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Immunology

Chimeric Antigen receptor-T (CAR-T) cells targeting Epithelial cell adhesion molecule (EpCAM) can inhibit tumor growth in ovarian cancer mouse model

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ABSTRACT. Ovarian cancer (OC) is one of the most lethal solid tumors with poor prognosis. In 2017, two chimeric antigen receptor-T (CAR-T) cell drugs were approved by the U.S. Food and Drug Administration (FDA), and continuously optimized CAR-T cells therapy might be the novel hope for OC patient. EpCAM are known to be over-expressed in OC cells and could be targeted by CAR-T cells. However, the feasibility of using EpCAM-CAR-T cells to treat OC still needs to be verified. We engineered the 3rd-generation EpCAM-CAR containing a single-chain variable fragment (scFv) EpCAM-scFv that targeting EpCAM, a CD8 transmembrane domain, the costimulatory domains from both CD28 and 4-1BB, and activating domain CD3ζ and then transduced the CAR into T-cells via lentivirus. In addition, the cytotoxicity and cytokine releasing ability of the EpCAM-CAR-T cells against OC cell SKOV3 were verified *in vitro*. The *in vivo* data also showed that EpCAM-CAR-T cells significantly reduced the tumor size in OC xenograft mouse models. The anti-tumor activity of EpCAM-CAR-T cells against OC *in vitro* and *in vivo* indicated that the CAR-T might provide a promising therapeutic approach to OC.

KEY WORDS: chimeric antigen receptor T cells, epithelial cell adhesion molecule, immunodeficient mice, immunotherapy, ovarian cancer

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Ovarian cancer (OC) is the 7th most frequent malignancy the most lethal gynecologic malignant tumor in women in the world and leads to 4% of cancer-related deaths [24]. Despite this is big progress in adjuvant chemotherapy and surgery, the overall 5-year survival rate of OC has only risen from 37 to 46% in the last 30 years. The poor prognosis of OC is mainly due to under-diagnosis in the early stage and the frequent occurrence of current chemotherapy regimens resistance [19, 26]. Although more than 80% of OC patients had a response to therapy at the early start, the majority of these patients ultimately recurred in OC [20]. Due to the drug or chemotherapy resistant, metastatic OC cells always stay dormant for years after traditional anti-tumor therapies and imperil one's life progressively [11]. Therefore, new therapy strategies and paradigms are needed for these OC patients.

In recent years, immunotherapy offers a promising treatment option for hematological and solid tumor [13]. Chimeric antigen receptor (CAR) T cell therapy cells are genetically engineered T cells, which characterized with tumor-specific, major histocompatibility complex-independent and immune-mediated cytolytic actions [29]. In 2017, two CAR-T cell therapies against acute lymphoblastic leukemia (ALL) and advanced lymphomas were approved by the U.S. Food and Drug Administration (FDA). Therefore, CAR-T therapy might be the novel hope for a cure for OC patient. response rates against solid cancer have been less successful to date. Nowadays, more and more attempts are to modulate the immunosuppressive tumor microenvironment and to enhance the CAR-T cell antitumor effects in solid cancers [17].

The CAR is a fusion protein incorporated of three components: an extracellular antigen recognition domain, which is a single-chain variable fragment (scFv) that recognize the tumor-related antigen (TAA); an intracellular signaling domains, such as, OX40, CD28 and 4-1BB (CD137), involved in the activation and the killing effect of T cells; a CD3ζ T cell activating domain. These components can help CAR-T cells target and wipe out the tumor cells [8]. Twenty years ago, the CAR-T treatment plan was

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proposed, but because the 1st generation structure only contained the CD3 activation domain and lacked the T cell co-stimulatory domain, T cells quickly apoptotic in the body and failed; in the follow-up study, the 2nd generation CAR structure was formed by improving the structure of CAR and adding a costimulatory signal domain. The products that have been marketed all adopted the 2nd generation CAR structure; on the basis of the 2nd generation CAR structure, a costimulatory signal is added to enable CAR-T cells to achieve stronger and longer-lasting immunity in the body. The structure is the 3rd generation CAR structure. Epithelial cell adhesion molecule (EpCAM) [16, 25] was originally found to be the main antigen of human colon cancer [9], and was a type I transmembrane glycoprotein, on a subset of normal epithelia [18] and numerous stem cells Expression [28]. And in some epithelial cancers [23], including ovarian cancer [30], the EpCAM antigen is also overexpressed in a heterogeneous manner. Although the detailed functions of EpCAM are still poorly understood, recent study have indicated that the role of EpCAM is not only correlated with cell adhesion, but also related to cell proliferation, differentiation and cell signaling [5, 9, 16]. In recent studies, EpCAM-targeted CAR-T cells have been used to treat various solid cancers [6, 31]. However, the possibility of using modified EpCAM-CAR-T cells to treat OC still needs to be revealed. In the present study, we constructed 3rd-generation EpCAM-CAR-Targeting EpCAM domain of tumor cells and verified the anti-tumor activities of the EpCAM-CAR-T cells *in vitro* and *in vivo*. Our study indicated that EpCAM-CAR-T cells might be the clinical application for the therapy of OC patients in the future.

MATERIALS AND METHODS

Construction of chimeric antigen receptor vector

The sequences of the 3rd-generation EpCAM-CAR contain the EpCAM-specifc target scFv with a CD8α signal peptide, a CD8 hinge and transmembrane sequence, as well as the intracellular signaling domain of 4-1BB, CD28 and CD3ζ in tandem. The full-length nucleotide sequence was synthesized (Shenggong, Shanghai, China) and inserted into a lentiviral vector pCDH-CMV-MCS-EF1-puro through EcoRI and NotI cloning sites.

Lentivirus packaging and production

Viruses were collected from the supernatants of HEK-293T cells transfected with the lentivirus vector and two helper packaging plasmids (psPAX2 and pMD.2G) using polyethyleneimine (Polyscience, Niles, IL, USA). After post transfection, lentivirus-rich supernatants were collected at 24, 48, and 72 hr respectively and filtered through a 0.45-μm filter.

Obtaining primary cells and CAR-T cells

Fresh blood was collected from healthy volunteers after informed consent under a protocol approved by the Ethics Committee of the First Affiliated Hospital of Dalian Medical University. Peripheral blood mononuclear cells (PBMC) were isolated from normal donor blood buffy coats using Ficoll (GE). T cells were separated from PBMCs using a mononuclear cell isolate tube (Sepmate, Stemcell, Vancouver, Canada). Primary cells were cultured in T Cell Medium made with GT-T551H3 (Takara, Kyoto, Japan) with 1% Penicillin-Streptomycin-Glutamine (Gibco, Grand Island, NY, USA). EpCAM-CAR-T cells were generated through the lentiviral transduction of normal donor T cells as described below. T cells isolated from normal donors were activated using microbeads coated with anti-human CD3 and anti-human CD28 antibodies (CytoCares, Shanghai, China) at a 1:1 bead: cell ratio, and then infected with lentivirus for 24 hr after stimulation at a multiplicity of infection (MOI) of 30. After infection, T cells were cultured in fresh media containing IL-2 (300 IU/ml) and added appropriate fresh media every 2–3 days.

Cells and culture conditions

HEK-293 T cells were maintained in Dulbecco's modified Eagle medium (Gibco). human ovarian cancer cell SKOV3 was obtained from Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and maintained in RPMI-1640 (Gibco). Both DMEM and RPMI-1640 complete cell culture medium contained 10% heat-inactivated FBS (Gibco/Life Technologies, Shanghai, China), 10 mM HEPES, 2 mM glutamine (Gibco/Life Technologies), and 1% penicillin/streptomycin. All cells were cultured at 37°C in an atmosphere of 5% carbon dioxide.

Western blot analysis

To confirm CAR expression in the transduced T cells, cell lysates were denatured, separated by SDS-PAGE, and transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA, USA). After blocking, the membranes were incubated with an anti-CD3ζ primary antibody (551033, BD, San Jose, CA, USA), followed by incubation with HRP-conjugated secondary antibodies. Protein bands were exposed to ECL (GE Healthcare, Pittsburgh, PA, USA) and detected by autoradiography. Untransduced T cells were used as NC-T.

Flow cytometry

Flow cytometry cell staining was performed at 4°C in PBS supplemented with 2% FBS unless otherwise indicated. The antibodies used for flow cytometry and functional studies included human EpCAM-biotin (Acro, Beijing, China), anti-biotin-APC (Biolegend, San Diego, CA, USA), anti-EpCAM-PE (Biolegend). Data were analyzed using NovaExpr software (FlowJo, LLC, Ashland, OR, USA).

In vitro cytotoxicity assays

Tumor cells were seeded in 96wells-E-plate (ACEA Biosciences, San Diego, CA, USA). After 6 hr, either control or EpCAM-CAR-T

cells added at a target ratio of 1.5:1. Untransduced T cells were used as NC-T and added to the control group. The group that did not add the effector cells was called Tumor Only group. The viability of target cells were detected by Real Time Cell Analysis (RTCA) system (xCElligence RTCA SP. ACEA, San Diego, CA, USA) according to the manufacturer's protocol. Cell index was recorded every 15 min.

Cytokine release assays

Cytokine release assays was carried out using human IFNγ ELISA kit (88-7316-88, eBioscience, Thermo Fisher Scientific, Shanghai, China). EpCAM-CAR-T cells were co-cultured with target cells at the ratio of 1.5:1. After 24 hr the supernatant was collected and detected Interferon-γ (IFNγ) levels according to manufacturer's protocol.

CDX models for EpCAM-CAR-T cell treatment

NOD-Prkdcem26IL2rgem26/Nju (NCG) mice were purchased from NBRI (Nanjing,China). Animal experiments were carried out in the Beautiful Life animal center. All mice were maintained in specific pathogen-free (SPF) grade facility and were provided clean food and water. The experiment was approved by the MeiLiRenSheng Animal Ethics Committee, and the approval number of this animal experiment is SHMLRS005. For constructing the SKOV3 cell line derived xenograft (CDX) models, 1×10^6 SKOV3 cells in 100 µl were injected subcutaneously into 6–8 weeks old NCG mice and injected a total of 15 mice. When tumor nodes were palpable, we selected 12 mice of relatively uniform size and randomly divided them into 3 groups (n=4), group- Phosphate buffer saline (PBS), group-NC-T, group-EpCAM-CAR-T. Untransduced T cells were used as NC-T. EpCAM-CAR-T cells (2.5×10^6CAR+), NC-T cells and PBS, resuspended in PBS contain IL2 (200 U/ml), were injected intravenously into tumor-bearing mice (200 µl/mice). When any of the following occurs, stop the experiment immediately and euthanize the mice. The size of the tumor exceeds 2,000 mm^3, the weight of the mouse has decreased by more than 25%, and the mice cannot eat for more than 48 hr. The tumor ulcerates or causes significant pain. The tumor affects the normal movement and behavior of the mice. Tumor size were measured twice a week with a caliper, the tumor volume was calculated by following equation: volume=(length×width^2)/2.

Immunohistochemistry

All tumor and benign tissue samples were acquired with informed consent from the patients. All patients who provided primary specimens gave informed consent to use the samples for research purposes. All procedures were approved by the Research Ethics Board of the First Affiliated Hospital of Dalian Medical University. Ovarian cancer tissue was obtained from a 68-year-old woman with ovarian serous adenocarcinoma, defined as malignant ovarian cancer. Tissue sections were fixed with 10% paraformaldehyde, embedded in paraffin, sectioned at a thickness of 4 µm and immunostained with antibodies specific for EpCAM (14452S,CST, Danvers, MA, USA) overnight at 4°C, followed by secondary staining with secondary goat anti-rabbit Ig (PV-9000) (ZSGB-BIO, Beijing, China). Images of all sections were obtained with a microscope (BX53; Olympus, Tokyo, Japan).

Ethics statement

Fresh blood was collected from healthy volunteers after informed consent under a protocol approved by the Ethics Committee of the First Affiliated Hospital of Dalian Medical University. All procedures were approved by the Research Ethics Board of the First Affiliated Hospital of Dalian Medical University. The mice experiment was approved by the MeiLiRenSheng Animal Ethics Committee and the approval number of this animal experiment is SHMLRS005.

Statistics

Statistical analyses were performed using GraphPad Prism 5.0 (GraphPad Software, Inc., San Diego, CA, USA). All experiments were repeated at least three times. Student's *t* test was applied to identify the statistical significance of differences between two groups, and $*P$ values <0.05, $**P$ values <0.01 or $***P$ values <0.001 were considered significant.

RESULTS

Expression of EpCAM on ovarian cancer samples and cell lines

The data of immunohistochemistry showed that the expression of EpCAM in ovarian cancer tissue is significantly higher than that of the para-cancerous tissue (Fig. 1A). SKOV3 cell line as ovarian cancer epithelial cells, through flow cytometry method, through the detection of EpCAM target (Fig. 1B), found that there is a large number of EpCAM target expression on the SKOV3 cell, so we selected SKOV3 cells as target cell in evaluating CAR-T cells anti-tumor activity *in vitro*, *in vivo*.

Construction of third-generation CAR-T cells

To kill ovarian cancer cells through EpCAM targets, we designed a third-generation CAR structure including scFv targeting EpCAM antigen, a CD8 transmembrane domain, the costimulatory domains from both CD28 and 4-1BB, as well as T cell activating domain CD3ζ (Fig. 2A).The well-constructed CAR plasmid combined with two helper packaging plasmids are packaged into lentivirus through 293T cells .We obtain EpCAM-CAR-T lentivirus from the supernatant of 293T and the liquid of lentivirus was filtered to infect activated T cells. 7 days after lentivirus infection, the flow cytometry test data of infected T cells showed that the positive CAR rate is 43.53%. After protein extraction, western blot (WB) detection of CD3ζ protein showed that CAR-T cell not only expressed endogenous CD3ζ, but also expressed the recombinant CAR protein which was consistent with the expected size (Fig. 2B, 2C). The results above showed that the CAR structures we designed were expressed correctly at protein and cellular levels.

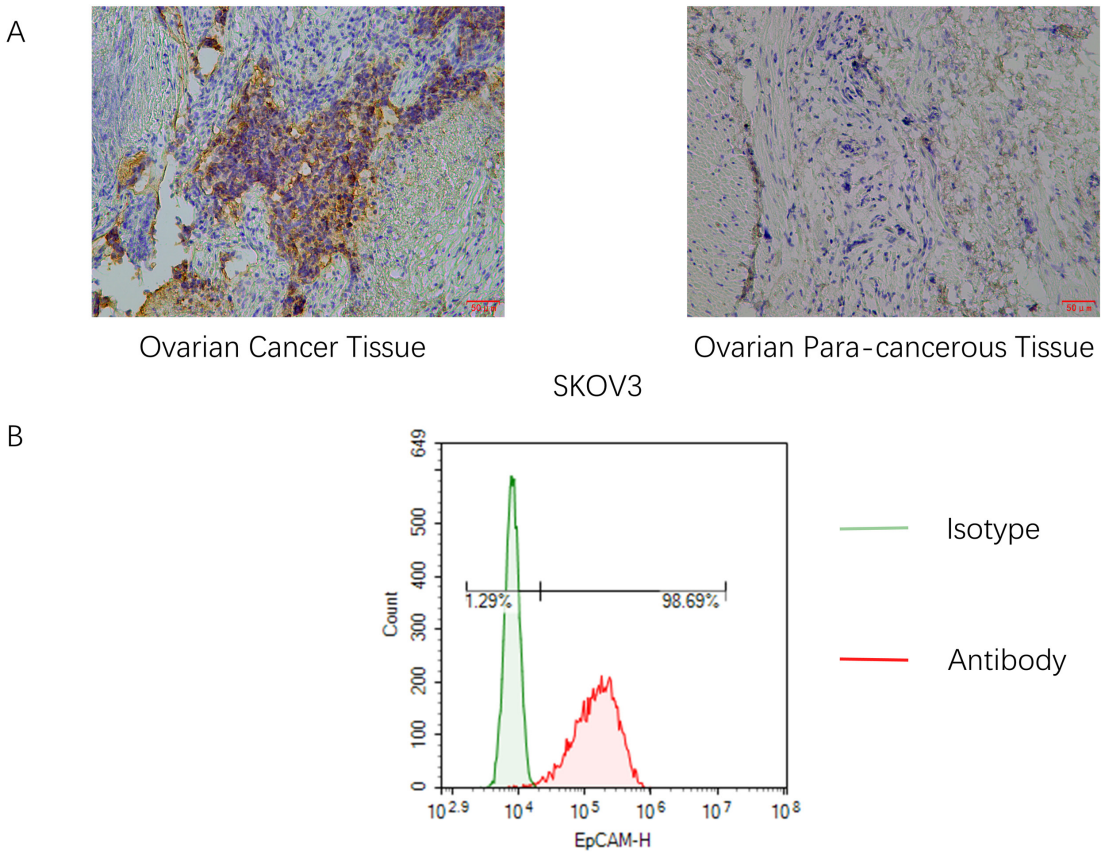

Fig. 1. Expression of epithelial cell adhesion molecule (EpCAM) on ovarian cancer samples and cell lines. A) EpCAM expression was detected in ovarian cancer (OC) and the para-cancerous tissue, scale bar=100 µm. B) The surface expression of EpCAM on OC cell line SKOV3 detected by flow cytometry.

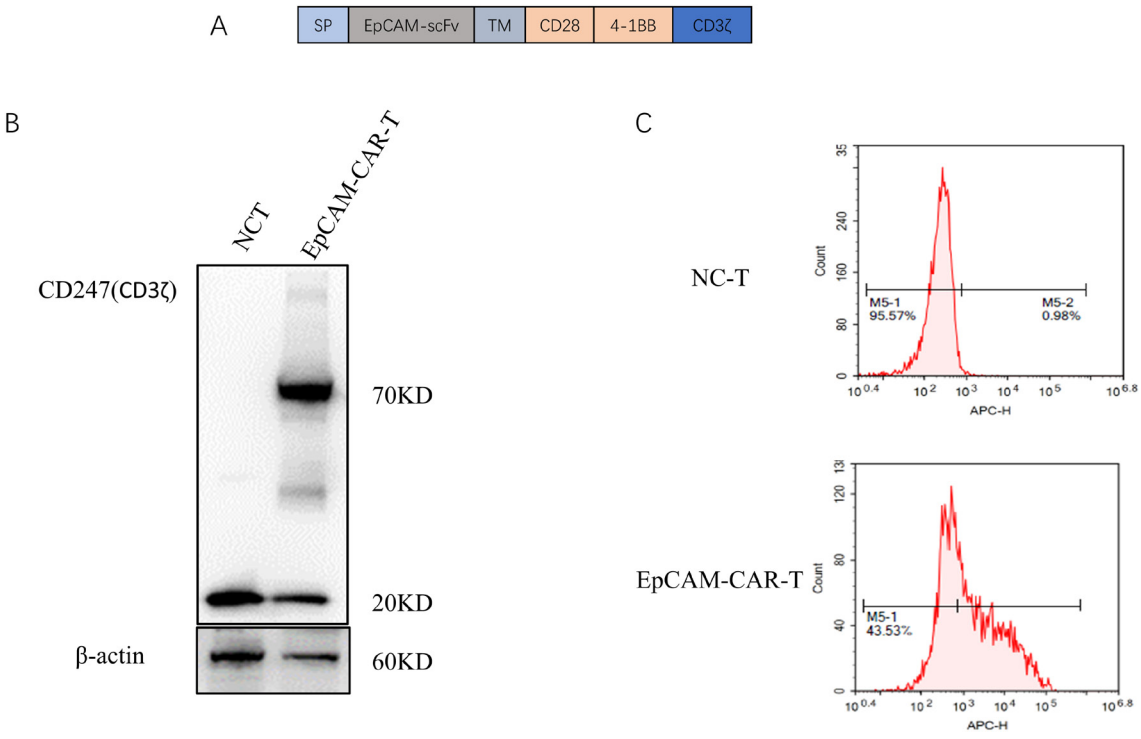

Fig. 2. Construction of third-generation chimeric antigen receptor-T (CAR-T) cells. A) Schematic diagram of the epithelial cell adhesion molecule (EpCAM)-CAR-T transgene. B) The CD3ζ level detected by Western blotting. C) Proportion of EpCAM-CAR infected primary human T cells determined by flow cytometry.

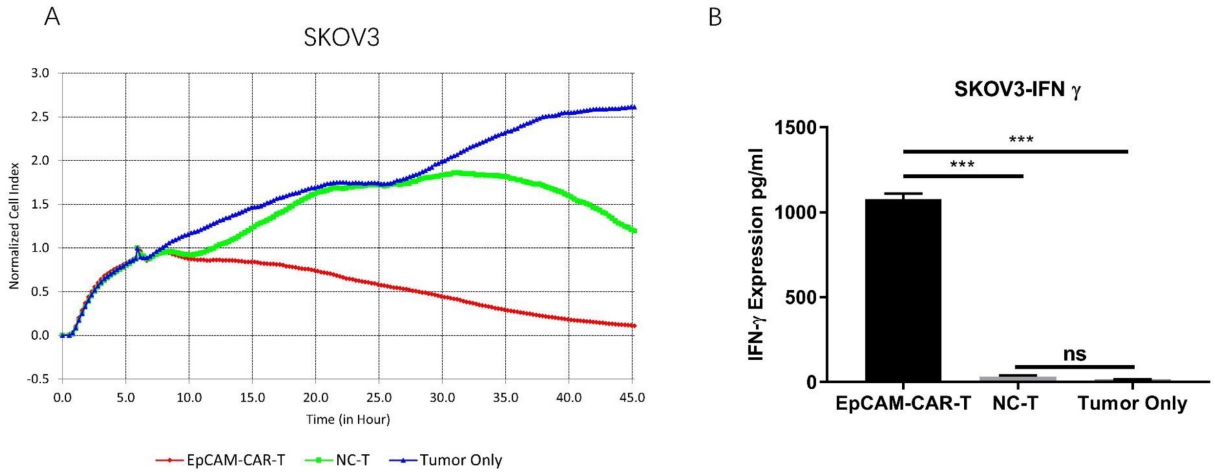

Fig. 3. Anti-tumor capability of epithelial cell adhesion molecule-chimeric antigen receptor-T (EpCAM-CAR-T) at the *in vitro* level. A) The cytotoxicity of EpCAM-CAR-T cells against SKOV3 cells was analyzed by Real Time Cell Analysis assay. B) Levels of Interferon-γ (IFNγ) released by EpCAM-CAR-T cells analyzed by ELISA after incubated with ovarian cancer (OC) cells for 24 hr. Untransduced T cells were used as NC-T. Error bars denote the s.e.m., and the results were compared with two-way ANOVA test. ***P<0.001.

Anti-tumor capability of EpCAM-CAR-T at the in vitro level

The data of flow cytometry and WB showed that the expression of CAR on EpCAM-CAR-T was well, and we used the Real Time Cell Analysis system to detect the real-time killing activity of EpCAM-CAR-T cells targeting the ovarian cancer cell line SKOV3. At E:T (Effector:Target)=1.5:1, EpCAM-CAR-T cells could kill the target cells when they were added to the SKOV3 cells, and at 40 hr, the target cells were close to total death. The control group cells did not respond within 24 hr after NC-T cell addition, which was consistent with the Tumor Only group. At 30 hr, the competitive cancer cell inhibition from CAR-T cells led to a decrease in detection values (Fig. 3A). In the same effector target ratio experiment, we incubated the cancer cells with CAR-T cells for 24 hr and the supernatant was collected for detecting the expression level of IFN-gamma using ELISA method. The ELISA data above showed that the IFNγ expression level of EpCAM-CAR-T group is significantly higher than that of other control groups, the IFNγ expression level of control groups is almost consistent with the baseline. Using two different methods, we prove that EpCAM-CAR-T cells had unique and fast *in vitro* killing activity targeting ovarian cancer cell line SKOV3.

Anti-tumor capability of EpCAM-CAR-T at the level of in vivo

The results of *in vitro* experiments proved that EpCAM-CAR-T had anti-tumor ability, and then we evaluated its anti-tumor ability in vivo using the mouse tumor model of SKOV3. First, we injected 1×10^6 SKOV3 cells in the subcutaneous part of each NCG mouse. One week later after cancer cell injection, we observed subcutaneous tumor formation in mice, which proved the success of the SKOV3-CDX model. We divided the mice into three groups containing PBS, NC-T, and EpCAM-CAR-T according to the tumor volume. Each SKOV3-CDX model of the EpCAM-CAR-T group was injected 2.5×10^6 CAR+ cells. In the corresponding control group, average tumor volume of the CDX-model is consistent with that of CAR-T group, and each mouse was injected with same number of T cells (Fig. 4A). Based on the data above, we found that the size of the tumor in the EpCAM-CAR-T group was much less than the size of the control group, especially in the 17-day-27-day period, the comparison between the EpCAM-CAR-T effect group and NC-T effective group was the most obvious, which showed that in the effective time of CAR-T cells anti-tumor effect of CAR-T cells was the most obvious in this special effective time. Twenty-seventh days later after T cell injection, EpCAM-CAR-T inhibition of tumors decreased and caused the tumor growth slowly. On the 41st day, we stopped the experiment. And using 2way ANOVA to analyze the data of the NC-T group and EpCAM-CAR-T group on day 41, the result showed that the P value of the two groups of data after comparison was <0.001, which proved the difference between the two groups of data at the end of the experiment is significant. (Fig. 4B).

DISCUSSION

Though various clinical trial including surgery, radiotherapy, chemotherapy, small molecule drugs, antibody drugs has applied to treat solid tumors, the therapies to improve the effect and prognosis of solid cancer patients are still poor [1, 27]. Ovarian cancer is one of the most lethal solid tumors with high incidence and fatality rate, and the median survival of ovarian cancer patients is only 18 months under the standard treatment of surgery followed by platinum-taxane chemotherapy [15]. Therefore, more effective and thorough treatments are still needed.

Although CAR-T therapy has made great progress in hematological tumors, CAR-T still has many problems and limitations in solid tumors [7]. In this study, we construct a CAR targeting EpCAM and demonstrated that PBMCs transduced with the EpCAM-CAR can kill the ovarian cancer cells skov3, both *in vitro* and *in vivo*. EpCAM is expressed on a variety of tumors [25, 30], and

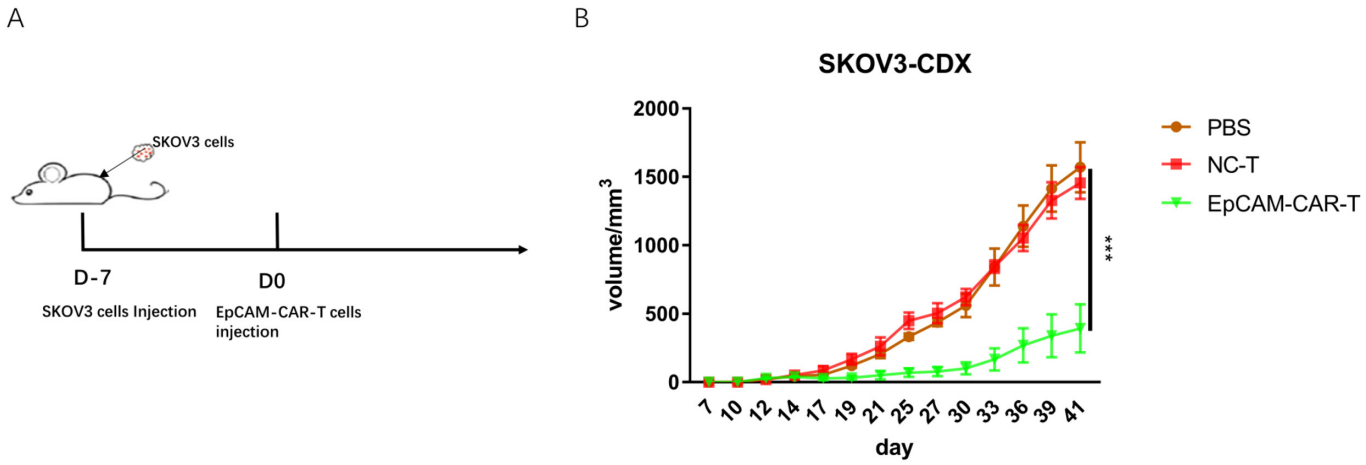

Fig. 4. Anti-tumor capability of epithelial cell adhesion molecule-chimeric antigen receptor-T (EpCAM-CAR-T) at the level of *in vivo*. A) Schema of the experimental events and nodes. In this process, model mice were injected with 1×10^6 ovarian cancer (OC) cells SKOV3. Seven days after injection, 12 mice were randomly divided into 3 groups: EpCAM-CAR-T cells treated group, control-T cells treated group and Phosphate buffer saline (PBS) of the same volume treated group. B) The tumor size variation with EpCAM-CAR-T cells, control T cells or PBS injection among the 41 days. Untransduced T cells were used as NC-T. Error bars denote the s.e.m., and the results were compared with two-way ANOVA test. ***P<0.001.

our results show that the EpCAM target is highly expressed in actual samples of ovarian cancer, which means that the use of EpCAM targets as a treatment method in ovarian cancer is theoretically feasible.

Beatty *et al.* used mRNA as the vector in a clinical Phase 1 trial, demonstrating the validity of MSLN-CAR T-cell therapy. However, mRNA has a short half-life in cells, and its long-term sustained effect in the body is poor, which is reflected in the results [4]. However, mRNA electroporated CAR was only transiently expressed in T cells, so that the anti-tumor responses may not be sufficiently durable to elicit a remission. In order to express the CAR gene for a longer time, we choose the lentiviral method for CAR gene transduction. We chose SKOV3, an ovarian cancer cell line with positive EpCAM expression, as the subject of this experiment *in vitro* and *in vivo*. In the *in vitro* experiment, we used RTCA technology to observe that when the effective target ratio was 1.5:1, the target cells added to the EpCAM-CAR-T group killed significantly and quickly after adding the cells. The target cells as the control group were not significantly killed. The cell index value began to slowly decrease 24 hr after the addition of NC-T cells. This decrease may be caused by the occupation of the cell space or the lack of nutrients required by the cells. In order to further demonstrate the specific killing ability of EpCAM-CAR-T cells on SKOV3 cells, we chose ELISA experiments for verification. According to the characteristics of CAR-T cells that release inflammatory factors to kill target cells after being activated [3], we found that the EpCAM-CAR-T cell group added EpCAM-CAR-T cell group specifically high IFN-γ expression in the target mixed cells, while the control group was at a very low level. These *in vitro* experiments prove that the EpCAM-CAR-T cells we designed can specifically kill the ovarian cancer cell line skov3 with high expression of EpCAM.

In the *in vivo* experiment, we injected EpCAM-CAR-T cells into mice injected with SKOV3 cells to form tumors and observed that the tumors of the mice injected with CAR-T cells were maintained in a small state compared to the control group. This completely shows that EpCAM-CAR-T has a good anti-tumor effect. After 27 days of injection, the tumors in the EPCAM group began to grow slowly. This may be caused by the decrease in the amount of CAR-T cells in the body. In subsequent experiments, we will carry out research around this point, larger CAR-T cell injection volume, multiple injections and changing administration methods are our next research directions. In addition, some current studies have shown that other functional elements should be incorporated into CAR carriers to promote CAR-T cell infiltration, maintain T cell effector activity, and enhance cooperation with bystander T cells or innate immunity, and further enhance the anti-tumor activity of CAR T cells. The current methods of implementing this strategy include the co-expression of immune-promoting cytokines [2, 10, 12], blocking the secretion of PD-1 receptor scFv [14, 21], and introducing dominant negative forms of inhibitory receptors in CAR [22]. These modifications to our CAR-T cells will be tested in future works to further improve the efficacy against large established ovarian cancer.

All these functional trials suggest that EpCAM-CAR-T cells may be a potential breakthrough in the treatment of ovarian cancer. More clinical attempts should be put into applying EpCAM-CAR-T cells to treat ovarian cancer in the future.

POTENTIAL CONFLICTS OF INTEREST. The authors have nothing to disclose.

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