



## Original Research

## CD166-specific CAR-T cells potently target colorectal cancer cells

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## ABSTRACT

Chimeric antigen receptor (CAR) T-cell therapy is emerging as an effective cancer treatment, such as for hematological malignancies, however its effectiveness as an approach to treat solid tumors, such as in colorectal cancer (CRC), remains to be better developed. One area of intense development has been in the identification and characterization of novel cancer-related ligand receptors for CAR design and evaluation. It is known that the CD6 receptors CD166 and CD318 are highly expressed in CRC, and several CAR-Ts have also been explored in pre-clinical and clinical studies for the treatment of CRC, with promising safety and efficacy findings. Here, we constructed a CAR based on the extracellular domain of CD6 and demonstrate its cytotoxic effect in target positive human CRC cell lines. Unexpectedly, we found that CD6-CAR-T cells targeted CD166 instead of CD318. Furthermore, CD6-CAR-T cells show robust cytotoxicity to CD166-positive cell lines in a dose-dependent manner with cytokine IFN- $\gamma$  significantly released. Particularly, CD6-CAR-T cells show potent cytotoxicity targeting CRC cancer stem cells (CSCs), highlighting that CD6-CAR-T is a promising approach for the therapy of CRC.

## Introduction

Despite increased screening rates and a concurrent decline in incidence and mortality among average risk for adults 50 years and older, the incidence of colorectal cancer (CRC) is increasing among younger patient populations, and is currently the third most common cancer among men and women, as well as the second most common cause of cancer-related death in the world [1]. Over the past few decades, progress has been made in traditional treatment options such as surgery, radiotherapy, chemotherapy, as well as combinations of such treatments; yet the 5-year survival rate of CRC patients remains low (less than 15%), and nearly 40% of CRC patients eventually relapse and suffer from late metastasis [2]. There is a clear need for novel treatment regimens that improve the survival rate for patients with CRC.

Immunotherapy is rapidly emerging as an effective treatment strategy for the treatment of cancer. Indeed, several CRC cancer vaccines are undergoing clinical trials, some of with promising results. For example, current clinical trial studies using the Poxviral vaccine regimen have reported that approximately 56% of patients show a significant immune response for metastatic carcinoma [3]. Another approach using monoclonal antibodies, consisting of tumor-targeting monoclonal antibodies

and immunomodulatory monoclonal antibodies, have also been shown to be effective to treat CRC tumor subtypes in patients [4,5]. A recent study reported that mismatch repair-deficient, locally advanced rectal cancer was highly sensitive to single-agent Programmed Cell Death Ligand 1 (PD-1) blockade, with such treatment leading to a clinical complete response across all 12 subjects [6]. Furthermore, as further indication of the importance of immunotherapy to treat colorectal cancer, PD-1 monoclonal antibody therapy is recommended regardless of first-line, second-line or third-line treatment for patients with high microsatellite instability/mismatch repair deficiency (MSI-H/dMMR), as set out by the Chinese Society of Clinical Oncology (2021) [7,8].

Of the various immunotherapy approaches to treating cancer, Chimeric antigen receptor (CAR) T-cell therapy is very promising, with demonstrated great success in hematological malignancies, yet is less well developed as a therapy for CRC [9,10]. Nevertheless, preclinical studies targeting a spectrum of tumor-associated antigens (TAAs) have so far reported promising results as a potential treatment for CRC, including TAAs targeting carcinoembryonic antigen (CEA), epidermal growth factor receptor (EGFR), mesothelin (MSLN), mucin 1 (MUC1), natural killer group 2 member D (NKG2D) and its ligands, human epidermal growth factor receptor 2 (HER2), guanylyl cyclase C

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(GUCY2C), epithelial cell adhesion molecule (EpCAM), tumor-associated glycoprotein (TAG)-72, c-met, and CD133. In addition to these preclinical studies, several clinical trials have reported promising safety and efficacy data for CAR-T in the treatment of colorectal cancer, however caution remains as to adverse effects of some treatment designs [11]. Nevertheless, current evidence supports CAR-T therapy as a potential, viable therapeutic strategy for patients with colorectal cancer.

It has been shown that the aggressiveness and metastatic properties of cancer cells is modulated by immune cell expression of CD6, a 105 kDa to 130 kDa type I transmembrane glycoprotein expressed almost exclusively by lymphocytes (including most mature T cells and approximately 50% of natural killer (NK) cells) and is a highly conserved scavenger receptor cysteine-rich amino acid superfamily [12,13]. In humans, CD6 is a potential therapeutic target relevant to the treatment of autoimmune diseases, while a recent study also demonstrated that UMCD6, an anti-CD6 monoclonal antibody, upregulated the expression of the activating receptor NKG2D and downregulated the expression of the inhibitory receptor NK cell lectin-like receptor subfamily C member 1 (NKG2A) on both NK cells and CD8+ T cells while increasing perforin and granzyme B production [14,15]. The combined capability of an anti-CD6 monoclonal antibody to control autoimmunity through effects on CD4+ lymphocyte differentiation, together with enhanced killing of cancer cells through distinct effects on CD8+ and NK cells altogether provides a potential new approach to cancer immunotherapy that suppresses rather than potentiates autoimmunity [16]. Interestingly, CD6, a known ligand for Activated leukocyte cell adhesion molecule (ALCAM/CD166), a 105 kDa transmembrane glycoprotein of the immunoglobulin superfamily found on the cell surface [17]. CD6, has also recently been reported as a ligand of the CUB-domain-containing protein 1 (CDCP1), a type I transmembrane protein also known as CD318 [18]. This is consistent with the observation that, of the three extracellular domains (SCRCs 1, 2, and 3) of CD6, the SCRC 1 domain of CD6 binds CD318, while the SCRC 3 domain binds CD166 [19].

The importance of CD318 in cancer is reflected in the finding that it is upregulated in malignancies of the breast, lung, colorectum, ovary, kidney, liver, pancreas, and hematopoietic system [20]. Furthermore, CD318 plays critical roles in tumorigenesis, as well as the development, invasion, and metastasis of tumors [21]. As prognostic marker, CD318 expression has also been associated with poor outcomes in cancers. In addition, it has also been suggested that some cancer tissues expressing membrane-localized CD318 have a poorer prognosis than those with the cytoplasmic expression of CD318 [22]. In the case of CRC, the expression of CD318 is significantly upregulated, and patients with high CD318 expression have overall lower survival and disease-free survival rates than patients with low CD318 expression [23]. Thus, CD318 could be target for the treatment of CRC.

Overexpression of the other CD6 interacting protein, CD166, is associated with various cancers, including colorectal, breast, gastric, liver, lung, prostate, pancreatic cancer, melanoma [24]. In addition, CD166 has been identified as a putative cancer stem cell (CSC) marker in some cancers, and is also found to be overexpressed in human CRC and CRC-CSC [25]. Furthermore, its expression is significantly and positively associated with poor cancer prognosis or early recurrence [26,27]. Notably, there is currently no CD166-based CAR reported for the treatment of CRC.

In this study, we investigated the possibility that CAR designed for CD6 may be effective for the tumorigenic growth of colorectal carcinoma cells. To investigate this, we designed three CARs based on the full length CD6 extracellular domain, the CD166-binding domain, as well as the CD318-binding domain. We then screened to identify a novel anti-CD166 CAR comprising an antigen recognition domain which is not scFv, but is instead an extracellular domain of the natural ligand CD6 that mimics the natural binding between CD6 and CD166 to construct second-generation CAR (CD6-CAR) T cells. These CD6-CAR-T cells were then tested for their capacity to influence colorectal cancer cells and

CSCs *in vitro*.

## Materials and methods

### Cell lines and culture

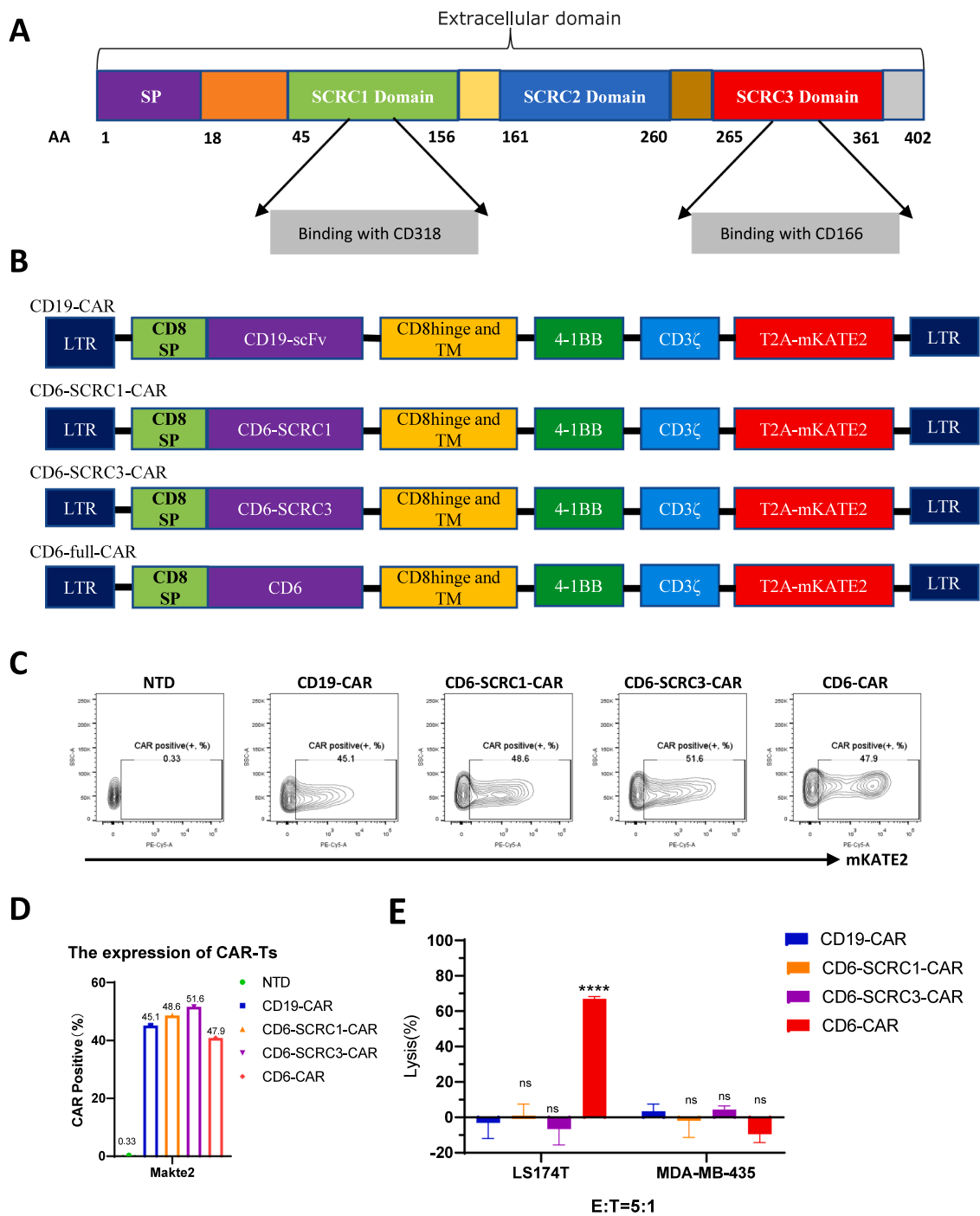
Various cell lines (HCT116, DLD1, MCF7, LS174T, MDA-MB-435, and MIApaca-2) were obtained from the China Infrastructure of Cell Line Resources (Kunming, Beijing, or Shanghai, China). HEK-293T cells were obtained from A. Lasorella (The Institute for Cancer Genetics, Columbia University Medical Center, New York, NY). HCT116, DLD1, MCF7, MDA-MB-435, MIApaca-2 and HEK-293T cells were cultured in DMEM (Life Technologies) supplemented with 10% FBS (Life Technologies), 100 U/mL penicillin (Life Technologies), and 100 mg/mL streptomycin sulfate (Life Technologies). LS174T cells were cultured in RPMI 1640 (Life Technologies) supplemented with 10% FBS, 100 U/mL penicillin, and 100 mg/mL streptomycin sulfate. LS174T suspended cell spheres, named LS174T-CSC, were established using serum-free neural stem cell medium composed of DMEM/F12, EGF (20 ng/ml, Life Technologies, USA), bFGF (20 ng/ml, Life Technologies, USA), and B27 (1x, Life Technologies, USA). All the cells were cultured at 37°C in a humidified incubator with 5% CO<sub>2</sub> and routinely confirmed to be mycoplasma free by PCR. The cells were passaged when they reached approximately 80% confluency.

### Plasmid construction and lentiviral packaging

The lentiviral vectors pTomo-pCMV-CD166-IRES-puro and pTomo-pCMV-CD318-IRES-puro, which were used for overexpression of CD166 and CD318 in HEK-293T, MIApaca-2 and MDA-MB-435 cells as described below, were constructed in a pTomo vector backbone (Addgene). Full-length human CD166 (accession\_NM\_001627.4) and CD318 (accession\_NM\_022842.5) cDNA sequences were amplified by PCR using PrimeSTAR HS DNA Polymerase (Takara) and cloned into the XbaI and AgeI sites of the plasmid (primers shown in Supplementary. 1-Table. S2). A codon-optimized targeting domain comprising the SCRC1 domain, the SCRC3 domain, the extracellular domain of human CD6, or CD19 scFv (Supplementary. 2) was synthesized (BGI Genomics Co., Ltd. Beijing, China) and fused to a CAR backbone comprising a human CD8 hinge spacer and transmembrane domain, 4-1BB costimulatory domain, and CD3ζ (Supplementary. 2). The entire encoding sequence of the CAR expression molecule (Supplementary. 2) was cloned into the lentiviral vector pTOMO (Addgene) between the XbaI and NheI restriction sites. For lentiviral packaging, the lentiviral plasmids were cotransfected into HEK293T cells with the packaging plasmids pCMVΔ8.9 and pMD2.G (Addgene) at a ratio of 10:5:2. Lentiviruses were harvested as described previously [28].

### CAR-T-cell preparation

Blood collected from healthy donors was used to isolate T cells using the RosetteSep™ Human T-Cell Enrichment Cocktail (STEMCELL, Canada), and the T cells were cultured in RPMI 1640 medium (Life Technologies, USA) supplemented with 10% FBS (Life Technologies, USA), 100 U/mL penicillin, 100 mg/mL streptomycin sulfate, and 200 U/ml IL-2 (PeproTech, USA). T-cells were activated by CD3/CD28 Dynabeads (Life Technologies, USA) according to the manufacturer's instructions. After 48 h, lentiviral particles were added to the cultures at a multiplicity of infection (MOI) of 100 in the presence of Lentibost (Sirion Biotech, 1:100). The CAR-T cells were counted on alternate days, and fresh medium was added to the cultures to maintain the cell density at  $1 \times 10^6$  cells/ml. Four days after T-cells were infected with CAR lentivirus, all were used for *in vitro* experiments.

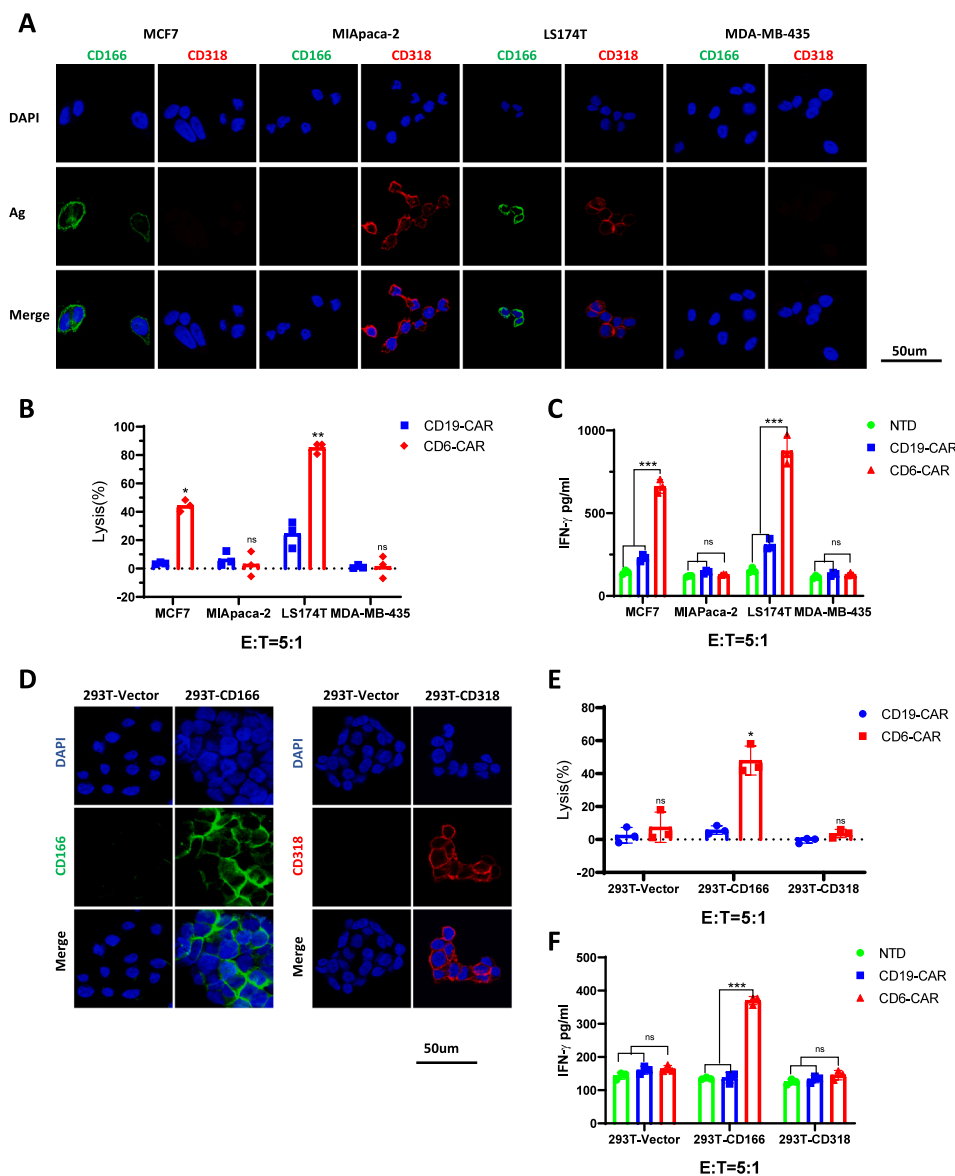


**Fig. 1.** Generation and screening of effective CAR-T cells *in vitro*. A, Schematic diagram of the ectodomain structure of CD6. SP: signal peptide; SCRC: scavenger receptor cysteine-rich; AA: amino acid. B, Schematic representation of CAR (CD19-CAR as a control group) constructs containing the CD3 $\zeta$  cytosolic domain in combination with the CD137 costimulatory module. LTR: long terminal repeat; SP: signal peptide; scFv: single-chain fragment variable; SCRC: scavenger receptor cysteine-rich; TM: transmembrane domain. C, D, A representative of CAR expression on human T cells transduced with lentivirus was analyzed using flow cytometry, which detected mKATE2 expression at day 4. The percentage of positive cells is detailed in the picture (C) and histogram (D). E, The CAR-T cells based on the ectodomain structure of CD6, and CD19-CAR-T cells were cocultured with LS174T and MDA-MB-435 cells at a 5:1 effector-to-target (E: T) ratio. Then, the cell cocultured wells were measured by luciferase cytotoxicity assay. Three independent experiments were performed. Data are presented as the mean  $\pm$  SD of triplicates and analyzed by one-way ANOVA with Dunnett's *t*-test with multiple correction testing. \*:  $p \leq 0.05$ ; \*\*:  $p \leq 0.01$ ; \*\*\*:  $p \leq 0.001$ ; ns: not significant.

**Cytotoxicity assay**

The specific cytotoxicity of the CAR-modified T cells was tested against various cancer cell lines at defined effector-to-target (E/T) ratios. After 24 hours of culture in RPMI 1640 (Life Technologies, USA)

supplemented with 10% FBS, 100 U/mL penicillin, and 100 mg/mL streptomycin sulfate, cytotoxicity was measured using a Cell-Mediated Cytotoxicity Fluorometric Assay Kit (Promega) according to the manufacturer's protocol.



**Fig. 2.** The cytotoxic effect of CD6-CAR-T cells was dependent on the expression of CD166 but not CD318. **A**, Expression of CD166 and CD318 in MCF7, MIApaca-2, LS174T and MDA-MB-435 cells by immunofluorescence staining; scale bar=50  $\mu$ m. **B**, CD6-CAR-T and CD19-CAR-T cells were cocultured with MCF7, MIApaca-2, LS174T and MDA-MB-435 cells at a 5:1 effector-to-target (E: T) ratio. Then, the cocultured wells were measured by luciferase cytotoxicity assay. Two independent experiments were performed. Data are presented as the mean  $\pm$  SD of triplicates and analyzed by Student's *t* test. \*:  $p < 0.05$ ; \*\*:  $p < 0.01$ ; \*\*\*:  $p < 0.001$ ; ns: not significant. **C**, The levels of IFN- $\gamma$  released by NTD, CD19-CAR and CD6-CAR-T cells were measured by ELISA after 24 h of coculture incubation at an E: T ratio of 5:1. Two independent experiments were performed. Data are presented as the mean  $\pm$  SD of triplicates and analyzed by one-way ANOVA with Dunnett's *t* correction. \*:  $p < 0.05$ ; \*\*:  $p < 0.01$ ; \*\*\*:  $p < 0.001$ ; ns: not significant. **D**, CD166 or CD318 expression in HEK-293T-Vector (293T-Vector) and HEK-293T-x (x: CD166 or CD318; 293T-CD166 or 293T-CD318) cells by immunofluorescence staining; scale bar=50  $\mu$ m. **E**, CD6-CAR-T and CD19-CAR-T cells were cocultured with 293T-Vector, 293T-CD166 and 293T-CD318 cells at a 5:1 effector-to-target (E: T) ratio. Then, the cell cocultured wells were measured by luciferase cytotoxicity assay. Two independent experiments were performed. Data are presented as the mean  $\pm$  SD of triplicates and analyzed by Student's *t*-test. \*:  $p < 0.05$ ; \*\*:  $p < 0.01$ ; \*\*\*:  $p < 0.001$ ; ns: not significant. **F**, The levels of IFN- $\gamma$  released by NTD, CD19-CAR and CD6-CAR-T cells were measured by ELISA after 24 h of coculture incubation at an E: T ratio of 5:1. Two independent experiments were performed. Data are presented as the mean  $\pm$  SD of triplicates and analyzed by one-way ANOVA with Dunnett's *t*-test with multiple correction testing. \*:  $p < 0.05$ ; \*\*:  $p < 0.01$ ; \*\*\*:  $p < 0.001$ ; ns: not significant.

### Cytokine release detection

For experiments with effector cells (NTD T cells, CD19-CAR, or CD6-CAR-T cells), these were cocultured with target cells for 24 h at a defined E:T ratio, and the supernatant was assessed for IFN- $\gamma$  secretion levels using enzyme-linked immunosorbent assay kits (Thermo Fisher Scientific, USA) according to the manufacturer's instructions.

### Flow cytometry

Cells were harvested, washed twice with  $1 \times$  PBS, and resuspended in cold PBS containing 2% FBS and 1% sodium azide (at a density of  $1 \times 10^6$  cells/ml). Subsequently, CD166 primary antibody (R&D system, AF1172) was added to the cell suspension according to the manufacturer's instructions and incubated for 2 h at 4  $^{\circ}$ C. Immediately after incubation, the cells were washed twice with ice-cold PBS and then incubated with Alexa Fluor $^{\circledR}$  647 secondary antibody (Jackson ImmunoResearch LABORATORIES LNC; 705-605-147) according to the manufacturer's instructions at room temperature for 1 h. The transduction rate of CAR into T-cells was detected by the mKATE2 signal. Flow cytometry was performed using a BD LSRFortessa cell analyzer (BD

Biosciences, USA). The data were analyzed using the FlowJo\_V10 analysis software package (TreeStar, USA).

### Immunofluorescence staining

Expression levels of CD166 and CD318 in tumor cell lines and HEK-293T cell lines were analyzed using immunofluorescence. To carry out immunofluorescence analysis, cells were grown in appropriate medium onto glass coverslips for 24 hours before fixation with 4% paraformaldehyde at 37  $^{\circ}$ C for 15 min. Fixed cells were then incubated at 4  $^{\circ}$ C overnight with the following primary antibodies: CD166 (R&D system, AF1172) and CD318 (Cell Signaling Technology, 4115). The next day, the cells were incubated with Alexa Fluor $^{\circledR}$  488- or Cyanine3-conjugated secondary antibodies (Abcam or Thermo; ab150129 or A10520) at room temperature for 1 hour. These slides were then mounted with Prolong Gold with 4',6-diamidino-2-phenylindole (DAPI, Invitrogen). Fluorescent images were taken using an Olympus IX71 epifluorescence microscope (Olympus, Japan).



### RNA isolation and real-time PCR

Total RNA was isolated using TRIzol (Sigma–Aldrich, USA), and DNA contaminants were removed using the TURBO DNA-free TM Kit (Life Technologies, USA). Next, 2 µg of total RNA was reverse transcribed using random primers and the RevertAid First Strand cDNA Synthesis kit (Thermo Scientific, USA). Quantitative PCR was performed in triplicate using the SYBR Green method (Life Technologies, USA) according to the manufacturer's instructions. The cycle threshold (CT) values of the target genes were normalized to those of 18S rRNA. All primers used in this study are shown in Supplementary.1-Table S1. Total RNA was isolated from the cell lines as previously described [29].

### Western blot analysis

Extract total proteins from cell lines as previously described [30]. A standard BCA assay kit was used to determine the protein concentration (Beyotime, China). Equal amounts of total protein were separated using standard SDS-polyacrylamide gel electrophoresis (PAGE). The membranes were then incubated 4°C overnight with anti-CD44, anti-CD133, anti-EPCAM, anti-E-cadherin, anti-β-actin (ProteinTech, USA), anti-CD166 (R&D system, AF1172), and anti-Vimentin (HuaBio, EM0401) antibodies diluted in 5% bovine serum albumin (BSA). The bands were detected using horseradish peroxidase (HRP)-conjugated secondary antibodies and the enhanced chemiluminescence (ECL) reagent according to the manufacturer's protocols (Millipore, USA).

### Statistical analysis

Experiments were repeated at least twice. All the data were analyzed using GraphPad Prism 8.0 statistical software and are presented as the mean ± SD. Statistical differences between two groups were analyzed using unpaired two-tailed Student's t tests. Statistical differences among three or more groups were analyzed by one-way analysis of variance (ANOVA) with Dunnett's test. Statistical significance was defined as \* $p \leq 0.05$ , \*\*  $p \leq 0.01$ , and \*\*\*  $p \leq 0.001$ .

## Results

### Generation and screening of CAR-T cells in vitro

To determine how full length CD6, as well as specific extracellular domains bound to CD166 and CD318 (Fig. 1a), we designed three CARs (CD6-SCRC1-CAR, CD6-SCRC3-CAR, and CD6-CAR) respectively (Schematic representation of CARs shown in Fig. 1b). Also, we used CD19-CAR as negative control. To determine the ability of the lentivirus vector to infect T cells, the surface expression of CAR on the T cells was observed by inversion fluorescence microscopy (Supplementary.1-Fig. S1) and measured by flow cytometry through the detection of mKATE2 (Fig. 1c and d). As shown in Supplementary.1-Fig. S1 as well as Fig. 1c and d, the results confirmed that all CARs had high levels of infectivity, with the frequency of CAR expression quantified at 45% or greater.

To determine whether CARs have a toxic effect on cells in culture, we performed cytotoxicity assays using the following cell lines: LS174T (which is target positive) and MDA-MB-435 (which is target negative). As shown in Fig. 1e, exposure of LS174T cells to CD6-CAR-T cells led to a significantly higher fraction of cells undergoing target-specific lysis compared with CD19-CAR cells in CD166- and CD318-positive cells (LS174T, Fig. 2a). In contrast, there was no significant increase in cell lysis in MDA-MB-435 cells, as shown in the CD166- and CD318-negative cell groups (MDA-MB-435, Fig. 2a).

**Table 1**

The expression conditions between CD166 and CD318 in cell lines.

CD318\CD166	+	-
+	LS174T	MIApaca-2
-	MCF7	MDA-MB-435

“+” represents the positive expression of CD166/CD318; “-” represents the negative expression of CD166/CD318.

### The cytotoxic effect of CD6-CAR-T cells was dependent on the expression of CD166 but not CD318

The entire CD6 extracellular domain, comprising SCRC3, which can bind to CD166, as well as SCRC1, which can bind to CD318, is essential for its binding specificity. To further explore the target of CD6-CAR-T cells, according to the expression of different combinations of CD166 and CD318, we selected four cell lines (MCF7; MIApaca-2; LS174T; MDA-MB-435), representing four expression condition (+/-, -/+, +/+, -/-; Table 1; Fig. 2a; Supplementary.1-Fig. S2c and d). The cells were then cocultured with CD6-CAR-T cells, and CD6-CAR-T cells only showed significant cytotoxicity to CD166-positive cell lines but not to CD166-negative cell lines. CD6-CAR-T cells exhibited significant cytotoxicity, but this was not significantly correlated with CD318 expression (Fig. 2b and c).

To further investigate whether the cytotoxicity of CD6-CAR-T cells was only positively correlated with the cell surface expression of CD166, lentiviral particles that induced CD166 or CD318 overexpression were constructed and used to infect HEK-293T cells (Fig. 2d; Supplementary.1-Fig. S2a and b). As shown, when CD6-CAR-T cells were cocultured with the overexpressed cells, we found that the presence of CD6-CAR-T cells was cytotoxic to HEK-293T-CD166 cells, but this effect was not detected in parallel experiments with HEK-293T-CD318 cells (Fig. 2e and f). Therefore, our data suggests that CD6-CAR exerts a cytotoxic effect by targeting CD166, yet may not respond to the presence of CD318. Data from similar experiments performed with additional tumor cell lines (MIApaca-2; MDA-MB-435) resulted in similar conclusions (Supplementary.1-Fig. S3).

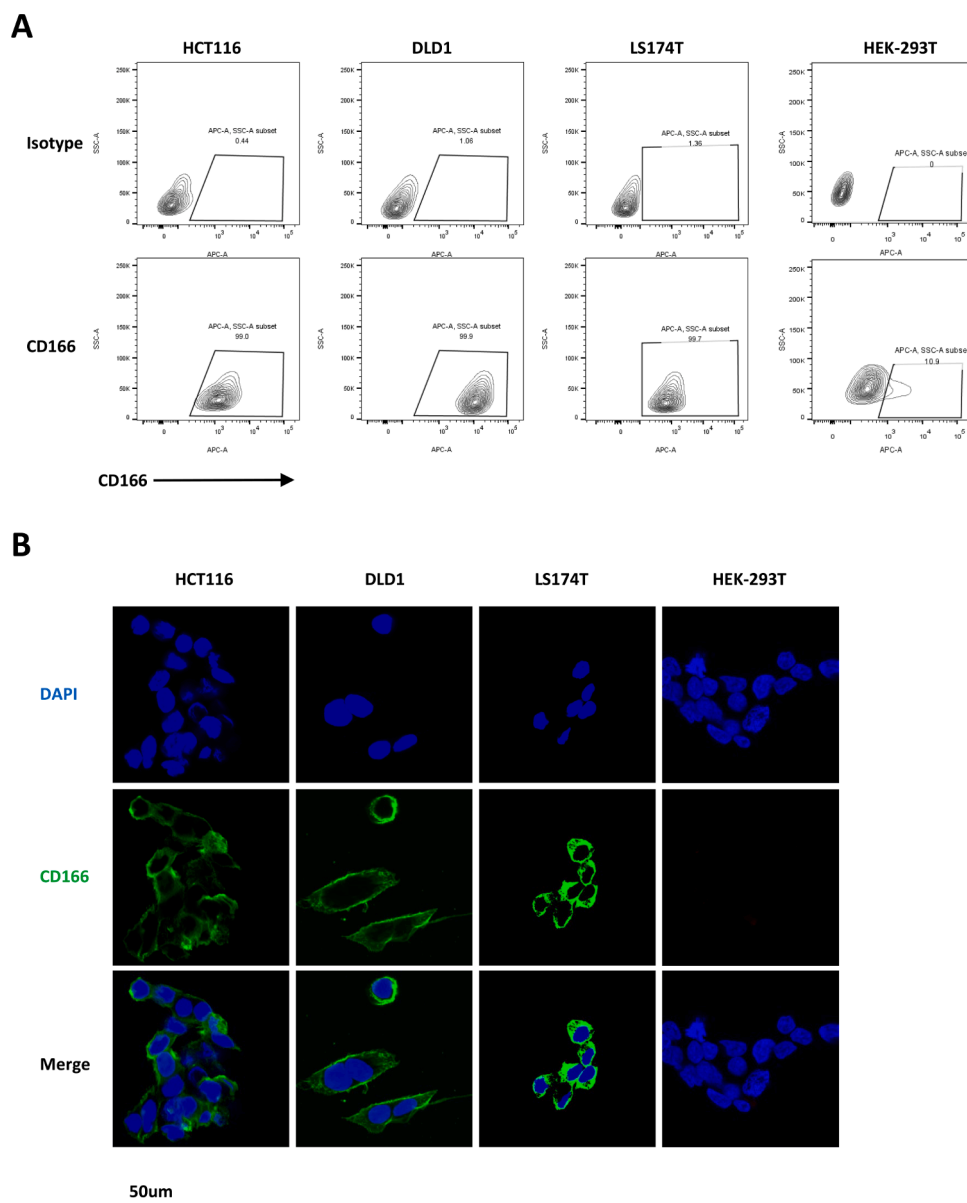
### CD166 expression in colorectal cell lines

To clarify the cell surface expression of CD166 on CRC cell lines, HCT116, DLD1, LS174T, and HEK-293T cells (as a negative control) were assessed by flow cytometry (Fig. 3a) and immunofluorescence staining (Fig. 3b). All the tested CRC cell lines expressed high levels of CD166, as expected. In addition, HEK-293T, which is not a CRC cell line but is an embryonic kidney cell line, has low-level expression. Therefore, our results clearly supported the possibility of using CD166 as a target in CRC therapy.

### CD6-CAR-T cells efficiently lysed CRC cells

In CAR-T treatment, the lysis of tumor cells by CAR-T cells is crucial to its use as an anticancer adoptive cell therapy. To test this in our CAR-T cells after lentiviral transfection, we incubated them with LS174T, HCT116, and DLD1 CRC cell lines at different E:T ratios via a standard luciferase cytotoxicity assay. HEK-293T cells served as the control cells. As shown, our results suggest that CD6-CAR-T cells efficiently lysed CRC cells but not HEK-293T cells. In addition, CD19-CAR-T cells showed very weak cytotoxicity (Fig. 4a-d).

Cytokine secretion by CAR-T cells targeting cancer cells represents a further indicator of the activation and specific cytotoxicity of T cells. Thus, we analyzed the levels of the classic cytokine IFN-γ to assess the cytokine profile when CAR-T cells were incubated with the target cells. As shown, the concentrations of tested cytokines were significantly elevated in the supernatant of the CD6-CAR-T-cell coculture system compared with those of target cells cocultured with CD19-CAR-T cells



**Fig. 3.** CD166 expression in CRC cell lines. A, Expression of CD166 in HCT116, DLD1, LS174T and HEK-293T cells by flow cytometry. The percentage of positive cells is detailed in the picture. B, Expression of CD166 in HCT116, DLD1, LS174T and HEK-293T cells by immunofluorescence staining; scale bar=50  $\mu$ m.

and NTD cells (Fig. 4e–h).

#### CD6-CAR-T cells had potent cytotoxicity targeting CRC CSCs

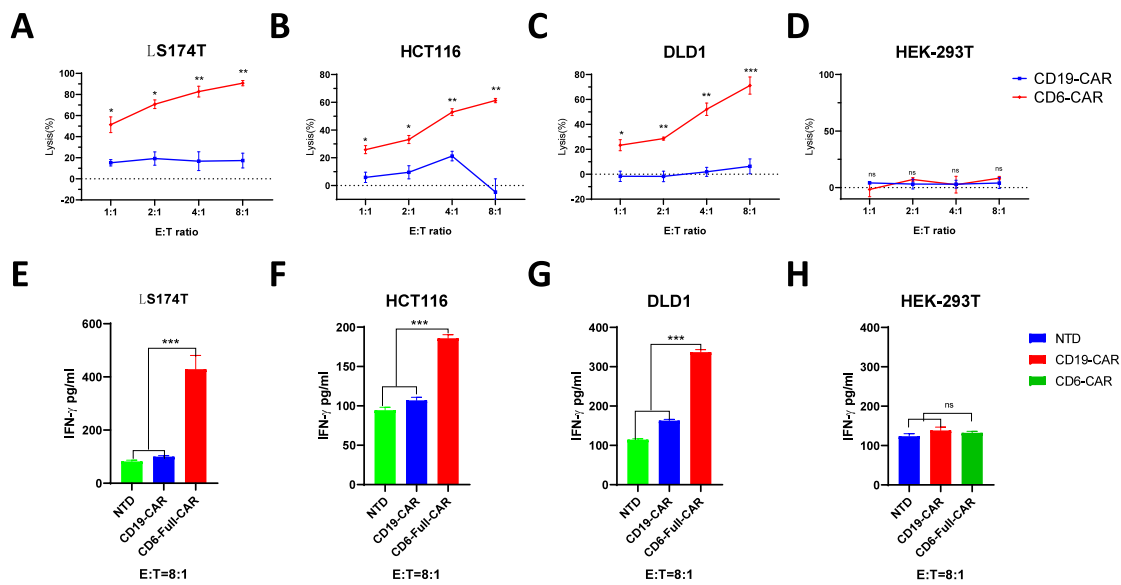
Next, we wanted to determine whether CD6-CAR-T cells possess specific activity against CSCs. To do this, we derived suspended cell spheres from LS174T cells and named them LS174T-CSCs. The expression of CSC and EMT markers were measured using real-time RT-PCR or Western blot (WB). Our results showed that CD44, CD133, CD166, EPCAM and Vimentin were significantly upregulated and E-Cadherin was markedly decreased in the tested spheres (Supplementary.1-Fig. S4), indicating that CSCs were enriched in these cell spheres. Next, the expression of CD166 in the suspended cell spheres was assessed by real-time RT-PCR (Fig. 5a) and flow cytometry (Fig. 5b). As shown, we detected robust cell-surface expression of CD166 in LS174T-CSCs. When CAR-T cells were added, we found that CD6-CAR-T group, but not CD19-CAR-T/NTD groups, exhibited potent cytotoxicity, strong cytokine release, and potent apoptosis when they were incubated with suspended cell spheres (Fig. 5c, d and f). What's more, compared with CD19-CAR-

T/NTD groups, suspended cell spheres after co-incubation with CD6-CAR-T cells have a lower expression in CSCs makers and E-Cadherin but a higher expression in Vimentin (Fig. 5e). Overall, the data suggest that CD6-CAR-T cells show potent cytotoxicity to CRC CSCs.

#### Discussion

Successful CAR-mediated therapies rely on the selection of appropriate TAAs for characterization and testing. Here, we focused on designed CARs to target CD166, a transmembrane glycoprotein from the immunoglobulin superfamily that is prominently expressed in tumor tissues, but not in non-tumor tissues, a finding that is consistent with our studies using CRC cell lines compared with HEK293T cells, a non-tumor cell line. Moreover, the efficacy and safety of targeting CD166 have been preliminarily confirmed by a scFv-based CAR targeting CD166 [31]. Here, our CD6-CAR-T studies, further supports the idea that CD166 targeting could represent a promising CAR-T therapeutic approach against CRC.

The potential therapeutic efficacy of CD166-specific CAR-T cells has



**Fig. 4.** CD6-CAR-T cells efficiently lysed CRC cells. A, The cytotoxicity of CD19-CAR and CD6-CAR-T cells against the indicated cell lines at different effector-to-target (E: T) ratios. Two independent experiments were performed. The results are presented as the mean volume  $\pm$  SD of triplicates and analyzed by Student's t test. \*:  $p \leq 0.05$ ; \*\*:  $p \leq 0.01$ ; \*\*\*:  $p \leq 0.001$ ; ns: not significant. B, The levels of IFN- $\gamma$  released by NTD, CD19-CAR and CD6-CAR-T cells were measured by ELISA after 24 h of coculture incubation at an E: T ratio of 8: 1. Two independent experiments were performed. The results are presented as the mean volume  $\pm$  SD of triplicates and analyzed by one-way ANOVA with Dunnett's t-test with multiple correction testing. \*:  $p \leq 0.05$ ; \*\*:  $p \leq 0.01$ ; \*\*\*:  $p \leq 0.001$ ; ns: not significant.

been tested in osteosarcoma (OS), but not in CRC [31]. In this study, we designed a novel CD6-CAR using the extracellular domain of CD6 instead of scFv, followed by 4-1BB and CD3 $\zeta$ , and successfully produced CD6-CAR-T cells that showed robust cytotoxicity against CRC cell lines and CRC CSCs *in vitro* but did not exhibit significant cytotoxicity against cell lines with low or weak CD166 expression, such as the human embryonic kidney cell line HEK-293T. The results demonstrated that CD6-CAR-T cells could specifically induce cytotoxic death of CRC cells as well as CRC CSCs through a mechanism that involves CD166 expression. Given that CD6 prominently binds CD166 through its SCRC 3 domain, our results suggest this approach could be effective to treat patients diagnosed with CD166-positive CRC.

Despite initially successful therapy, some CRC patients will experience treatment failure due to multi-drug resistance (MDR) and minimal residual disease (MRD). Both MDR and MRD could be attributed to a subpopulation of tumor cells with the capacity for self-renewal and differentiation, known as CRC CSC, and they play a key role in proliferation, metastasis, drug resistance, and tumor recurrence. Many surface markers have been used to identify CRC CSCs, such as CD44, CD133, CD166, EPCAM, Lgr5, and BMI1 etc. [32–34]. However, effective treatments that specifically target CRC CSCs are lacking. Currently, CD133-, HER2-, EPCAM- and NKG2D-directed CAR-T cells have been shown to lyse CSCs in gastric cancer, prostate cancer, and glioblastoma, indicating that the strategy of using CAR-T cells to eliminate CRC CSCs could be a promising treatment approach for CRC [35–38]. Given the previous finding that CD166 is a cancer stem cell marker in CRC, we confirm, through our investigation here, that targeting CD166 CAR-T cells can lyse CSCs in an *in vitro* model [39].

Several studies have shown that the cell-biological programme termed epithelial-to-mesenchymal transition (EMT) may be the culprit that leads to differences between CSCs and non-CSCs. In addition, overexpression of epithelial-mesenchymal transformation (EMT) transcription factors not only promotes EMT, but also calls up stem cells and enhances the tumor-causing potential of cell lines [40,41]. Here, we found that suspended cell spheres had a lower expression of CRC CSC makers (CD44, CD133, CD166, and EPCAM) and E-Cadherin when they were incubated with CD6-CAR-T cells, rather than CD19- CAR-T/NTD cells, but had a higher expression of Vimentin. The results suggest that

CRC CSC makers and EMT makers would decrease during cytotoxicity, which further confirm that CD6-CAR-T could exhibit potent cytotoxicity to CRC CSCs.

The effectiveness and low toxicity of CAR-T-cell therapy largely depends on the specificity of the target antigen through which it is designed. In our study, CARs were designed based on the full-length ectodomain sequence of CD6, which binds CD166 via the SCRC3 domain, as well as binding to CD318 through its SCRC1 domain. We confirmed that only CD6-CAR-T cells containing both SCRC1 and SCRC3 could exert significant cytotoxicity, while CD6-SCRC1-CAR containing SCRC1 and CD6-SCRC3-CAR containing SCRC3 did not exhibit significant cytotoxicity. Moreover, CD6-CAR-T cells only showed significant cytotoxicity to CD166-positive cells but not to CD318-positive cells. Therefore, our results indicate that CD6-CAR-T cells induce lysis of cancer cells through targeting their expression of CD166, not CD318. Experiments with CD6-SCRC1-CAR and CD6-SCRC3-CAR could suggest the entire ectodomain is essential to CD166 binding.

Given the effectiveness and safety of CD166-specific CAR-T cells in the treatment of osteosarcoma, we predict that CD6-CAR-T cells may be an effective therapeutic treatment option for CRC in the clinic.

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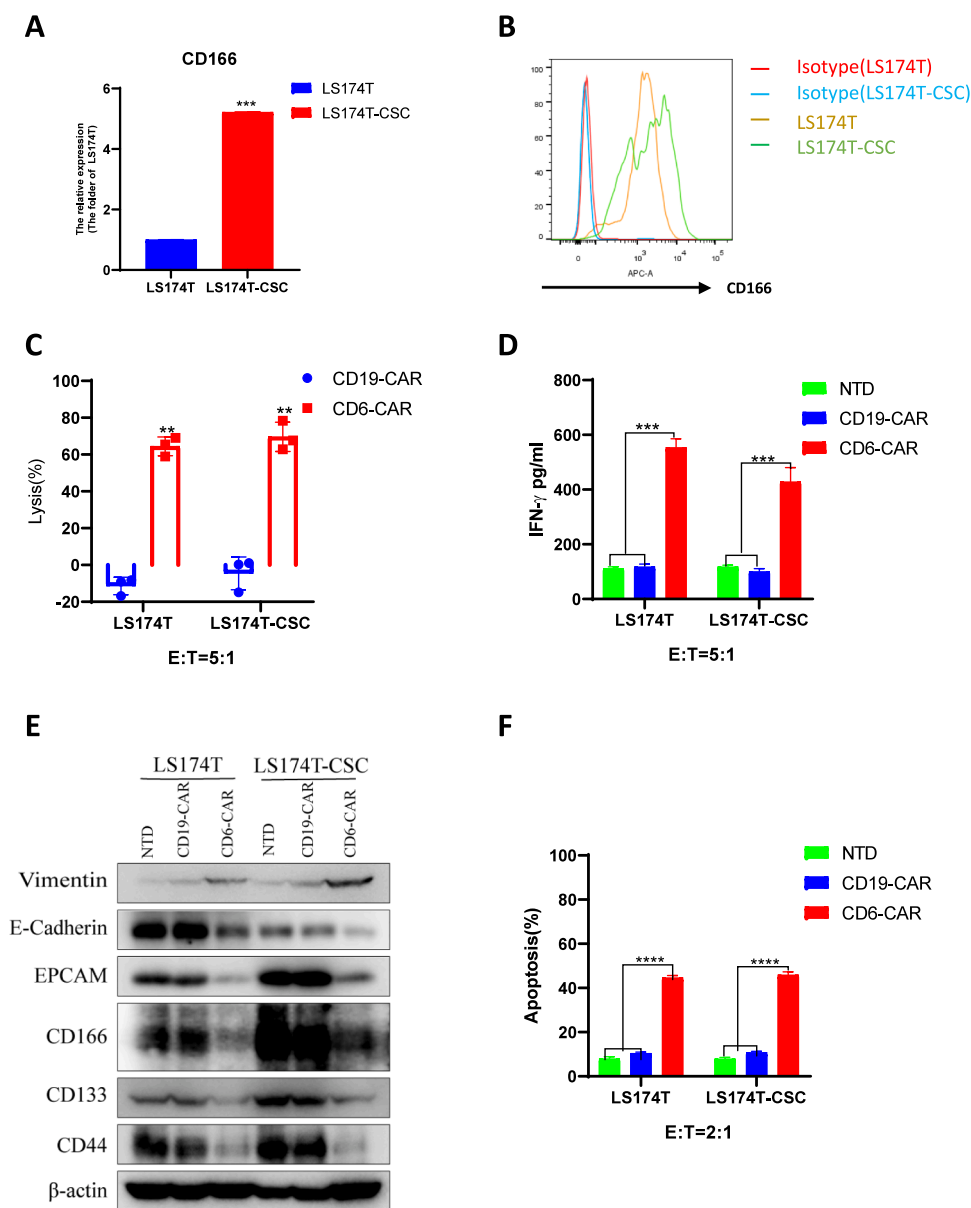
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#### CRedit authorship contribution statement

**Shuai He:** Writing – original draft, Investigation, Data curation, Formal analysis, Writing – review & editing. **Shirong Li:** Investigation, Data curation, Formal analysis. **Jing Guo:** Investigation. **Xiaozhu Zeng:** Investigation. **Dandan Liang:** Investigation. **Yongjie Zhu:** Investigation. **Yi Li:** Investigation. **Dong Yang:** Data curation, Formal analysis. **Xudong Zhao:** Writing – original draft, Writing – review & editing.

#### Declaration of Competing Interest

The authors declare that they have no known competing financial



**Fig. 5.** CD6-CAR-T cells had potent cytotoxicity targeting CRC CSCs. A, The expression of CD166 was detected by real-time PCR. Two independent experiments were performed. The results are presented as the mean volume  $\pm$  SD of triplicates and analyzed by Student's *t*-test. \*:  $p \leq 0.05$ ; \*\*:  $p \leq 0.01$ ; \*\*\*:  $p \leq 0.001$ ; ns: not significant. B, Expression of CD166 in LS174T and LS174T-CSC cells by flow cytometry. The percentage of positive cells is detailed in the picture. C, Cytotoxicity of NTD, CD19-CAR and CD6-CAR-T cells against cancer stem cells at effector to target (E: T) ratios of 5: 1. Two independent experiments were performed. The results are presented as the mean volume  $\pm$  SD of triplicates and analyzed by Student's *t* test. \*:  $p \leq 0.05$ ; \*\*:  $p \leq 0.01$ ; \*\*\*:  $p \leq 0.001$ ; ns: not significant. D, The levels of IFN- $\gamma$  released by NTD, CD19-CAR and CD6-CAR-T cells were measured by ELISA after 24 h of coculture incubation at an E:T ratio of 5: 1. Two independent experiments were performed. The results are presented as the mean volume  $\pm$  SD of triplicates and analyzed by one-way ANOVA with Dunnett's *t* correction. \*:  $p \leq 0.05$ ; \*\*:  $p \leq 0.01$ ; \*\*\*:  $p \leq 0.001$ ; ns: not significant. E, The CSCs makers (CD44, CD133, CD166 and EPCAM) and EMT makers (E-Cadherin and Vimentin) were analyzed by WB after 48 hours cocultured with CAR-T cells at an E: T ratio of 2:1,  $\beta$ -actin was the loading control. F, LS174T or LS174T-CSC cells were cocultured with CAR-T cells at an E: T ratio of 2:1 for 20 hours. Annexin V-fluorescein isothiocyanate (FITC)/PI double-staining assays using flow cytometry. The percentage of annexin V+/PI+ or annexin V+/PI- cells were measured. Two independent experiments were performed. The results are presented as the mean volume  $\pm$  SD of triplicates and analyzed by one-way ANOVA with Dunnett's *t* correction. \*:  $p \leq 0.05$ ; \*\*:  $p \leq 0.01$ ; \*\*\*:  $p \leq 0.001$ ; ns: not significant.

interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Supplementary materials

Supplementary material associated with this article can be found, in the online version, at [doi:10.1016/j.tranon.2022.101575](https://doi.org/10.1016/j.tranon.2022.101575).

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