Therapeutic Effect of Dual CAR-T Therapy Targeting PDL1 and Anti-MUC16 Antigens on Ovarian Cancer cells

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Abstract

**Background:** More favorable treatment against epithelial ovarian cancer (EOC) is urgently needed because of its insidious nature at an early stage and a low rate of five-year survival. The primary treatment, extensive surgery combined with chemotherapy, exhibit few benefits for improving prognosis. Chimeric antigen receptor T (CAR-T) cell technology as novel immunotherapy has made breakthrough progress in the treatment of hematologic malignancies, and there were also benefits in a partial solid tumor in previous research. Therefore, CAR-T cell technology may be a promising candidate as an immunotherapeutic tool against EOC. However, there are some weaknesses in targeting one antigen from the previous preclinical assay, such as on-target off-tumor cytotoxicity. Thus, the more specific dual-target CAR-T cell may be a better choice.

**Methods:** We constructed tandem PD1-antiMUC16 dual-CAR, PD1 single-CAR, and anti-MUC16 single-CAR fragments by PCR and genetic engineering, followed by preparing CAR-T cells via lentiviral infection. The expression of CAR molecules on single and dual CAR-T cells detected by flow cytometry. The killing ability and activation of CAR-T cells were measured by cytotoxic assays and cytokines release assays in vitro. The therapeutic capacity of CAR-T cells was assessed by tumor-bearing mice model assay in vivo.

**Results:** We successfully constructed CARs lentiviral expression vectors and obtained single and dual CAR-T cells. CAR-T cells demonstrated robust killing ability against OVCAR-3 cells in vitro. Meanwhile, CAR-T cells released plenty of cytokines such as interleukin-2 (IL-2), interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α). Besides, CAR-T cells indicated a therapeutic benefit against OVCAR-3 tumor-bearing mice models and significantly prolonged survival time of mice. Dual CAR-T cells were proved to be two to four times more efficacious single CAR-T cells on survival time.

**Conclusion:** Dual CAR-T cells exhibited a similar ability as single CAR-T cells against OVCAR-3 cells in vitro. However, dual CAR-T cells verified more outstanding capacity against OVCAR-3 cells than single CAR-T cells in vivo. Furthermore, it significantly prolonged the survival time of tumor-bearing mice models. Thus, PD1-antiMUC16 CAR-T cells have more potent antitumor activity than single CAR-T cells in vitro and in vivo, and it could be applied in the treatment of EOC.
Background
Epithelial ovarian cancer (EOC) occupies approximately 90% in Ovarian cancer (OC), which is the fifth most typical tumor in female malignancies [1-2]. Meanwhile, EOC is classified by tumor cell histology as serous, endometrioid, mucinous, clear cell, and unspecified type[3]. Serous carcinoma, more than a half [4], is the primary type of EOC, it is diagnosed at stage III (51%) or stage IV (29%) due to the absence of specific early symptoms[3]. Owing to the inadequate screening and detection methods in early-stage, more effective and less recrudescent therapies are urgently needed. The primary treatment of EOC is extensive surgery combined with platinum-based and taxane-based chemotherapy to date, however, there are few benefits for improving prognosis [2-4]. Therefore, novel therapeutic methods are a promising direction for development.

CAR-T cell therapy strategies, one of the representative adoptive immunotherapies, has made unprecedented significant progress in the treatment of hematologic malignancies. At present, the Food and Drug Administration (FDA) has approved CD19 CAR-T products for leukaemia and lymphoma. In contrast, it is hard that patients with solid tumors received benefits because of the deficiency of tumor-specific targets and physiologic barrier[5].

Surprisingly, some researchers engineered multiple CAR-T cells on OC in many studies have demonstrated desirable results, such as the NKG2D-CAR-T cell can specifically recognize and kill the OC cells expressing NKG2DL antigen [6]. CAR-T cells recognize and combine with the tumor cells expressing specific antigen via extracellular scFv fragment[7]. After recognizing the target cells, CAR-T cells release many cytokines such as IL-2, IL-6, TNF-α, and IFN-γ to activate T cells, stimulate NK cells, and promote the secretion of various factors for starting a series of killing effect [8]. However, some weaknesses were exposed, most CAT-T cell has one specific CAR molecule that targets one antigen of the tumor cells, it may cause the on-target off-tumor toxicity, difficulty in homing, absence of sustaining T cell effect, and lead cytokine release syndrome (CRS) in vivo [9-10]. Besides, single CAR-T cannot improve the tumor microenvironment. The immune escape caused by the influence of the tumor microenvironment cannot be avoided [11]. Overall, considering the previous good lethal effect and the deficiency of single-target CAR-T technology in many carcinomas, we hypothesized a
higher specificity CAR-T, dual-target CAR-T, would address the weakness and exhibit an outstanding lethal effect on EOC. For structuring valiant dual-target CAR-T, selecting specific antigens as targets are the crucial point.

Mucin 16 (MUC16), as the glycoprotein with the most massive molecule weight in the mucin family, is also a critical biomolecule to maintain the intracellular balance and protect the epithelium [12]. It is expressed in a variety of tumor cells and involved in the proliferation and metastasis of tumor cells. Studies have shown that 80% EOC express MUC16, and its extracellular segment is cut and released in the peripheral blood, becoming a well-known tumor marker, namely CA125 [13]. Therefore, MUC16 is an ideal antigenic target for CAR molecules.

Programmed cell death-1 (PD1) is an immunosuppressive molecule widely expressed on the surface of activated T cells, B cells, antigen-presenting cells, and macrophages. It belongs to the CD28/cytotoxic T lymphocyte-associated antigen-4 (CTLA-4) family [14]. PD-1 and its ligand PDL1 constitute the PD1/PDL1 signaling pathway, which plays an inhibitory role in T cell immunity. Current research suggests that T cells can secrete many cytokines such as IL-10, IFN-γ to induce the generation of CTLA ligand, such as PD1 expressing on OC cells by connecting with OC cells. At the same time, PD1 induces expression and combines with inhibitory receptors on the surface of T cells, thereby reducing the anti-activity of effector T cells, and guiding T cell reposition or making T cell failure to achieve immune escape [15–19]. In the experiment of melanoma-bearing mice, it was found that the up-regulated expression of PDL1 in the tumor microenvironment led to the suppression of anti-tumor immune escape on T cells. After intraperitoneal injection of the PD1 antibody to block the PD1 pathway, T cell significantly increased infiltration [20–22]. There is also a study that shows that the five-year survival rate of patients with low expression of PDL1 is significantly higher than that of patients with high expression of PDL1 [21, 23–24]. From the above information of PD1, we infer PD1 would be another ideal target.

In this study, we developed a novel tandem-specific CAR-T cell that targets MUC16 and PDL1 antigens and investigated whether the extracellular domain of PD1-antiMUC16 CAR-T can effectively recognize the targeted antigens, and further kill tumor cells, release cytokines and prolong the survival time of
tumor-bearing mice.

Methods

Cell lines

Lenti-X 293T cell line was provided by TaKaRa (Cat#632180, Osaka, Japan). OVCAR-3 cell line was purchased from Fuheng (Cat#FH0726, Shanghai, China). Umbilical blood mononuclear cell (UBMC) was obtained from healthy donors in Beijing Obstetrics and Gynecology Hospital. Lenti-X 293T cells were used to construct lentiviral expression vectors and were cultured in high glucose DMEM medium (Hyclone, Logan, United States) containing 5% fetal bovine serum (FBS, Hyclone, Logan, United States) and 1% penicillin-streptomycin solution (Hyclone, Logan, United States). OVCAR-3 cells were marked as OVCAR3-Luc cells by luciferase, culturing in RPMI-1640 medium (Gibco, California, United States) supplemented with 10% FBS, 1% penicillin-streptomycin solution, and 0.1% insulin (Gibco, California, United States). After being activated by anti-CD3/CD28 magnetic beads (Novoprotein, Shanghai, China), UBMC was cultured in the GT-T551 H3 medium (TaKaRa, Osaka, Japan) containing 5% FBS and 40 IU/ml IL-2 (Novoprotein, Shanghai, China). All cells were cultured in an incubator (ESCO, Portland, United States) at 37°C and 5% CO₂. This study was approved by Medical Ethics Committee, Beijing Obstetrics and Gynecology Hospital, Capital Medical University.

Construction of CAR molecule

After designing the sequences, primers and templates were synthesized by Sangon Biotech. According to the PCR principle, the single chain antibody fragment (scFv) fragments of PD1, anti-MUC16, PD1-antiMUC16 CAR were obtained. The main structure of PD1 and anti-MUC16 was PD-1etco and 4H11-VH-(Gly4Ser)3-4H11-VL. The primary structure of PD1-antiMUC16 was the tandem of PD1 and anti-MUC16. The three scFv fragments were cloned into the pLVX-EFlα-IRES-mCherry plasmid (Clontech, TaKaRa, Osaka, Japan) through EcoR I and Mlu I cloning sites and named PD1-antiMUC16 CAR, anti-MUC16 CAR, and PD1 CAR, respectively. The plasmid has been genetically engineered to be a second-generation CAR containing CD8a hinge region, CD8 transmembrane region, 4-1BB co-stimulation domain, and CD3ζ domains. The plasmids were amplified in bacterial solution, and the positive samples were selected by agarose gel electrophoresis and verified via sequencing analysis.

Lentivirus packaging
Set experiment groups (pLV-PD1-anti-MUC16, pLV-anti-MUC16, and pLV-PD1) (Addgene, Massachusetts, United States) and Control group (control T). In each group, 13.7µg plasmid was taken and mixed with packaging plasmid containing 3.43µg PMD2.G, 3.43µg PMDLgPRRE, 3.43µg PRSV-REV to make DNA-mix. 7 × 10^6 cells Lenti-X 293T were added into the DNA-mix and the same volume of polyethylenimine (PE1, Polyscience, Pennsylvania, United States), then cultured in the incubator. Fresh virus packaging medium containing Opti-MEM (Gibco, California, United States), 5% FBS, 1% L-glutamine (Gibco, California, United States), 1% sodium pyruvate (Gibco, California, United States), and 0.2% penicillin-streptomycin solution was supplemented after 6 hours of culturing. After 24 hours, we collected supernatant and obtained virus concentrate (210 ul), from which 6 ul was taken to infect 293 T cells again for virus titer detection. The rest of the virus concentrate was stored in a 4°C refrigerator for the preparation of CAR-T cells. After 48 hours of infection, the infected cells were placed into 12-well plates (Corning, New York, United States). 5 ul Percp-cy5.5 antihuman PD-1 antibody (BD, New Jersey, United States), and 10 ul FITC-Protein L antibody (ACRO, Delaware, United States) were added to each well, and incubated in the dark for 30 minutes. After that, flow cytometry (NovoCyte Advanteon, ACEA, Hangzhou, China) detected the positive rate of PD1 and anti-MUC16 in Lenti-X 293T cells and calculated viral titers according to the formula:

T cell transduction
Set experiment groups (pLV-PD1-anti-MUC16, pLV-anti-MUC16, and pLV-PD1) and Control group (control T). After thawing, 2.5 × 10^6 UBMC were added 10 times the volume of phosphate buffer saline (PBS, Hyclone, Logan, United States), centrifuged at 1000 rpm for 15 minutes (BT-320C, Baiyang, Beijing, China), and an appropriate T cell complete medium and 25 ul magnetic beads were added and cultured in the incubator for 48 hours. Lentiviral supernatants were collected and taken 200 ul into the 12-well plate coated with 200ug NovoNectin (Novoprotein, Shanghai, China) per well overnight. Meanwhile, control T well was added with 200 ul GT-T551 H3 medium. All wells were added 800 ul UBMC (2.5 × 10^5 cells/well) and cultured in an incubator. After culturing for 6 hours, 2 ml T cells complete growth medium (GT-T551 H3 + 5%FBS + 40 IU/ml IL-2) were added. The following day, T cells were re-infected. After the second infection for 96 hours, every well was taken out of 1 ×
10^6 cells, washed twice with PBS, removed of the magnetic beads, and stained with 5 ul Percp-cy5.5 antihuman PD-1 antibody and 10 ul FITC-Protein L antibody for 30 minutes in the dark, followed by washing with PBS twice, and then detection of the positive rate of CAR structure by flow cytometry.

Preparation of target cells
After thawing, OVCAR3-luc cells were cultured in RPML1640 medium containing 20% FBS, 1% glutamine, 1% sodium pyruvate, 1% penicillin and streptomycin, 0.01 mg/ml insulin, and seeded in T25 bottle at density 5 x 10^5 /4 cm^2 in an incubator (37°C, 5% CO2). MUC16 plasmid and PDL1 plasmid (Juventas, Tianjin, China) both mixed with PEI-DNA mix to finish lentiviral packaging by PEI transfection, and then were harvested for MUC16 lentiviral expression vector, PDL1 lentiviral expression vector, and MUC16-PDL1 lentiviral expression vector. After 24 hours and 48 hours, the lentiviral concentrate was harvested, followed by infecting OVCAR3-luc cells. Flow cytometry measured the positive rate of MUC16 and PDL1 on the OVCAR3-luc cell surface after 96 hours of re-infection.

Flow cytometry
One million CAR-T cells were stained with 5 ul Percp-cy5.5 antihuman PD-1 antibody and 10 ul FITC-Protein L antibody for an antigen-antibody binding reaction. After 30 minutes of dark incubation, dissociative antibodies uncombined with antigens were washed by PBS. CAR molecule expression was measured for fluorescence intensity by flow cytometry. This method was used to detect viral titer and transfection rate of T cell. To evaluate the cytotoxicity of CAR-T cells via detecting the luciferase expression of tumor cells after stained with 70 ul Steady-Glo. Simultaneously, evaluating the transfection efficiency of tumor cells via detecting green fluorescent protein (GFP).

Enzyme-linked immunosorbent assay (ELISA)
The human IFN-gamma ELISA kit, IL-2 ELISA kit, and TNF-alpha ELISA kit (all kits, Dakewe, Beijing, China) were used to measure the concentrations of IFN-γ, IL-2, TNF-α, respectively. According to the instructions of the ELISA kit, three samples were processed, and the standard curve was prepared. Then the fluorescence value was measured by the enzyme-labeled instrument (TECAN, Mannedorf, Switzerland), and the cytokines quantity was calculated.

Cytotoxicity Assay And Cytokine Release Assay
Target cells: OVCAR3-luc cells, OVCAR3-PDL1-luc cells, OVCAR3-MUC16-luc cells, and OVCAR3-MUC16-PDL1-luc cells; Effector cells: PD1-antiMUC16 CAR-T cells, antiMUC16 CAR-T cells, PD-1 CAR-T cells, control T cells (negative group). Target cells was adjusted for a density of $2 \times 10^5$ cells/ml by GT-T551 H3, and seeded into the black flat 96-well U-bottomed plate (50 ul/well). Effector cells were adjusted for a density of $3.2 \times 10^6$ cells/ml by GT-T551 H3, and seeded into the same 96-well plate (50 ul/well) at effector to target (E/T) ratios of 1:1,4:1,8:1,16:1, respectively. Simultaneously, target cells (50 ul/well) were cultured alone and with 50 ul GT-T551 H3 medium. The plate was put in the incubator at 37°C for 4 hours. After that, all cells were stained with 70 ul Steady-Glo (Promega, Wisconsin, United States) per well for 20 minutes in the dark. Flow cytometry detected the fluorescence value and calculated the killing rate of various CAR-T cells on tumor cells according to the formula:

The CAR-T cells were co-cultured with target cells, at 1:1 ratio ($1 \times 10^4$ T cells and $1 \times 10^4$ target cells) in a V-bottomed 96-well plate for 48 hours in the incubator, followed by harvesting supernatant through centrifugation (2500 rpm, 5 minutes), and detected for the release of IFN-γ, IL-2, TNF-α by ELISA kit, respectively.

Xenograft mice models for in vivo treatment

All animal studies were approved by the Ethics Committee of Capital Medical University (2018-KY-026-01). In-house bred NPG mice (NOD.Cg-PrkdcsidIl2rgtm1Vst/Vst) were obtained from Beijing Vitalstar Biotechnology Co., Ltd. Twenty healthy NPG mice (females, 35–41 days old, 18–21 g in weight) were raised in specific pathogen-free (SPF) conditions and provided autoclaved food and water. For the xenograft models, NPG mice were intraperitoneally injected with $5 \times 10^5$ OVCAR3-MUC16-GFP-PDL1-luc cells and 50 ul Matrigel (Corning, New York, United States). After 48 hours, mice were intraperitoneally injected with 100 ul D-Luciferin, Postassium Salt (Sciencelight, Shanghai, China) for 6 minutes, followed by being put in an anesthetic box containing isoflurane (100 ml, RWD, Shenzhen, China) for 2 minutes. After that, tumor burden were measured by IVIS Spectrum and analyzed by Living Image, version 4.3, software (Perkin Elmer) and distributed randomly to four groups ($n = 5$) on
day 0, followed by intraperitoneally injected with CAR-T cells (1 x 10^6 cells per mouse). Imaging was performed on days 7,14,21,28 to monitor the tumor changes and recorded the survival time of each mouse, followed by drawing the survival curve by GraphPad Prism 8.3.0 software.

Statistical analysis
Statistical analysis was performed using SPSS 23.0 software. Data are showed as mean ± SD. In vitro killing assay, the significance of different groups was determined using nonparametric tests. For the in vivo assay, the Student’t test was used to distinguish the difference between groups. The value for which P < 0.05 was considered significant. The mice survival curve was drawn using GraphPad Prism 8.3.0 software.

Results
Construction of dual-target CAR-T cells by lentiviral vector transduction
According to the above protocol, we designed the PD1-antiMUC16 CAR molecule structure, which comprised PD1-antiMUC16 or PD1 or anti-MUC16 extracellular scFv fragment, a hinge region, a transmembrane domain, followed by intracellular 41BB co-stimulation domain and CD3ζ domain. Besides, the scFv fragment contained three parts, PD1 ecto, 4H11-VH (heavy chain), and 4H11-VL (light chain), these three parts were connected by the linker peptide (Gly4Ser)3 and constructed the 1932 bp dual-target CAR molecule (Fig. 1a). Through gene recombination, CAR molecule sequences combined with the 9367 bp lentiviral vector, which were digested by two enzymes (EcoR I and Mlu I )(Fig. 1b). Tested by agarose gel electrophoresis, PD1, anti-MUC16, PD1-antiMUC16, and plasmid skeleton fragment bands were observed at 510 bp, 1422 bp, 1932 bp, and 7435 bp respectively(Fig. 2a). Meanwhile, the sequence of the positive samples was analyzed and affirmed entirely consistent with the designed one (Fig. 2b). The plasmids were transferred into 293T cells to package lentivirus by polyetherimide (PEI) transfection. Anti-MUC16 and PD1 antigen were combined with 10 ul FITC-Protein L antibody and 5 ul Percp-cy5.5 antihuman PD-1 antibody, respectively, followed by detecting the positive rate of CARs through flow cytometry. Thereout, we harvested the favorable rates of PD1-antiMUC16 CAR, PD1 CAR, and antiMUC16 CAR on the surface of 293T cells were 10.45%, 3.56%, and 18.54%, respectively (Fig. 3), and obtained respectively three viral titers 6.27 x 10^7 TU/ml, 2.14 x 10^7 TU/ml, and 1.11 x 10^8 TU/ml from the formula(1). After
utilizing the same detection methods, the infection rates of PD1-antiMUC16 CAR-T cells, PD1 CAR-T cells, and anti-MUC16 CAR-T cells can be obtained (52.36%, 46.03%, and 86.24%, respectively) (Fig. 3). It is indicated that CAR-T cells with single- and dual-targets were successfully constructed.

Overexpressing MUC16 and PDL1 antigens of target cells

According to the above principles and methods, including PCR, genic recombination, lentiviral vector transduction, we acquired the positive rate of PDL1 molecule on the surface of the LentiX-293T cell was 4.40% by flow cytometry after combining with 5 ul Percp-cy5.5 antihuman PD-1 antibody for 30 minutes in the dark. The lentiviral titer was $2.64 \times 10^7$ TU/ml, according to the previous formula (1). After that, the infection rate of the PDL1 structure on OVCAR3-luc cell was acquired (40.72%). The population that consists of OVCAR3-PDL1-luc cells and unstructured cells was named a pool. The positive samples were cultured and proliferated, followed by purifying to 100%(No.3 monoclonal sample)(Fig. 4a). Meanwhile, the lentiviral titer of MUC16 in the MUC16 group was $1.24 \times 10^8$ TU/ml, the positive rate of MUC16-GFP in the OVCAR3-MUC16-GFP-luc pool which was selected based on the expression GFP was 94.70%, and the MUC16 monoclonal sample purified to 99.93% (Fig. 4b). Moreover, we created MUC16-PDL1 antigen via structuring the PDL1 directly on the monoclonal sample of MUC16. The lentiviral titer of PDL1 in MUC16-PDL1 group was $2.52 \times 10^7$TU/ml, the positive rate of PDL1 on OVCAR3-MCU16-GFP-luc pool was 84.68%, and the PDL1 depurated to 99.75% (Fig. 4c). OVCAR3-luc cell lines overexpressing MUC16 and PDL1 antigens were successfully constructed, and the positive rate of tumor cell surface antigen was more than 99%.

Functional activity of CAR-T cells in vitro

To ascertain the cytotoxicity of CAR-T cells against MUC16 or PDL1 positive cancer cells in vitro, we co-cultured CAR-T cells and cancer cells ($1 \times 10^4$cells/well) at 1:1, 4:1, 8:1, 16:1 ratio. We performed 4 hours of killing assay of PD1-antiMUC16, PD1, and anti-MUC16 CAR-T cells on various target cells. The assay displayed that the dual-target CAR-T cells exhibited more potent cytotoxicity than control T cells against any target cell (P < 0.05), and the capacity enhanced with the increase of E/T ratio (P < 0.05). The killing rates of PD1-antiMUC16 CAR-T cell on OVCAR3-MUC16-GFP-PDL1-luc cells was $[12.03 \pm 1.98\%], [38.29 \pm 0.13\%], [65.16 \pm 0.95\%], [84.96 \pm 0.53\%]$ at 1:1, 4:1, 8:1, and 16:1 E/T ratios, respectively. Comparatively, the killing rates of the control T cells was $[3.90 \pm 2.76\%], [9.74 \pm 0.13\%], [12.20 \pm 0.95\%], [17.56 \pm 0.75\%]$, respectively. Meanwhile, single-target CAR-T
cells, PD1 and antiMUC16 CAR-T remained almost the same cytotoxicity efficacy with dual CAR-T cell (Fig. 5), except in specified assay. In the CAR-T versus OVCAR3-luc cell assay, PD1 CAR-T cell exhibited outstanding cytotoxicity efficacy on target cells than other CAR-T cells(P < 0.05)(Fig. 5a). In the other CAR-T versus target cells assay, the dual CAR-T cells always demonstrated not debased cytotoxicity efficacy than single CAR-T cells.

In cytokine release test, to assess whether CAR structure enhanced the anti-tumor activity of T cells, co-cultures were established between CAR-T cells and target cells at 1:1 ratio (1 × 10⁴ T cells versus 1 × 10⁴ cancer cells) in a V-bottomed 96-well plate for 48 hours in incubator. The results revealed all CAR-T cells exerted a more robust capacity of secreting IL-2, IFN-γ, and TNF-α (Fig. 6), which harvested from supernatants and measured by ELISA kits. Disappointingly, dual CAR-T cells did not reveal higher levels of cytokines production than single CAR-T cells.

**Functional activity of CAR-T cells in vivo**

In order to determine the efficacy of CAR-T cells against ovarian cancer cells in vivo, we established intraperitoneal tumor-bearing models using NPG mice, which were injected OVCAR3-MUC16-GFP-PDL1-luc cells(5 × 10⁵ cells) and 50 ul Matrigel into the abdominal cavity and raised 48 hours. As shown in Fig. 7a, all mice appeared well-distributed and stable size tumors, and measured fluorescence values by IVIS Spectrum and analyzed by Living Image as the pre-therapeutic evidence of tumor.

Tumor-bearing mice models were randomized into four groups (n = 5) and injected CAR-T cells (1 × 10⁶ cells) into the abdominal cavity on day 0, after that measured fluorescence values weekly to monitor the progress of the tumor. Miraculously, dual CAR-T exhibited significant regression of ovarian cells as detected on day 7 to day 14 (Fig. 7bc), followed by slow proliferation. However, two single CAR-T groups did not show the excellent therapeutic effect as dual CAR-T cell instead of restraining the rapid progress of the tumor. From all models, we could discover that the first week after injecting CAR-T cells exhibited the best treatment efficiency(Fig. 7d). It could help set the dose and frequency of subsequent clinical trials.

As time passed, all four groups of mice died for ovarian cancer; however, their tumor-bearing survival time was different. Dual CAR-T group demonstrated exceptionally longer survival time of mice than single CAR-T groups and control group, their mean survival time unexpectedly reached 80.6 ± 10.33days, compared with two single CAR-T groups, PD1 CAR-T group was 45.2 ± 6.34 days, and antiMUC16 CAR-T group was 23.0 ± 1.55 days, and the control group had the shortest survival time, only 19.8 ± 2.14 days (Fig. 7e). From the perspective of extending the
lifetime of tumor-bearing mice, the dual CAR-T group demonstrated the further capacity of prolonging the survival time of mice than others (P < 0.01).

Discussion
The aggressive ovarian cancer, exceptionally high-grade serous carcinoma, should be deemed an urgent unsolved problem in the 21st century in the field of malignancies, because of the low five-year survival time, the rapidly invasive progression and high recurrence rate. CAR-T technology has exhibited practical antitumor activities in hematologic malignancies, also showed potential in the treatment of OC. However, the paucity of specific antigens and the immune escape of OC is the primary obstacle.

In our study, for increasing the target specificity, reducing immune escape, we adopted a tandem structure to design CAR molecule for two antigenic targets using second-generation CAR-T design conception. Meaningfully, the results verified both two CARs played antitumor activity rather than interacting with each other, which may be caused by the hinge domain supplying space for scFv folding. MUC16 and PDL1 are undoubtedly ideal target antigens for CAR-T technology against OC in our study. Additionally, both dual CAR-T cells and single CAR-T cells have favorable cytotoxic efficiency against various devised OVCAR-3 cells in vitro, especially at a high E/T ratio. However, the destructive effect of dual CAR-T cells is not superior to that of the single CAR-T cells. Although there is no apparent difference between the dual CAR-T cell and single CAR-T cell in cytotoxicity and cytokines production in vitro, dual CAR-T demonstrated remarkable tumor therapeutic effect in vivo and prolonged survival time of tumor-bearing mice models than single CAR-T cells.

Why are the dual CAR-T cells exhibited disparity in vivo and in vitro? We assume that the critical point is that PD1, which recognizes target antigen correlated with the tumor microenvironment, hard to exert the highest capacity in vitro. This conjecture was verified in the assay. PD1 CAR-T exhibited potent cytotoxicity in mice models and significantly prolonged the survival rather than the malaise performance in vitro, especially against OVCAR3-PDL1-luc cells and OVCAR3-MUC16-GFP-PDL1-luc cells. The results demonstrated that the surrounding environment showed a significant impact on the attack capability of CAR-T cells against target cells. This finding was due to the design of experimental and control groups, PD1-antiMUC16 CAR-T were constructed as the experimental group, PD1 CAR-T and anti-MUC16 CAR-T were used as the positive control, and control T without any CAR structure was used as the negative control. Meanwhile, due to the natural deficiency of PDL1 instead of
induced expression by activated T cells, we established a variety of tumor cells, OVCAR-3 cells, OVCAR3-PDL1-luc cell, OVCAR3-MUC16-GFP-luc cell, and OVCAR3-PDL1-MUC16-GFP-luc cells for better simulate antigenic expression in OC patients.

Nonetheless, there is still some unknown knowledge left, such as how to reduce the CRS, improve homing, and keep persistently in OC patients. Previously, some researchers have proposed that selecting target antigens with high specificity, optimizing the function of CAR-T cells, and blocking immunosuppressive molecules, such as PD1/PDL1 signal, could reduce the occurrence of CRS [28–32]. Moreover, combining higher specific CAR-T cells with chemokine receptors may improve homing and enhance therapeutic activity[5]. Nevertheless, it still needs to be verified by more cellular experiments, animal assay, even clinical trials. Simultaneously, natural killer (NK) cells may be another right candidate for CAR drivers because they can recognize and killing tumor cells directly[33].

**Conclusion**

We demonstrated that PD1-antiMUC16 dual-target, and single-target CAR-T cells possess cytotoxicity against OVCAR-3 cell line expressing PDL1 and MUC16 antigens and induce cytokines release in vitro. Dual CAR-T cells verify an excellent therapeutic effect on OVCAR3-MUC16-GFP-PDL1-luc tumor-bearing mice and significantly prolong their survival time. Single CAR-T cells inhibit tumor cell proliferation in tumor-bearing models and prolong the survival time too. Thus, PD1-antiMUC16 CAR-T cells have a therapeutic effect on OC and can be used in subsequent clinical trials.

**Abbreviations**

EOC: Epithelial ovarian cancer; OC: Ovarian cancer; CAR-T: Chimeric antigen receptor T; IL-2: Interleukin-2; IFN-γ: Interferon-γ; TNF-α: Tumor necrosis factor-α; CRS: Cytokine release syndrome; MUC16: Mucin 16; PD1: Programmed cell death-1; PDL1: Programmed cell death-ligand 1; CTLA-4: Cytotoxic T lymphocyte-associated antigen-4; UBMC: Umbilical blood mononuclear cell; FBS: Fetal bovine serum; scFv: Single chain antibody fragment; FDA: Food and Drug Administration; PBS: Phosphate buffer saline; PEI: Polyetherimide; GFP: Green fluorescent protein; SPF: Specific pathogen-free.

**Declarations**

**Ethics approval and consent to participate**

This study was approved by the ethics committee of Capital Medical University (2018-KY-026-01). Healthy donors
were not required to give informed consent to the study because they were all anonymous donors.

**Consent for publication**

Not applicable.

**Availability of data and material**

Data supporting the results in the article are available from the corresponding author upon reasonable request.

**Competing interests**

The authors declare that they have no conflict of interest.

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**Authors’ Contributions**

Conceptualization and statistics by TL, Methodology and design of experiment by JDW; Review and editing by JDW and TL; Project Administration by JDW. All authors read and approved the final manuscript.

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Supplementary Files

Additional file 1: Figure S1. Full-length gels of dual-target CAR.

a, 1KB marker was used as a standard marker. anti-MUC16 F fragment and anti-MUC16 R fragment were utilized for constructing the anti-MUC16 fragment. PD1-M was performed as a mock form of PD1. The left side of the clipping line was Figure 2. The base length of anti-MUC16 F, anti-MUC16 R, PD1-M, and PD1 was 813bp, 510bp, 700bp, and 510bp, respectively. b, All bands were anti-MUC16 fragments and the length about 1500bp. The two bands on the left, more evident than the others, were displayed for subsequent experiments. c, The 8000bp band and 10000bp band in standard bands were not wholly distinguished. Mock marked a 7000bp band. PLV-PD1-antiMUC16 plasmid consisted of a dual CAR structure with a 2000bp band and a base skeleton with 7435bp. d, After amplifying in bacterial solution, PD1-antiMUC16 was measured by agarose gel electrophoresis. Mock marked 1500bp band. All images of gel were performed by DNA sequence analysis of
Figure 1

Construction of dual CAR molecule and PD1-antiMUC16 plasmid. a, Schematic diagram of dual-target CAR molecule transgene. PD1-antiMUC16 CAR structure shows the five parts, extracellular PD1-
antiMUC16 scFv, a CD8a hinge, a transmembrane region, 41BB co-stimulation domain, and CD3ζ domain, among the structure antiMUC16 that consist of 4H11-VH and 4H11-VL. b, Schematic diagram of dual-target CAR expression vector. PD1-antiMUC16 plasmid structure shows base sequence sites and the sites of enzyme digestion.

Figure 2

Detection results of PD1-antiMUC16 CAR by agarose gel electrophoresis and sequencing. a, CARs detected by agarose gel electrophoresis, M represents 1Kb marker, the right bands of PD1, anti-MUC16, and PD1-antiMUC16 CARs were circled in red. b, The base sequence of the PD1-antiMUC16 CAR structure is quite correct. The full-length gels are presented in Supplemental Figure S1.
Detection results of the positive rate of single and dual CARs on T cells. a, The testing results of viral titer and infection rate of anti-MUC16 CAR-T cell. AntiMUC16 CAR-T cells stained with 10ul FITC-Protein L antibody for 30 minutes in the dark, followed by detecting via flow cytometry. The green peak is the control group, and the red peak is the experimental group. b, The detecting results of viral titer and infection rate of PD1 CAR-T cell. PD1 CAR-T cells stained with 5ul Percp-cy5.5 antihuman PD-1 antibody for 30 minutes in the dark, followed by detecting via flow cytometry. c, The testing results of viral titer and infection rate of PD1-antiMUC16 CAR-T cell. Control T showed both negative results stained with two antibodies. However, PD1-antiMUC16 CAR-T showed both positive results with two antibodies.
Detection results of target cells overexpressing PDL1 and(or) MUC16. a, Detection results of PDL1 antigen on OVCAR3-PDL1-luc cells. OVCAR3-PDL1-luc cells stained with 70μl Steady-Glo for 20 minutes in the dark. b, Detection results of MUC16 antigen on OVCAR3-MUC16-GFP-luc cells. GFP is used to mark the MUC16. c, Detection results of MUC16 and PDL1 antigens on OVCAR3-MUC16-GFP-PDL1-luc cells. OVCAR3-MUC16-GFP-PDL1-luc cells stained with 70μl Steady-Glo as the above methods. Control groups are the green peak; Experimental groups are the red peak. The top pictures show the titer test results, and the bottom two show the cell pools and the monoclonal samples.
Figure 5

The antitumor activity results in single and dual CAR-T cells against various target cells. There are four kinds of tumor cells expressing PDL1 and(or) MUC16 antigen or not, and four different T cells expressing PDL1 and(or) antiMUC16 or not. The CAR-T cells co-cultured for 4 hours with target cells (1×10^4) at E/T of 1:1, 4:1, 8:1, and 16:1 in a total volume of 100ul, after that stained with 70ul Steady-Glo for 20 minutes and detected by flow cytometry. Data show the mean±SD, and the results were analyzed with the nonparametric test. Error bars represent the SD. ns: P>0.05, *: p<0.05.
Three cytokines production by effector cells in response to various OVCAR-3 cell lines. Four effector cells (PD1-antiMUC16 CAR-T cells, PD1 CAR-T cells, antiMUC16 CAR-T cells and T cells without CAR molecule) co-cultured with four kinds of OVCAR-3 cells (OVCAR3-luc cells, OVCAR3-MUC16-GFP-luc cells, OVCAR3-PDL1-luc cells, and OVCAR3-MUC16-GFP-PDL1-luc cells) at 1:1 ratio for 48 hours to explore the different secretion capacities of IL-2, IFN-γ and TNF-α between four T cells. These cytokines tested by ELISA kits. Only T: without target cells, O: OVCAR3-luc cell, O-M: OVCAR3-MUC16-GFP-luc cell, O-P: OVCAR3-PDL1-luc cell, O-M-P: OVCAR3-MUC16-GFP-PDL1-luc cell. The groups covered by horizontal line are compared in pairs. Data show the mean±SD, and the results were analyzed with the nonparametric test. Error bars represent the SD. ns: P>0.05, *: p<0.05.
Figure 7

Therapeutic efficacy of CAR-T cells on the xenograft mice model. a, The Vivo imaging results of OVCAR3-MUC16-GFP-PDL1-luc tumor-bearing mice before treatment. NPG mice were intraperitoneally injected with OVCAR3-MUC16-GFP-PDL1-luc cells (5×10^5) and 50ul Matrigel for 48 hours, followed by measuring via IVIS Spectrum and analyzed by Living Image. b, The Vivo imaging results of tumor progression in mice at four points (day 7, day 14, day 21, and day 28) in time, and were randomized divided into four groups. The black area and empty spaces indicate the mice have died. c, The therapeutic effects of the four T cells are compared at the same point in time. d, The therapeutic effect of each T cell group is compared at different points in time. e, The survival curve of tumor-bearing mice. All groups below the horizontal line have been compared, the two groups pointed by the arrow line are compared. Data show the mean±SD, and the results were analyzed with Student’t test. Error bars represent the SD. The survival curve was drawn by GraphPad Prism 8.3.0 software. ns: P>0.05, *: P<0.05, **: P<0.01.
Supplementary Files

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