Construction of chimeric antigen receptor-modified T cells targeting EpCAM and assessment of their anti-tumor effect on cancer cells

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Abstract. Colon cancer is a common malignancy worldwide and there is an urgent requirement to develop effective treatment strategies. In recent years, tumor immunotherapy has become a new method of effectively treating tumors. Chimeric antigen receptor (CAR) T cell technology combines the precise targeting specificity of monoclonal antibodies with the strong toxicity and persistence of cytotoxic T cells to specifically recognize tumor-associated antigens and promote tumor cell death efficiently and permanently, without depending on major histocompatibility complex restriction. In the present study, epithelial cell adhesion molecule (EpCAM)-targeting CAR T cells (EpCAM-CAR-T) were developed, and their ability to kill cancer cells *in vitro* was assessed. Firstly, an EpCAM-CAR plasmid was constructed using molecular biology techniques, and transfected into T cells to obtain EpCAM-CAR-T cells. Transfection efficiency was assessed using reverse transcription-quantitative PCR and flow cytometry. Next, the expression levels of EpCAM in five colon cancer cell lines were examined by western blotting and flow cytometry. Finally, the effect of EpCAM-CAR-T cells on cancer cell death was examined *in vitro* via co-culture experiments. T cells stably expressing EpCAM-CAR were successfully obtained, and the transduction efficiency according to flow cytometry was 50.4%. *In vitro* experiments showed that EpCAM-CAR-T cells exhibited a significantly higher apoptotic effect on cancer cells compared with untransfected T cells. Analyses also demonstrated that this effect was dependent on the ratio of EpCAM-CAR-T cells to tumor cells, and the expression of surface EpCAM. Similarly, the ELISA results showed that interleukin (IL)-2 IL-6 and interferon-γ levels were significantly elevated following exposure to EpCAM-CAR-T cells compared to exposure to untransfected T cells, and were dependent on the number of EpCAM-CAR-T cells and the amount of EpCAM expressed on the surface of tumor cells. The present study provided a basis for the clinical application of CAR-T cell therapy against solid tumors, and a provided a new strategy for the treatment of colon cancer.

Introduction

Globally, colorectal cancer ranks third in cancer incidence and fourth in cancer-associated mortality. It is recognized as one of the most severe malignant tumors worldwide, exhibiting high incidence and mortality (1-3), and it has become a major global health problem. At present, traditional treatment methods for colon cancer include surgery, radiotherapy and chemotherapy (1). Although traditional therapy improves the survival rate of patients with colon cancer, its invasiveness and biological toxicity considerably affect patient quality of life (4). Therefore, it is necessary to develop new and more effective methods for treating colon cancer.

In recent years, with advancements in tumor biology and immunology, cell-based cancer immunotherapy has become a potential method of tumor treatment (5-8). Typical immunotherapies include the use of tumor-infiltrating lymphocytes, T cell receptor-engineered T cells and chimeric antigen receptor (CAR)-modified T cells (9-11). CARs are fusion molecules that couple antibody molecules that recognize tumor antigens with T cell activation signal (12). CARs are composed of the extracellular antigen recognition region through the transmembrane region, including the hinge region and the intracellular signal region (5). The precise targeting specificity of monoclonal antibodies allied with the strong toxicity and persistence of cytotoxic CAR-modified T cells allow these cells to specifically recognize tumor-associated antigens without relying on major histocompatibility complex (MHC) restriction, thereby efficiently and permanently killing tumor

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cells (13). This immunotherapy technology has opened new avenues for the treatment of colon cancer.

Epithelial cell adhesion molecule (EpCAM), which promotes the proliferation and metastasis of tumor cells, is one of the strongest and most ubiquitous tumor surface antigens, and has potential as a target for tumor immunotherapy (14). Since the 1990s, EpCAM-specific monoclonal antibodies (mAbs) have been used in the treatment of human colon cancer, increasing the 5-year survival rate of patients by 30% and reducing the recurrence rate by 27% within 7 years of treatment (15). It was recently reported that a new treatment for colon cancer involving single-chain fragment variable (scFv) antibody-truncated protamine-small interfering RNA, which recognizes and binds to colon cancer cells through EpCAM antigen activity (16). This RNA specifically inhibits Wnt/β-catenin signaling, effectively interrupting the functional cycle between EpCAM and Wnt/β-catenin signaling, thus providing a new strategy for the effective treatment of colon cancer (16).

In the present study, EpCAM-targeting CAR-T cells were constructed and their apoptotic effect on EpCAM+ colon cancer cells was evaluated. EpCAM-CAR-T cells were transfected with a recombinant lentivirus carrying the EpCAM-CAR gene expression cassette and tested for their killing efficacy against colon cancer cells *in vitro*. The results indicated that EpCAM-CAR-T cells may be able to induce EpCAM+ colon cancer cell apoptosis, and this ability may be dependent on the expression of EpCAM on the surface of colon cancer cells and on the number of T cells. In summary, the EpCAM-CAR-T cells developed in this study exhibited antitumor potential and may serve as a basis for further research and development of colon cancer treatment.

Materials and methods

Cell culture. All cell lines (SW620, SW480, HCT116, LoVo, HT-29 and 293T; preserved by the Department of Digestive Tumor Microenvironment of the First Affiliated Hospital, Sichuan, China) were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS; Gibco; Thermo Fisher Scientific, Inc.).

Short tandem repeat (STR) profiling. In total, 20 STR loci, plus the gender determining locus amelogenin, were amplified using the commercially available PowerPlex® 21 System from Promega Corporation. The amplified products were processed using the Applied Biosystems 3730xl DNA Analyzer and data were analyzed using GeneMapper 5.0 software (Applied Biosystems; Thermo Fisher Scientific, Inc.). Appropriate positive and negative controls were run and confirmed for each sample submitted.

Blood donor samples. For all experiments, blood samples were collected with informed consent from healthy volunteers using a protocol approved by the Ethics Committee of the First Affiliated Hospital of Chengdu Medical College (Chengdu, China). Between March and May 2017, peripheral blood samples were collected from 3 healthy volunteers (2 males and 1 female), aged 20-35 years. The lymphocyte density gradient centrifugation kit (GE Healthcare Life Sciences) was used

to isolate peripheral blood mononuclear cells (PBMCs) from blood. Briefly, peripheral blood was slowly added to the upper layer of the equal volume lymphocyte liquid and centrifuged at 400 x g for 30 min at room temperature. Then, the mononuclear cell layer was collected and washed with PBS buffer.

Construction of EpCAM-CAR. The EpCAM scFv was cloned from the vector pET-26b-EpCAM (Novagen, Inc.), which contained the sequence for the scFv antibody for EpCAM. This vector was established in our laboratory as previously described (17). Then, the EpCAM-scFv was linked the CD8 α hinge-transmembrane region with 4-1BB co-stimulatory domain and CD3 ζ chain, and the DNA sequence encoding this cassette was digested by *Hind*III and *Xho*I, and cloned into the lentiviral backbone pCLK-EF-1 (Invitrogen; Thermo Fisher Scientific, Inc.) vector as previously described (18). The plasmids pCLK-EpCAM-CAR, psPAX-2 (Invitrogen; Thermo Fisher Scientific, Inc.) and pMD2.G (Addgene, Inc.) were transfected into *Escherichia coli* HD5a (cat. no. CD201-01; Beijing Transgen Biotech Co., Ltd.); 1 μl plasmids (1 μg/μl) were incubated with *E. coli* DH5a on ice for 30 min, heated for 90 sec in a water bath at 42˚C and incubated on ice for 2 min. DH5a were cultured in 900 μl LB liquid medium (cat. no. 12795027; Invitrogen; Thermo Fisher Scientific, Inc.) on 37˚C for 1 h at 200 RPM. Then, the mixture was coated on the surface of LB solid medium (cat. no. 22700025; Invitrogen; Thermo Fisher Scientific, Inc.) plates and static-cultured at 37˚C for 16 h. Mono-bacteria were selected and cultured for plasmid extraction. The plasmid extraction kit (cat. no. 12381; Qiagen, Inc.) was used to extracted these three plasmids for detection and lentiviral packaging. Then, a 0.5% agarose gel was used to detect the size of plasmid (1 μl DNA/lane). Goldview was used as the visualization reagent.

Packaging and concentration of the lentivirus. A lentiviral supernatant was generated from 293T cells transfected with PCLK-EF-1-CAR, pMD2.G and psPAX-2. 293T cells were cultured and used for packaging lentivirus at 70-80% confluence. The vectors (9 μg PCLK-EF-1-CAR; 9 μg pMD2.G; 4.5 μg psPAX-2) were transfected into 293T cells using the calcium phosphate method. Following transfection, 293T cells were cultured at 37˚C with 5% CO$_2$ for 72 h. The lentivirus suspension was collected and filtered with a 0.22-μm filter. Then, the lentivirus suspension was ultracentrifuged at 70,000 x g at 20˚C for 2 h to concentrate the virus. Reverse transcription-quantitative PCR (RT-qPCR) was used to determine the titer of concentrated virus.

Transduction and expansion of T cells. Human PBMCs were cultured in RPMI 1640 medium with 10% FCS, and activated with CD3 antibodies (cat. no. MA1-10175; 50 ng/ml; Invitrogen; Thermo Fisher Scientific, Inc.) and interleukin (IL)-2 (cat. no. 0208AF12; 300 U/ml; PeproTech, Inc.) for 24 h (19). Then, T cells were transduced with the concentrated lentiviral at a multiplicity of infection of 4 on RetroNectin-coated plates (Takara Bio, Inc.). Transduced cells were cultured with IL-2 (300 U/ml) for 14 days before subsequent analysis. Non-transduced T cells were used as negative controls and were cultured under the same conditions. In all trials, the functions of transduced and non-transduced T cells obtained from the same donor were compared.

Flow cytometry. In order to detect the expression of EpCAM on the cell surface, the colon cancer cell lines SW620, SW480, HCT116 and LoVo, and the colorectal cancer cell line HT-29, were incubated with EpCAM antibody (1:500; cat. no. 2929; Cell Signaling Technology, Inc.) at 37˚C for 30 min. Then, cells were washed and incubated with a Cy3-conjugated fluorescent secondary antibody (1:100; cat. no. SA00009-1; ProteinTech Group, Inc.) at 37˚C for 30 min in the dark. In order to detect the expression of CAR on the surface of T cells, T cells were incubated with an antigen-binding fragment 2 [F(ab)2] antibody (1:200; cat. no. NBP1-51900; Novus Biologicals, Ltd.) at 37˚C for 30 min. Cells were then washed, and incubated with a Cy3-conjugated fluorescent secondary antibody (1:100; cat. no. SA00009-4; ProteinTech Group, Inc.) at 37˚C for 30 min in the dark. Cells were washed with PBS and detected using a flow cytometer. BD Accuri™ C6 software was used for data analysis (BD Biosciences).

Western blotting. Total proteins were obtained using RIPA buffer (Beyotime Institute of Biotechnology) and quantified using a bicinchoninic acid protein assay kit (Beyotime Institute of Biotechnology). Proteins (30 μg/lane) were then transferred to PVDF membranes (Beyotime Institute of Biotechnology) after separation by 10% SDS-PAGE. Membranes were blocked using 5% nonfat milk for 1 h at 26˚C. In order to detect the expression of EpCAM, membrane-bound proteins from colon cancer cells were incubated with an anti-EpCAM primary antibody (1:1,000; cat. no. 2929; Cell Signaling Technology, Inc.) at 4˚C overnight. Membranes were reacted with secondary horseradish peroxidase-conjugated antibody (1:2,000; cat. no. 7076; Cell Signaling Technology, Inc.) for 2 h at 37˚C. Membranes containing T cell-derived proteins were instead incubated with an anti-F(ab)2 antibody primary (1:5,000; cat. no. NBP1-51900; Novus Biologicals, Ltd.) at 4˚C overnight, and then with secondary horseradish peroxidase-conjugated antibody (1:1,000; cat. no. HAF109; R&D Systems, Inc.) for 2 h at 37˚C. After washing, protein bands were measured using an enhanced chemiluminescence assay kit (EMD Millipore) and imaged with a chemiluminescence detection system (Bio-Rad Laboratories, Inc.). The relative expression of a target protein was determined as the ratio of the grayscale value of the target protein to that of β-actin (1:1,000; cat. no. 58169; Cell Signaling Technology, Inc.) by Image Lab (version 4.0; Bio-Rad Laboratories, Inc.). All experiments were repeated three times.

Gene expression analysis by RT-qPCR. Total RNA was isolated from lentiviruses or EpCAM-CAR-T cells and untransfected T cells using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.). The primers used for viral titer determination were: Lentiviral Rev response element forward, 5'-TTTGTTCCTTGGGTTCTTGGG-3' and reverse, 5'-GAT TCTTGCCTGGAGCTGCTT-3'. For CAR mRNA expression analysis, the primers were: Forward, 5'-CAAGATTAC ACTCAGGAGTCCC-3' and reverse, 5'-GTGGGTATTACT GGATGGTGGG-3'. The primers used to measure GAPDH were: Forward, 5'-TGACTTCAACAGCGACACCCA-3' and reverse, 5'-CACCCTGTTGCTGTAGCCAAA-3'. RNA was reverse transcribed using a PrimeScript™ RT reagent kit (cat. no. RR047A; Takara Bio, Inc.) to obtain cDNA as follows: 37˚C for 15 min, 85˚C for 5 sec and 4˚C for 1 h. qPCR was performed using a TB Green® Premix Ex Taq™ II kit (cat. no. RR820A; Takara Bio, Inc.). The thermocycling conditions were as follows: 95˚C for 30 sec, then 39 cycles of 95˚C for 5 sec and 60˚C for 30 sec, followed by 95˚C for 10 sec, then 65˚C for 5 sec, and finally 95˚C for 0.5 sec. The Cq values of the target genes were normalized to that of GAPDH (20).

Cytotoxicity assay. The antitumor effect of EpCAM-CAR-T cells on colon cancer cells was measured using a LDH-Glo™ Cytotoxicity Assay (J2380, Promega). Briefly, EpCAM-CAR-T cells and untransfected T cells were added as effector cells to each well, followed by the addition of the target colon cancer cells (10^5; SW620, SW480, HCT116, LoVo or HT-29). The final Effector: Target (E:T) ratios were 0.5:1, 1:1, 2:1, 4:1, 8:1 or 16:1. The cell mixtures were incubated at 37˚C under 5% CO_2 for 4 h. Collecting 50 μl culture supernatant, mixture with 50 μl LDH Detection Reagent, then transferred to fresh 96-well flat-bottom plates. Record luminescence after incubate for 60 min at room temperature. The percentage of cell lysis was calculated as: Specific lysis (%)=(Effector spontaneous release-Target spontaneous release)/(Target maximum release-Target spontaneous release) x100. Each assay was performed in triplicate.

Cytokine production analysis. To measure cytokine production *in vitro*, colon and colorectal cancer cells were co-cultured with EpCAM-CAR-T cells or untransfected T cells at the ratios of 0.5:1, 1:1, 2:1, 4:1, 8:1 or 16:1 at 37˚C under 5% CO_2. After 24 h, the supernatant was collected, and the levels of IL-2 (cat. no. EK0397), IL-6 (cat. no. EK0410) and IFN-γ (cat. no. EK0373) were analyzed by ELISA (all Boster Biological Technology).

Statistical analysis. Data are expressed as the mean ± standard deviation from at least three independent experiments. Differences between different treatment groups were analyzed using one-way ANOVA analysis. P<0.05 was considered to indicate a statistically significant difference. All statistical analyses were performed using SPSS 16.0 (SPSS, Inc.).

Results

Construction and identification of EpCAM-CAR plasmid. Firstly, the primary plasmid EpCAM-CAR for lentiviral packaging, as well as the helper plasmids psPAX-2 and pMD2.G, were constructed. The structure of the main plasmid EpCAM-CAR is shown in Fig. 1A and B. Then, the plasmids were identified by agarose gel electrophoresis. As shown in Fig. 1C, the sizes of the plasmids EpCAM-CAR, psPAX-2, and pMD2.G were 10.8, 11.0 and 5.8 kb, respectively.

Packaging and concentration of lentivirus. The viral-packaged master plasmid (EpCAM-CAR) and the helper plasmids (psPAX-2 and pMD2.G) were transfected into 293T cells using the calcium phosphate method to obtain a recombinant plasmid carrying the EpCAM-CAR gene expression cassette. Cells were also transfected with a construct whereby the main plasmid was replaced with a fluorescent plasmid with the same
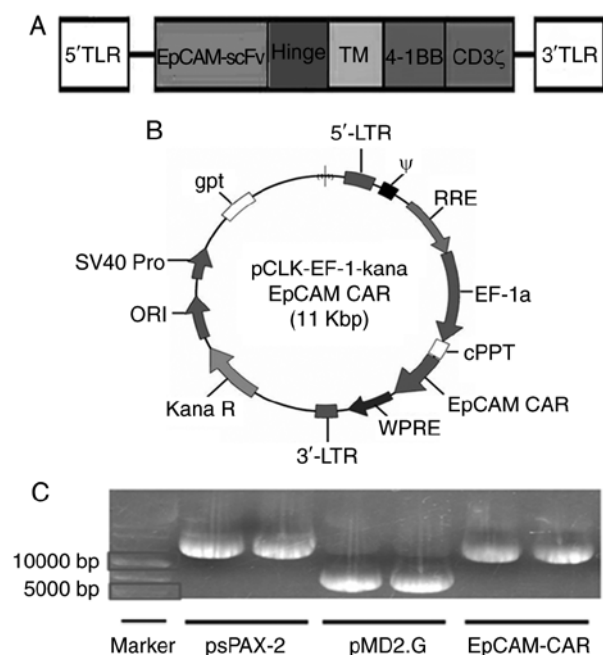
Figure 1. Plasmid carrying the EpCAM-CAR gene. (A) The EpCAM gene consists of EpCAM-scFv, along with a hinge region, a TM region and a signal region. Its signal variable region includes the 4-1BB costimulatory molecule and CD3ζ activation domain. (B) EpCAM-CAR is a pCLK-EF-EpCAM-CAR plasmid construct carrying the EpCAM-CAR gene, obtained by cloning the EpCAM fragment into the pCLK-EF-1 vector containing the lentiviral packaging component. (C) 0.5% agarose gel was used to detect the size of plasmid (1 μl DNA/lane). The sizes of the plasmids EpCAM-CAR, psPAX-2 and pMD2.G were 11.0, 11.0 and 5.8 kb, respectively, which matched the expected molecular weights. EpCAM, epithelial cell adhesion molecule; CAR, chimeric antigen receptor; scFv, single-chain fragment variable; 4-1BB, TNF superfamily member 9; TM, transmembrane; LTR, long terminal repeat; RRE, Rev response element; WPRE, woodchuck hepatitis virus post-transcriptional response element; cPPT, central polypurine tract.
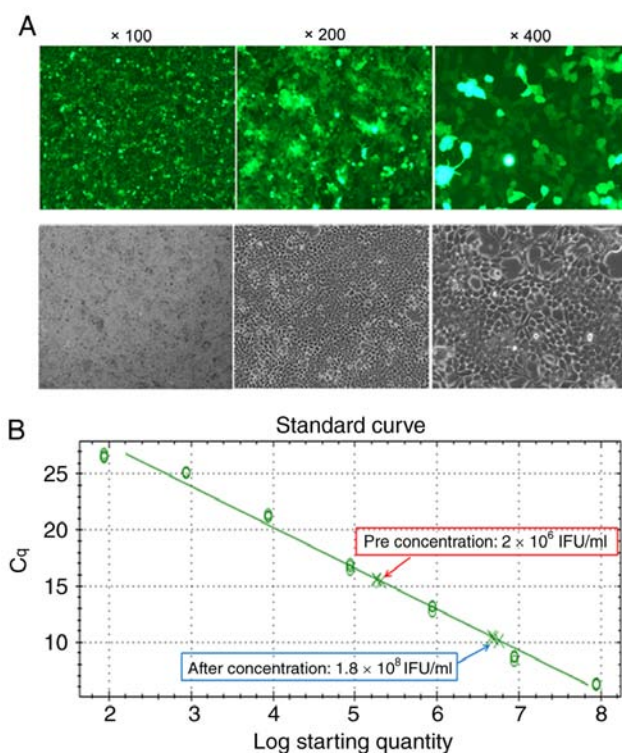

Figure 2. Packaging and concentration of EpCAM-CAR lentivirus. (A) Plasmids EpCAM-CAR, psPAX-2 and pMD2.G were transfected into 293T cells using calcium phosphate. After 48 h, some cells had shrunk and stopped growing (magnifications, x100, x200 and x400). (B) The concentrated lentiviral titer was detected by reverse transcription-quantitative PCR. The lentiviral titers before and after concentration were $2x10^6$ and $1.8x10^8$ IFU/ml, respectively, and the concentration efficiency was >90%. EpCAM, epithelial cell adhesion molecule; CAR, chimeric antigen receptor; IFU, infection function units.

fragment size and skeleton for use as a positive control. After 48 h, the fluorescence of the positive control 293T cells was ~90% (Fig. 2A), indicating that the lentivirus was successfully packaged. Then, virus supernatants were concentrated by ultracentrifugation. The concentrated lentiviral titer was assessed by RT-qPCR, and a standard curve was drawn according to the Cq value and the copy number. The lentiviral titers before and after concentration were $4.3x10^6$ infection function units (IFU)/ml and $1.8x10^8$ IFU/ml, respectively (Fig. 2B). Based on these results, the recombinant lentivirus carrying the EpCAM-CAR gene was considered to have been successfully packaged and concentrated.

Expression of EpCAM in cancer cell lines. The present study used the colon cancer cell lines SW620, SW480, HCT116 and LoVo, and the colorectal cancer cell line HT-29, as target cells to detect the antitumoral effects of EpCAM-CAR-T cells. All cell lines were authenticated using STR profiling, and all cells had a matching degree >95%. Western blotting and flow cytometry were used to detect EpCAM expression in these cancer cell lines. The results of the western blotting indicated that EpCAM protein expression was observed in all five cancer cell lines, and the molecular weight of the protein was 40 kDa. Among the lines, SW620 exhibited the highest expression, while HT-29

exhibited the lowest expression of EpCAM (Fig. 3A and B). The results of the flow cytometry showed that the expression rates of EpCAM on the surfaces of HCT116, HT-29, LoVo, SW620 and SW480 cells were 78.3, 67.3, 75.4, 97.5 and 85.4%, respectively (Fig. 3C).

Construction of T cells stably expressing EpCAM-CAR. To construct T cells that stably expressed EpCAM-CAR, T cells were transfected with recombinant lentivirus. The co-stimulatory domain 9/4-1BB and CD3 chain in the CAR can activate T cells and promote the proliferation of T cells following transfection of CAR into T cells. The results indicated that the number of cells increased over time, and T cells tended to become activated (Fig. 4A). On the 14th day following viral transfection, the expression of CAR mRNA in T cells was detected by RT-qPCR, confirming that CAR expression levels were higher in transfected cells than in control cells (Fig. 4B). In addition, the expression of CAR in T cells evaluated by protein immunoblotting confirmed that CAR was only expressed in transfected T cells, and its molecular weight was 53 kDa (Fig. 4C), which was consistent with the theoretical molecular weight (21). The expression of CAR on the surface of T cells facilitates recognition of and binding to antigens on the surface of tumor cells. Therefore, the expression of CAR on the surface of T cells was evaluated by flow cytometry. The results showed that CAR was expressed on the surface
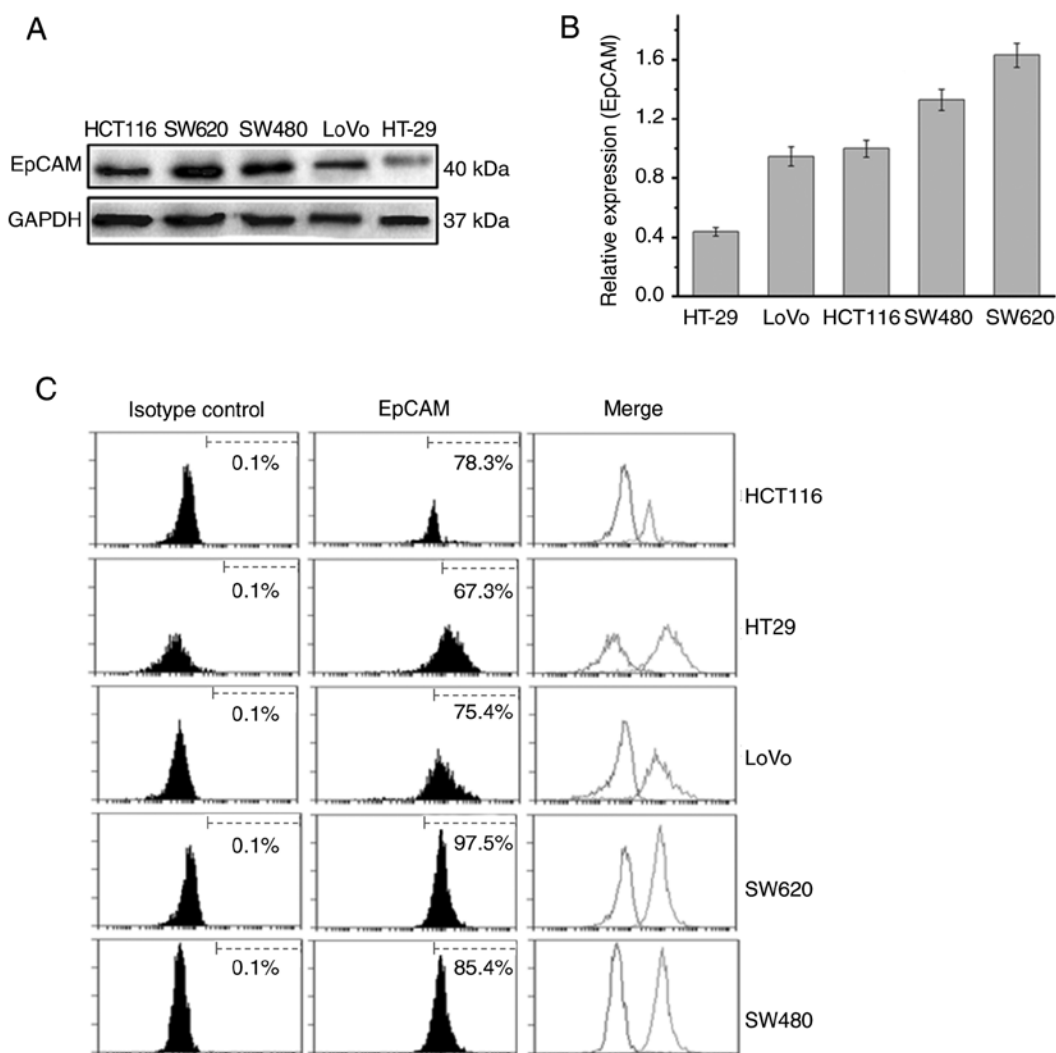
Figure 3. Expression of EpCAM in cancer cell lines. (A) Western blot analysis of EpCAM in HCT116, SW620, SW480, LoVo and HT-29 cells. (B) Relative grayscale levels of EpCAM protein in HCT116, SW620, SW480, LoVo and HT-29 cells (n=3/line). (C) Flow cytometry was used to detect the expression of EpCAM on HCT116, HT-29, LoVo, SW620 and SW480 cells. The expression rates were 78.3, 67.3, 75.4, 97.5 and 85.4%, respectively. EpCAM, epithelial cell adhesion molecule; CAR, chimeric antigen receptor.

of 50.4% of transfected T cells (Fig. 4D), indicating that the transfected T cells stably expressed EpCAM-CAR.

In vitro cytotoxicity effect of EpCAM-CAR-T on cancer cells. In order to evaluate the antitumoral effect of EpCAM-CAR-T cells on cancer cells, SW620, SW480, HCT116, LoVo and the colorectal cancer cell line HT-29, all of which exhibit, different EpCAM expression rates, were used as target cells for EpCAM-CAR-T cells and untransfected T cells (effector cells). First, T cells were co-cultured with colon cancer SW620 cells, which exhibited the highest EpCAM expression, at E:T ratios of 0.5:1, 1:1, 2:1, 4:1, 8:1, and 16:1 for 4 h. The supernatant was then extracted for analysis of the cytotoxic effects of T cells at different effector ratios using an LDH release assay kit. The results showed that as the E:T ratio increased, the LDH release of the EpCAM-CAR-T cell group also increased, indicating that the antitumor effect of EpCAM-CAR-T cells was dependent on the number of EpCAM-CAR-T cells (Fig. 5A). Studies have shown that IL-2, IFN-γ and IL-6 have antitumor roles in tumor immunotherapy by regulating immune responses (22-24).

Therefore, levels of the inflammatory cytokines IL-2, IL-6 and IFN-γ in the co-culture supernatants of EpCAM-CAR-T cells and SW620 cells were evaluated by ELISA. The results showed that the levels of these inflammatory cytokines released by EpCAM-CAR-T cell co-culture were significantly higher than those in the control group. In addition, cytokine levels increased as the E:T ratio increased (Fig. 5B-D).

The results indicated that the antitumor effects of EpCAM-CAR-T cells were strongest, and the release of cytokines was highest at an E:T ratio of 16:1 when T cells were co-cultured with colon cancer SW620 cells. Therefore, the remaining cell lines (SW480, HCT116, LoVo and HT-29) were co-cultured with T cells at an E:T ratio of 16:1 for 4 h. Compared with the control T cells, EpCAM-CAR-T cells exhibited a stronger antitumor effect on the four cancer cell lines (Fig. 6A). Furthermore, the levels of IL-2, IL-6 and IFN-γ released by EpCAM-CAR-T cells were also significantly higher than those released by control T cells (Fig. 6B-D). Moreover, the antitumor effect and inflammatory cytokine release may also be associated with the
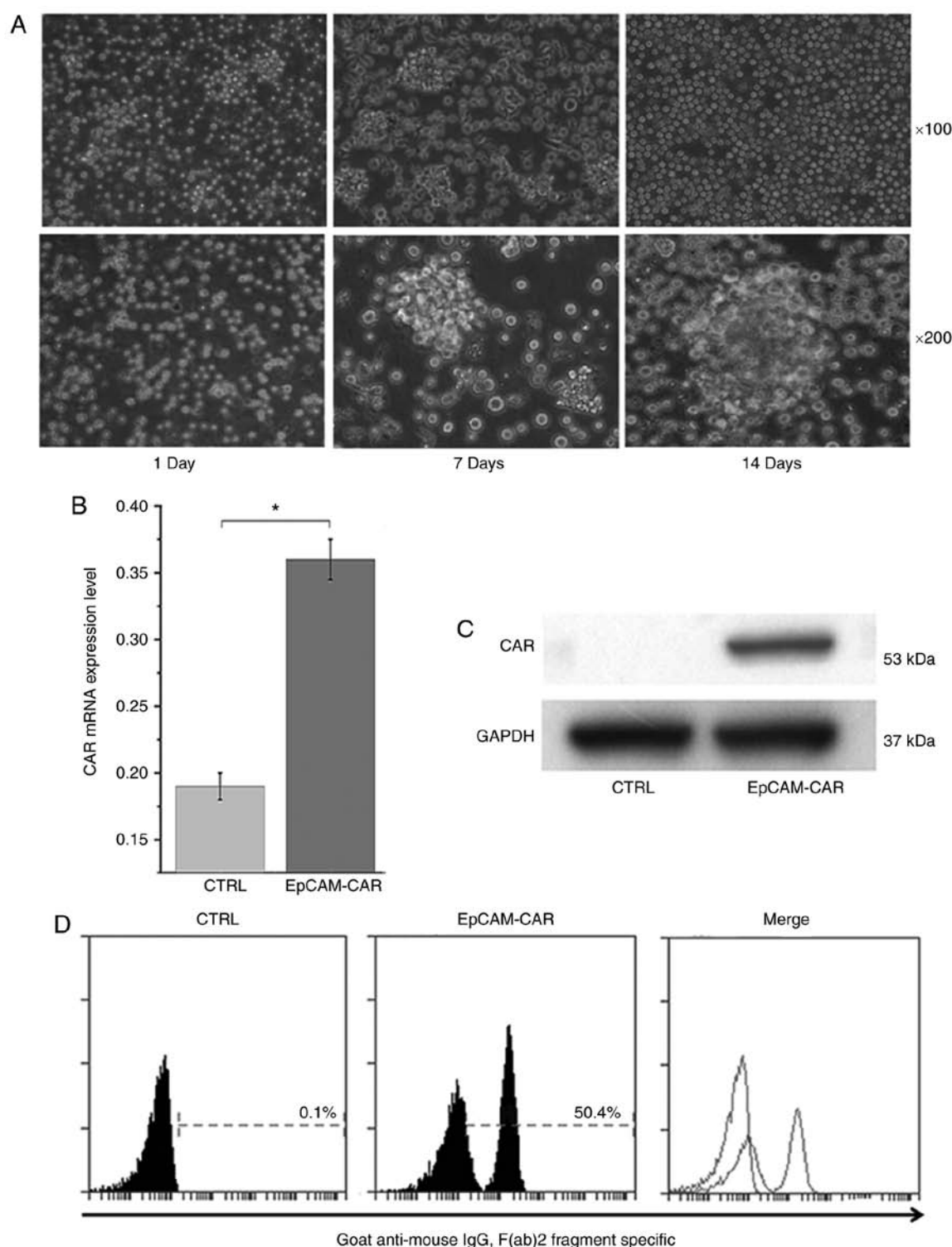
Figure 4. T cells transfected with EpCAM-CAR lentivirus stably present CAR on their surface. (A) T cells were cultured after transfection with EpCAM-CAR. The T cells on days 1, 7 and 14 were observed under a microscope (magnification, x100 and x200). After transfection, T lymphocytes became larger and some T cells grew as colonies. The number of T lymphocytes appeared to increase over time. (B) Reverse transcription-quantitative PCR was used to detect the expression level of EpCAM-CAR in T cells, showing significantly higher expression of CAR in transfected cells. *P<0.05 vs. CTRL. (C) Western blotting was used to detect the protein levels of EpCAM-CAR in T cells, demonstrating higher levels of CAR in transfected cells. (D) Flow cytometry was used to detect the expression of F(ab)2 in the EpCAM-CAR structure on the surface of T cells. EpCAM, epithelial cell adhesion molecule; CAR, chimeric antigen receptor; F(ab)2, antigen-binding fragment 2; CTRL, control.

expression of EpCAM on the surface of cancer cells, as cell lines with higher expression rates of EpCAM appeared to exhibit more potent antitumor effects and to release higher levels of inflammatory cytokines (Fig. 6).

Discussion

Tumor immunotherapy is a method for treating malignant tumors by regulating the immune status of the body (25). CAR-T
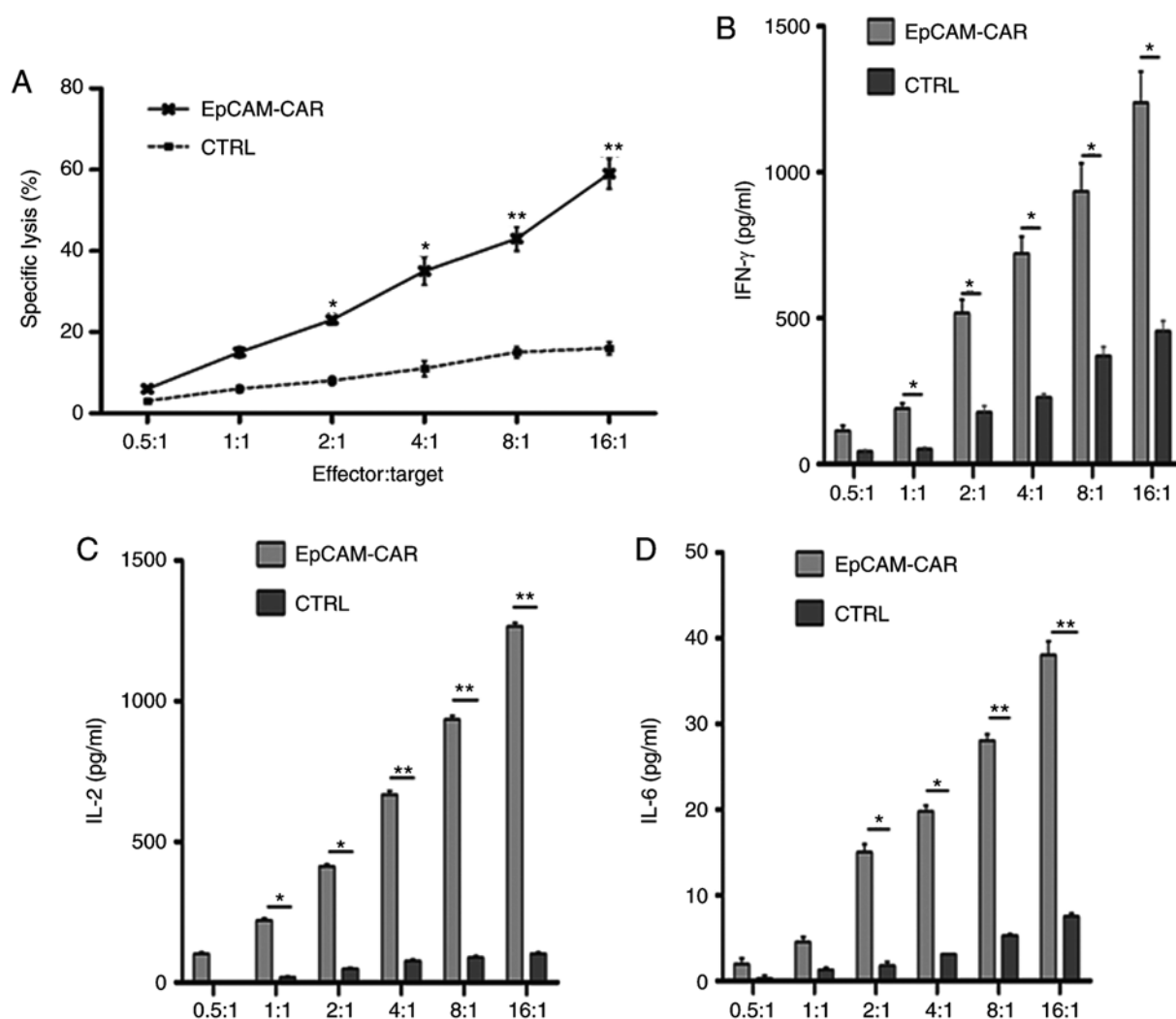
Figure 5. Antitumoral effect of EpCAM-CAR-T cells on SW620 cells. EpCAM-CAR-T and control T cells were co-cultured with SW620 cells in different ratios. (A) A lactate dehydrogenase assay was used to measure the antitumoral effect of T cells on SW620 cells, showing higher levels of cell lysis with higher ratios of T cells relative to target SW620 cells. ELISA was also used to assess the levels of (B) IFN-γ, (C) IL-2 and (D) IL-6 in the supernatants of T cells co-cultured with SW620 cells, also showing higher release of cytokines with increasing ratios of T cells to target SW620 cells. *P<0.05 and **P<0.01 vs. CTRL. EpCAM, epithelial cell adhesion molecule; CAR, chimeric antigen receptor; IFN-γ, interferon γ; IL, interleukin; CTRL, control.

cell therapy is also known for its role in the treatment of B cell hematological malignancies (26). As a promising treatment modality, CAR-T cell therapy offers the following advantages: i) Binding to tumor surface antigens in a non-MHC-restricted manner; ii) simultaneous recognition of multiple antigens; and iii) large-scale *ex vivo* acquisition of CAR-T cells (4). Studies have shown that immune cell therapy has achieved successful results in acute lymphoblastic leukemia (ALL), chronic lymphocytic leukemia and non-Hodgkin's lymphoma (26-29). In 2017, the US Food and Drug Administration approved a CAR-T cell therapy, which has been used in B-cell ALL (30), inspiring further development of various CAR-T cells for immunotherapy in the future.

Studies have shown that EpCAM may play a role in cell proliferation, migration, differentiation and morphogenesis (31). Overexpression of EpCAM is associated with progression and poor prognosis in gastric cancer (32) and pancreatic cancer (33). In a large retrospective study, truncated EpCAM was observed to be associated with several factors related to cancer stem cell formation and epithelial-mesenchymal transition, such as poor differentiation, vascular and limb invasion and lymph node metastasis (34). In addition, EpCAM is considered to be a hallmark of numerous cancer stem cells (35). Tumor stem cells are characterized by high tumorigenicity and high drug resistance (35). Traditional treatment of tumors is unable to remove tumor stem cells, resulting in limited antitumor efficacy and recurrence (36). In this study, the expression of EpCAM was assessed in four colon cancer cell lines and one colorectal cancer cell line using western blotting analysis and flow cytometry. EpCAM was selected as both a marker of cancer cells and a target for CAR-T cells. EpCAM-CAR-T cells can specifically recognize cancer cells expressing EpCAM, thereby achieving greater efficacy in killing cancer cells.

After successful construction of EpCAM-CAR-T cells, their lysis efficiency was initially evaluated on the co-cultured colon cancer cell line SW620, which had the highest expression of EpCAM *in vitro*. This was tested at different E:T ratios (ranging between 0.5:1 and 16:1) for 4 h. The LDH release assay results showed that EpCAM-CAR-T cells were capable
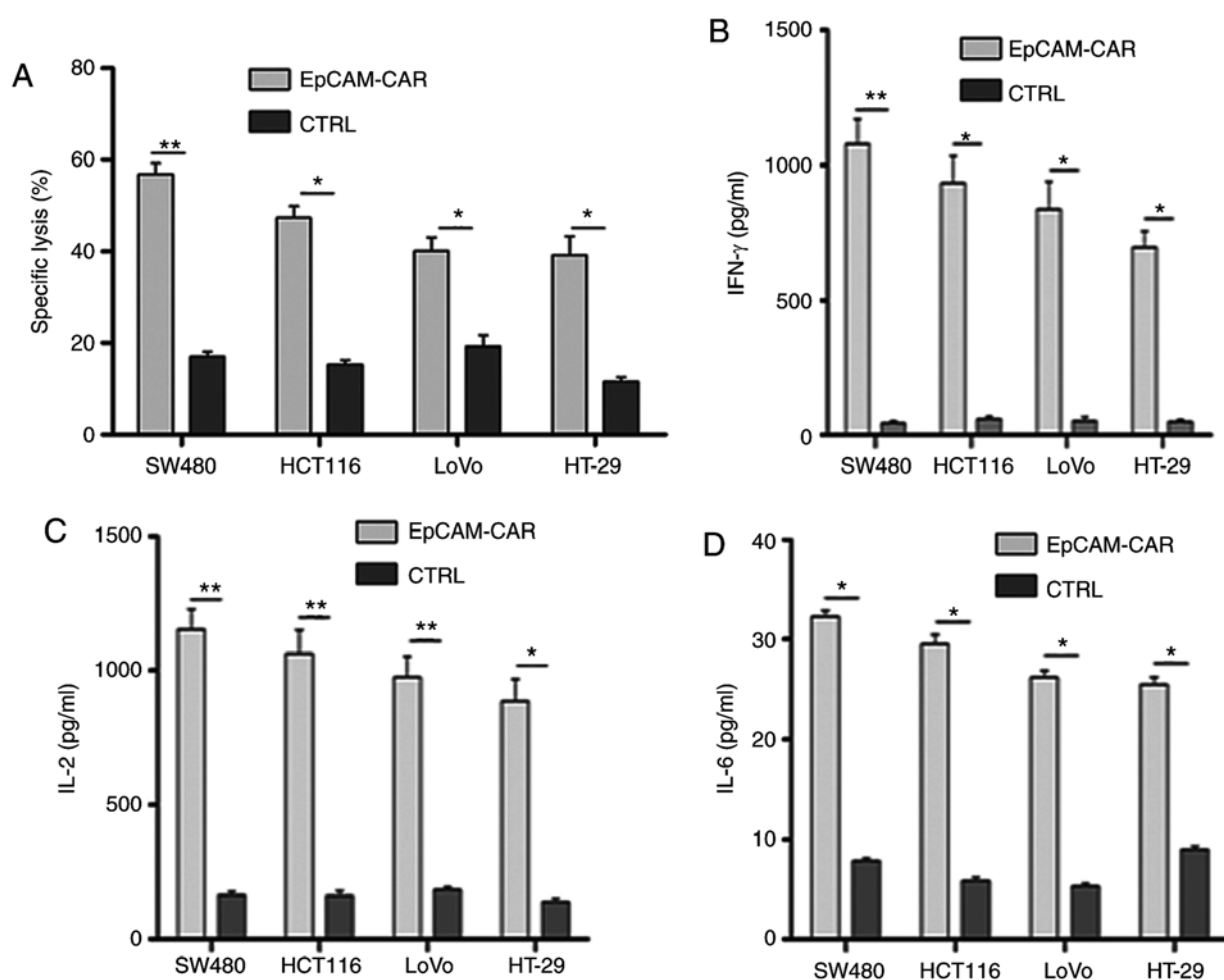
Figure 6. Antitumoral effect of EpCAM-CAR-T cells on other cancer cell lines. EpCAM-CAR-T and control T cells were co-cultured with the remaining cancer cell lines at a ratio of 16:1. (A) A lactate dehydrogenase assay was used to detect the antitumor effect of T cells on the tested cancer cells. ELISA was also used to assess the levels of (B) IFN-γ, (C) IL-2 and (D) IL-6 in the supernatants of T cells co-cultured with cancer cells. *P<0.05 and **P<0.01 vs. CTRL. EpCAM, epithelial cell adhesion molecule; CAR, chimeric antigen receptor; IFN-γ, interferon γ; IL, interleukin; CTRL, control.

of causing SW620 cell lysis, and this effect was dependent on the number of EpCAM-CAR-T cells. The lysis efficiency of EpCAM-CAR-T cells was also evaluated in the other four cancer cell lines (SW480, HCT116, LoVo and HT-29), which exhibit different expression levels of EpCAM, at an E:T ratio of 16:1. The results showed that the killing efficiency of EpCAM-CAR-T cells may have been dependent on the levels of EpCAM expressed by the different cell lines, with higher levels of LDH being detected in cell lines that express higher levels of EpCAM.

Studies have shown that IL-2, IFN-γ and IL-6 play an antitumor role in tumor immunotherapy by regulating immune responses (37-39). Therefore, the secreted levels of IFN-γ, IL-2 and IL-6 were examined in the co-culture supernatants of EpCAM-CAR-T cells and EpCAM+ tumor cells. The secretion of these cytokines was found to be increased with larger E:T ratios and with higher expression of EpCAM on the surface of target cancer cells. These results indicated that the antitumoral effect and inflammatory cytokine release by EpCAM-CAR-T cells may be associated with the levels of EpCAM on the surface of cancer cells, with higher expression rates of EpCAM leading to more potent antitumor effects and more inflammatory cytokine release.

Studies have found that the main side effect of CAR-T cell therapy is cytokine release syndrome (CRS), which primarily manifests as fatigue, high fever, hypotension and hypoxia, and may even cause cardiopulmonary dysfunction, organ failure and mortality in some cases (40). The secretion of a large number of pro-inflammatory cytokines by activated T cells may lead to this adverse reaction. It has been reported that the use of tropizumab, an IL-6 receptor antagonist, may help control severe CRS without impairing T cell efficacy (41). Beyond cytokine use, certain studies have reported that steroids may be used to control CRS (42,43). In addition, it has also been reported that the severity of CRS may be associated with the tumor load when CAR-T is injected. Therefore, early reinfusion of CAR-T cells or drug pretreatment to reduce the tumor burden can significantly reduce the occurrence of severe CRS (42).

In summary, the present study described the successful construction of an EpCAM-CAR plasmid and the subsequent establishment of EpCAM-CAR-T cells that target EpCAM expressed on the surface of colon and colorectal cancer cells. EpCAM-CAR-T cells effectively killed cancer cells, and significantly promoted cytokine release *in vitro*, indicating that CAR-T cells targeting EpCAM may have the

potential to treat colon or colorectal cancer. Future studies should aim to conduct experiments to test the antitumor effects of EpCAM-CAR-T cells *in vivo*. Overall, these findings indicate a new avenue for the treatment of cancer by immunotherapy.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

YZ and XAL designed the study. PW, MML and YQL performed the experiments. YZ and PW analyzed and interpreted the data, and drafted the manuscript. All authors critically revised the manuscript, and read and approved the final version of the manuscript.

Ethics approval and consent to participate

All experimental protocols were approved by the Ethics Committee of the First Affiliated Hospital of Chengdu Medical College (Chengdu, China). All participants provided written informed consent.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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