other treatment control groups (200 ×). $^*P < 0.05$ represented that the difference was statistically significant.

to the manufacturer's instruction. Images were obtained by a microscope (Nikon, Japan).

2.8. Statistical analysis

After the data were input into excel database, a statistical analysis was performed through GraphPad Prism 5.0 (GraphPad, La Jolla, CA).Count data were expressed as mean ± standard deviation. ANOVA was used to assess the differences between groups, and Kaplan-Meier curve to describe the survival of each group. At the same time logarithmic rank test was performed. Statistical significance was defined as $P < 0.05$.

3. Results

3.1. The CD105 × PD-1 CAR T cell was constructed successfully

The CD105 × PD-1 CAR molecular was as shown in Fig. 1. For construction of lentiviral vector code CD105 Nb, CD8a hinge region, 4-1BB, CD3ζ, and PD-1 scFv, cleavable sequence P2A was supplemented based on CD105 CAR, The primary PBMC in human peripheral blood was extracted and separated for CAR transduction. For CAR expression, the transfection rates in CD105 CAR T cell and CD105 × PD-1 CAR T cell were 47.9% and 47.4% respectively, as shown in Fig. 2A. The secretion of anti-PD-1 antibodies in the supernatant of CD105 × PD-1 CAR T cell was further detected by SDS-PAGE, and the results showed that a large amount of PD-1 antibodies was secreted in the supernatant(Fig. 2C). To evaluate the binding force of T cell and PD-1 antibodies, the supernatant of CD105 × PD-1 CAR T cells, CD105 CAR T cells and PBMC were taken for incubation with activated T cells, and then, the secreted PD-1antibodies was detected by flow cytometric anti-His-tag mAb and anti-PD-1mAb. The results showed that PD-1antibodies bound PD-1 on the surface of T cells after incubation(Fig. 2B, D).

3.2. Increased secretion of PD-1 antibodies enhanced the activation and proliferation of CAR T cell in vitro

To evaluate the proliferation of CD105 × PD-1 CAR T cell after antigen - specific activation, UTD, CD105 CAR group and CD105 × PD-1 CAR T group of cells were taken for 48 h of incubation with irradiated HepG2 cells respectively. The results showed that CD105 CAR group and CD105 × PD-1 CAR T group of cells proliferated significantly after CD105 antigen stimulation. Furthermore, the proliferation rate of CD105 × PD-1 CAR T group was higher than that of CD105 CAR group(Fig. 3A).

CD69, CD62L and TIM-3, as T cell surface activated molecule, memory molecule, and depletion molecule, play an important role in the proliferation and survival of CAR T cell. To evaluate the effect of PD-1 antibodies secreted by CD105 × PD-1 CAR T cell on the expression of CD69,CD62L, and TIM-3, groups of CAR T cells were performed 12 h of incubation with HepG2, and the results that the expression rates of CD69 and CD62L in the CD105 × PD-1 CAR T group were significantly higher than in the CD105 CAR group, while there was no significant difference in TIM3 expression between the two groups(Fig. 3B), suggesting that increased PD-1 antibodies secretion enhanced the proliferation and activation of CAR T cells.

3.3. Increased secretion of PD-1antibodies enhanced the capability of CAR T cell for specific $CD105^+$ target cell killing

In cytotoxicity assay, we found that the CD105 × PD-1 CAR T demonstrated significant cytotoxic activity against CD105-positive SMMC7721 and HepG2 except for CD105-negative MHCC97H cells(Fig. 4C and D). The ELISA resaults showed that IL-2 and TNF-α levels in the CD105 × PD-1 CAR T group were higher than in the CD105 CAR group(Fig. 4B). ELISPOT assay also showed that the number of IFN-γ -secreting positive cells in the CD105 × PD-1 CAR T cell group was higher than in the CD105 CAR T cell group, suggesting that increased PD-1 antibodies secretion enhanced the capability of CAR T cells for specific $CD105^+$ target cells(Fig. 4A).

3.4. Increased secretion of PD-1antibodies enhanced the antitumor activity of CAR T cell in vivo

To evaluate the antitumor effect of group of CAR T cells, a NOD/SCID mouse xenograft subcutaneous tumor model of HepG2 was established. After treatment, the experimental results showed that CD105 × PD-1 CAR T cell significantly prolonged the survival period of mice, and reduced the tumor growth volume of mice, thus demonstrating an excellent tumor suppression effect, and this tumor suppression capability was enhanced relative to CD105 CAR T cell(Fig. 5A and B). Pathological tests also showed that CD105 × PD-1 CAR T cell induced tumor cell apoptosis and decreased microvascular density in mice(Fig. 5C and D).

4. Discussion

For CAR T cell therapy promising in the anticancer field, remarkable achievements have been made in the treatment of acute leukemia and non-Hodgkin lymphoma, and the therapy has been approved by FDA [18]. However, unlike application in hematologic tumor treatment, the application of CAR T cell therapy in treatment of solid tumors is faced with a complex and impressive dilemma. Relevant limitations in therapeutic effect were mainly reflected in the selection of tumor antigens that can be targeted, inhibition of CAR T cell function by immunosuppressive microenvironment, duration of CAR T cells in vivo, and toxic and side effects caused by off-target effect [19,20]. The physiological barrier of solid tumor matrix may also prevent CAR T cells from maximizing relevant efficacy, thus causing poor outcomes. In addition, binding of PD-L1 (an immune checkpoint inhibitory molecule expressed on tumor

cell) to PD-1 on CAR T cell negatively regulates CAR T cell activity, and inhibits proliferation of T cell, production of cytokines such as IL-2 and INF-γ, proliferation and differentiation of B cell, and secretion of Ig. Current research shows that the impaired of anti-tumor immune surveillance in the liver microenvironment promotes the development of tumors, and PD-1/PD-L1 signaling pathway plays an important role in this process [21,22]. Since the high expression of PD-L1 in HCC cells inhibits the activity of T cells in the liver tumor microenvironment, the high expression of PD-L1 in tumor cells has been identified as a predictor of relapse in HCC patients [23]. Some studies showed that PD-1 expression of CAR T cells increased after antigen-specific activation, and blocking PD-1 enhanced CAR T cell function and IFN-γ production [24,25]. In conclusion, the application of CAR T cell in solid tumors is faced with double inhibition of tumor cells and stroma. Current major challenge in research on CAR T cell therapy is how to design and optimize the CAR T cell for effective intervention of solid tumors, overcoming the immunosuppressive microenvironment to live longer in the body for anti-tumor therapy.

As a specific transmembrane protein expressed in tumor endothelial cells, after specific binding with TGF-β, CD105 can promote the production of VEGF, proliferation and migration of tumor cells, and formation of extracellular matrix [26].The high expression of CD105 in liver cancer is closely related to the prognosis of patients, and a test showed that injection of monoclonal antibody into mouse models of liver cancer may inhibit tumor growth and reduce microvascular density [27]. In a previous study, we designed a CD105 CAR T cell for anti-tumor effect investigation, which was proved to be feasible for inhibiting tumor neovascularization [12]. This study focused on whether increased PD-1 antibodier secretion enhanced CAR T cell function or not. The PD-1 ScFv secreted by CD105 × PD-1 CAR T bound to PD-1 on the surface of T cell and blocked the interaction of PD-1/PD-L1 signaling pathway, thereby relieving the inhibition of T cell activity. In the design of CAR structure, we selected CD105 Nb as the extracellular segment of CAR, binding CD8a as the transmembrane region, 4-1BB as the costimulatory molecule, CD3ζ as the intracellular segment, and added PD-1 scFv to construct CD105 × PD-1 CAR T cell. The CD105 × PD-1 CAR T cells stimulated by $CD105^{+}$ tumor cells produced more CD69 and CD62L, which were considered as molecules involved in cellular activation and anti-tumor memory. CD105 × PD-1 CAR T cells also secreted more inflammatory cytokines IL-2 and TNFα under a transfection efficiency similar to that of CD105 CAR, the numbers of IFN-γ-secreting spot forming cells were significantly greater than CD105 CAR, showing higher killing efficiency for target cells and cytotoxic ability.

After in vivo CAR T cell adoptive therapy, the experimental results were consistent with our in vitro data, which supported the enhancement of CD105 × PD-1 CAR T cell for antitumor effect. Compared with CD105 CAR T cell, CD105 × PD-1 CAR T cells had more significant effects in inhibiting tumor growth of mice and prolonging survival period of mice. In terms of tumor suppression mechanism, after injection of tumor model, CD105 × PD-1 CAR T cells further inhibited the proliferation of tumor cells, promoted the apoptosis of tumor cells in vivo, and prevented the formation of tumor micro-vessels. Current study failed to clarify whether the secreted PD-1Nb affected other immune cells in tumors in addition to adoptive CAR T cells. Considering the persistent regulatory effect of PD-1 blockers on tumor microenvironment, the ability of CAR T cell that secretes PD-1Nb to clear solid tumors under immune conditions (such as gene mouse model and PDX model mouse) is to be explored for further improving the treatment of solid tumors.

In summary, wehave developed a novel CAR T cells-targeting CD105 and has the function of PD-1 antibodies secretion. we provide preclinical evidence for the therapeutic potential of CD105 × PD-1 CAR T cells. These CAR T cells can selectively lytic CD105-positive cells both in vivo and vitro, and it seems to be better than CD105 CAR. This optimally designed CAR T therapy may prove advantageous for the treatment of malignant solid tumors by destroying the stroma. Thus, it has the potential to improve current immunotherapeutic approaches for malignant solid tumors.

Declarations

Author contribution statement

Xi Wang; Qinghui Sun: Analyzed and interpreted the data; Wu Wang: Conceived and designed the experiments; Wenli Yang: Performed the experiments; Wrote the paper; Zhiheng Lai; Yanyang Pang: Performed the experiments.

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Data availability statement

No data was used for the research described in the article.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.heliyon.2022.e12688.

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