Intraperitoneal administration of carcinoembryonic antigen-directed CAR-T cells is a robust delivery route for effective treatment of peritoneal carcinomatosis from colorectal cancer

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Abstract

**Background:** Peritoneal carcinomatosis from colorectal cancer is a highly challenging disease to treat, and as such constitutes a pressing issue in medicine. Adoptive transfer of chimeric antigen receptor-T (CAR-T) cells has shown impressive efficacy in hematologic malignancies, though the approach has been less effective in solid tumors when delivered via intravenous (i.v.) routes. We explored whether intraperitoneal administration of CAR-T cells could provide an effective and robust route for treatment of peritoneal carcinomatosis from colorectal cancer.

**Methods:** To generate carcinoembryonic antigen (CEA)-specific CAR-T cells, we constructed a lentiviral transfer plasmid encoding a second-generation CAR composed of the single-chain variable fragment (scFv) from BW431/26, the CD8 alpha hinge region, 4-1BB co-stimulatory domain, and the CD3zeta T cell activation domain. We established various animal models of peritoneal carcinomatosis with intraperitoneal (i.p.) and extraperitoneal metastasis. Tumor-bearing animals were treated by i.p. or i.v. administration of CEA CAR-T cells. Treatment efficacy was evaluated and kinetic expansion and tissue distribution of CAR-T cells were studied.

**Results:** CEA-directed CAR-T cells showed high tumor cell cytotoxicity in vitro. Intraperitoneally administered CAR-T cells exhibited superior antitumor activity compared to systemic i.v. cell infusion in an animal model of peritoneal carcinomatosis. In addition, i.p. administration conferred a durable effect and protection against tumor recurrence and exerted strong antitumor activity in an animal model of peritoneal carcinomatosis with metastasis in intraperitoneal or extraperitoneal organs. Our data further indicate that when compared to systemic delivery, i.p. transfer of CAR-T cells could provide increased antitumor activity in extraperitoneal tumors without PC. This phenomenon was further confirmed in an animal model of pancreatic carcinoma after intraperitoneal administration of our newly constructed prostate stem cell antigen (PSCA)-directed CAR-T cells. On a mechanistic level, our data evidenced rapid and high CAR-T cell expansion and high persistence in peripheral blood following i.p. cell administration.

**Conclusions:** Intraperitoneal administration of CAR-T cells could be an effective and robust route for therapy of peritoneal carcinomatosis with metastasis in intra- and extraperitoneal organs. These findings hold great promise for CAR-T cell therapy in patients with peritoneal carcinomatosis.

Background

Colorectal cancer (CRC) is the third most frequent cancer worldwide and the second leading cause of cancer-related death (1). The peritoneum is the most common site of colorectal tumor dissemination after the liver (2,3). Approximately 40% of patients with colorectal cancer develop peritoneal carcinomatosis (PC) throughout its natural history (4,5), and up to 10% of PC cases are diagnosed synchronously with the primary tumor (6). This type of metastatic disease has a high mortality rate, with a median survival of 6 months if left untreated (7).

Currently, cytoreductive surgery combined with hyperthermic intraperitoneal chemotherapy (HIPEC) is the standard treatment for selected patients, showing moderate results in overall survival but also high morbidity (5,8). The aim of cytoreductive surgery is to surgically remove all macroscopic disease as measured by an optimal completeness of cytoreduction score; subsequently, HIPEC is administered to eliminate all
microscopic disease (9). Cytoreductive surgery combined with HIPEC has been demonstrated to improve patient survival more than systemic chemotherapy alone, achieving a mean survival of approximately 22-35 months vs 12-17 months, respectively (8,10–12). However, the role of HIPEC has recently become a topic of controversy, as it appears only select patients benefit from this approach (13). Hence, there is an urgent clinical need to develop new strategies and therapeutic approaches to treat this devastating disease, especially in those patients who develop peritoneal metastases that are unfit for surgical resection or in cases of incomplete cytoreduction.

Rapid advances in immunotherapy over recent decades has led to many breakthroughs, including the development of therapy approaches based on chimeric antigen receptor-T cells (CAR-T cells). Most published studies evaluating CAR-T cell therapy have been carried out in hematologic malignancies and with more than surprising efficacy, achieving complete response in 70% to 90% of patients (14–18). In contrast, CAR-T cell based approaches have fallen short of expectations in studies involving solid tumors. Certain key differences between solid and hematologic malignancies explain these uneven results: tumor-associated antigens in solid tumors are more heterogeneous, which complicates the process of antigen selection (19); CAR-T cells have a more limited capacity to migrate to and penetrate solid cancers (20); and lastly, the tumor microenvironment in solid cancers acts as a physical and immunosuppressive barrier, consisting of tumor stroma, immunosuppressive cells and metabolites (21,22).

Carcinoembryonic antigen (CEA) is a glycoprotein related to gastrointestinal cancer used in clinical practice as a sensitive biomarker to identify patients with gastrointestinal cancer, estimate disease severity, and to detect recurrence during follow-up. CEA is not detected in healthy adult tissues except on the luminal side of gastrointestinal cells, and thus remain invisible to immune cells. After neoplastic transformation, however, malignant cells lose this polarity, causing them to express CEA on the entire cell surface, thereby making them an attractive target for CAR-T cell therapy (23,24).

CAR-T cell therapy was first used to target CEA in a clinical trial in patients with adenocarcinoma liver metastasis expressing CEA and was administered through the hepatic artery. Though 5 of 6 patients had progressive disease, the authors confirmed the safety of local delivery and reported presence of CAR-T cells in liver and tumor tissue (25). Another clinical trial, this time using systemic administration of CAR-T cells targeting CEA in patients with metastatic colorectal cancer, found that CAR-T cells are both well-tolerated, even at high dose levels (up to $10^8$ cells/kg), and helped to control the disease. Seven of 10 patients achieved stable disease status after the treatment, 2 of whom remained stable for more than 30 weeks, and another 2 showed tumor shrinkage (26).

Previous studies have demonstrated that locoregional delivery of CAR-T cells achieves better results than systemic administration in terms of tumor reduction and effect duration in tumor-bearing animals and in the clinical setting (27–29). Data such as these have led us to explore whether i.p. administration of CAR-T cells could be a new approach to treat PC from CRC. In this preclinical study, we use tumor models of CEA+ colorectal PC to test the efficacy of intraperitoneally administered CAR-T cells targeting CEA. To confirm the results obtained for the i.p. route, we subsequently evaluated the efficacy of CAR-T cells targeting PSCA in a murine model of PC.
Our results confirm that i.p. infusion of CEA CAR T cells is superior to systemic tail vein (TV) administration for treatment of PC. i.p. administration revealed not only higher tumor reduction in peritoneal metastases, but also a exhibited a greater effect in other intraabdominal metastases and induced tumor regression in extra-abdominal metastases. TV administration, in contrast, was ineffective in controlling tumors. Moreover, the beneficial effect of i.p. administration was found to be durable, protecting the mice against tumor rechallenge. We further demonstrate that i.p. delivery for treatment of extra-abdominal tumors achieves better antitumor effects. These results indicate that CAR-T cell therapy can be initiated in patients with PC.

Materials And Methods

Cell lines

The human colorectal adenocarcinoma cell line DLD-1 (purchased from ATCC) was maintained in RPMI 1640 medium (Gibco) supplemented with 10% fetal bovine serum (ExCell Bio). The human pancreatic adenocarcinoma epithelial cell line HPAC (purchased from ATCC) and BxPC3 cell line (purchased from ATCC) were maintained in DMEM (Gibco) supplemented with 10% fetal bovine serum (ExCell Bio). DLD-1, HPAC, and BxPC-3 cells were transduced using a lentivirus encoding FLuc-F2A-GFP, and GFP-expressing cells were isolated by fluorescence-activated cell sorting (FACS) to obtain DLD-1-Luc-GFP cells, HPAC-Luc-GFP cells, and BxPC-3-Luc-GFP cells. DLD-1-Luc-GFP cells were subsequently infected with lentivirus to overexpress human CEA, and then sorted and confirmed to greater than 95% purity by FACS (FACS Aria III, BD) with anti-human CEA antibodies (14-0669-82; Invitrogen); the resulting cells were named DLD-1-CEA-Luc-GFP. All cells were cultured in a humidified incubator at 37 °C with 5% CO₂. All cell lines were STR-fingerprinted and validated to be mycoplasma-free by PCR.

Lentivirus production

Lentivirus was collected from the supernatants of 293T cells transfected with the lentivirus vector and helper plasmids (PMD2.G, pMDLg/pRRE, and pRSV-Rev) as described above. After harvesting the supernatant, the lentivirus was mixed with 50% PEG and 4M NaCl at a 6:2:1 ratio and centrifuged at 10,000 × g at 4 °C for 1 hour. The supernatant was discarded following centrifugation and the precipitate was dissolved in an appropriate volume of saline. For all experiments related to lentiviral transfection, a multiplicity of infection (MOI) of 2-3 was used.

CAR-T cell manufacturing

Peripheral blood mononuclear cells (PBMCs) were isolated from healthy donor blood by means of apheresis using Lymphocyte Separation Medium (LST1077; TDBscience Tianjin, China). Before lentiviral transduction, PBMCs were activated by CD3/CD28 Dynabeads (40203D; Gibco) for 24h. T cells were transduced in 6-well plates (1 × 10^6 cells per well at 3-5 MOI) using CEA or PSCA CAR lentivirus; polybrene was used to improve transduction efficiency. At day 2 after T cell transduction, the CD3/CD28 Dynabeads were removed and T cells were expanded by RPMI 1640 medium (Gibco) containing 10% FBS (ExCell Bio), recombinant human IL-7 (AF-200-07; peproTech), recombinant human IL-15 (AF-200-15; peproTech), and recombinant human IL-21 (AF-200-21; peproTech) for approximately 8–10 days. T cell purity and positive rate of CAR were measured by FACS with anti-human CD3 (300400; Biolegend) and Protein L (11044-H07E-P; Sino biological) on day 7-8.
**In vitro cytotoxicity assay**

CEA CAR T cells/Control T cells and the targeted tumor cells were co-cultured at the designated effector to target (E:T) cell ratio in RPMI 1640 medium, without phenol red (Gibco, 11835030) with 10% FBS on 96 wells for 24h at 37 °C. After incubation, the medium was discarded and luciferin (150 μg/ml, Promega) was added to the wells. Luminescence values were measured using a microplate reader (Thermofisher Scientific), and the values for luminescence were normalized to the target cells with medium only.

**Luciferase-based Cytotoxic T Lymphocyte (CTL) assay**

In a 96-well U-bottom plate (NEST, USA), CEA CAR-T cells/control T cells and target cells were co-cultured at 37 °C for specified time spans at various E:T ratios, and the cell targets were seeded at a density of 1 × 10^4 cells/well. Supernatants were harvested for cytokine secretion detection following centrifugation of the plate. The substrate was added with the DPPIV-Glo™ Protease Assay (Promega, USA) and immediately centrifuged and detected. Results are reported as the percentage of target-cell killing as indicated by luciferase activity in the wells with tumor cells but without T cells [% killing=100−(RLU from well with effector and target-cell coculture)/(RLU from well with target cells) × 100%].

**ELISA assays**

The culture supernatant collected from CTL assay or blood and ascites fluid collected from mice was stored at -80°C before testing. The Human IFN-γ ELISA Set (555142; BD Biosciences) and Human Interleukin-2 (IL-2) Uncoated ELISA Kit (88-7025-88; Thermofisher Scientific) were used to detect the release of cytokines during the CTL assay. Samples were diluted at an appropriate ratio based on the instruments included in the ELISA kits, and each sample was assayed in duplicate or triplicate. Data analysis was conducted according to the related protocol and algorithm by Varioskan LUX (Thermofisher Scientific). All data were within the range of the calibrated curves.

**In vivo research**

Six-to-eight-week-old Severely Immunodeficient NCG mice (NOD/ShiltJGpt-Prkdc<sup>em26Cd52</sup>Il2rg<sup>em26Cd22</sup>/Gpt) were purchased from Gmpharmatech Co., Ltd. For the DLD-1 i.p. xenograft model, DLD-1-CEA-Luc-GFP cells (1 × 10<sup>6</sup>) were injected intraperitoneally into NCG mice on day -7. For the DLD-1 s.c. and i.p. xenograft model, DLD-CEA-Luc-GFP cells (1 × 10<sup>6</sup>) were subcutaneously injected into NCG mice on day -13, then DLD-1-CEA-Luc-GFP cells (1 × 10<sup>6</sup>) were intraperitoneally injected on day -7. For the DLD-1 liver/spleen orthotopic and i.p. xenograft model, DLD-1-CEA-Luc-GFP cells (1 × 10<sup>6</sup>) were orthotopically injected into the liver/spleen of NCG mice on day -17, and DLD-1-CEA-Luc-GFP cells (1 × 10<sup>6</sup>) were intraperitoneally injected on day -7. CEA CAR-T cells were injected by i.v. or i.p. administration on day 0 (low dose for 1 × 10<sup>7</sup> copies/mouse, high dose for 3 × 10<sup>7</sup> copies/mouse). For the HPAC i.p. xenograft model, HPAC-Luc-GFP cells (1 × 10<sup>6</sup>) were intraperitoneally injected into NCG mice on day -7. Then, PSCA CAR-T cells were injected by i.v. or i.p. administration on day 0 (low dose for 1 × 10<sup>6</sup> copies/mouse, high dose for 3 × 10<sup>6</sup> copies/mouse). In all in vivo experiments, activated untransduced T cells (Control T) were used as controls, and the cell number is equal to the highest dose in the other groups tested. Luminescence imaging was performed weekly using the Spectrum in vivo imaging system (IVIS, PerkinElmer) 15 min after i.p. injection of 150 mg/kg D-luciferin, sodium salt (Gold...
Biotechnology). Subcutaneous (s.c.) tumors were measured twice weekly by Vernier caliper. Tumor volume was calculated as follows: \( V(\text{mm}^3) = \frac{[(\text{width})^2 \times (\text{length})]}{2} \).

**Tissue collection and RT-PCR analysis**

For continuous detection of CAR-T cells *in vivo*, blood samples were collected by submandibular bleeding weekly, DNA was extracted with the QIAamp DNA Blood Mini Kit (QIAGEN) then detected by qPCR. For endpoint detection of CAR-T, mice were euthanized, after which we harvested the heart, liver, spleen, lung, kidney, stomach, small intestine, large intestine, mesentery, gonad, brain, skeletal muscle, tumor, bone marrow, and ascites, and then used these for DNA extraction. A TaqMan primer set of forward primer 5'-CAGAAGAAGGAAGGAGGATGTG-3', reverse primer 5'-TACTCTCTCTCGTCTAGATTG-3', and probe 5'-FAM-CTGAGAGTGAAGTTC-3' was used for CAR copy-number detection. The TaqMan method was performed in accordance with the published protocol (10) in a QuantStudio Real-Time PCR System (Thermofisher Scientific).

**Statistical analysis**

All statistical analyses were performed using IBM SPSS Statistics software version 25. Data are presented as means ± SD or SEM as stated in the figure legends. Results were analyzed by unpaired Student’s t test (two-tailed), one-way ANOVA with Tukey’s correction for multiple comparisons, or ANOVA for repeated measurement, where applicable. Significant differences are marked on figure legends as *P<0.05, **P<0.01. Survival curves were analyzed with the log-rank test. Statistical significance was defined as \( P < 0.05 \).

**Results**

**CEA-directed CAR-T cells present effective cytotoxicity *in vitro***

To generate CEA-specific CAR-T cells, we constructed a lentiviral transfer plasmid encoding a second-generation CAR composed of the single-chain variable fragment (scFv) from BW431/26, the CD8 alpha hinge region, 4-1BB co-stimulatory domain, and the CD3zeta T cell activation domain (Figure 1A). CEA CAR lentivirus was used to transduce human healthy donor-derived PBMCs and obtain CEA CAR-T cells, as described above. DLD-1 human colon-cancer cells were first transduced with a lentivirus expressing GFP and luciferase (DLD1-Luc-GFP) for sorting and *in vivo* imaging (Figure 1B). As this cell line had very low surface expression of CEA, we transduced another lentivirus expressing CEA to generate DLD1-CEA-Luc-GFP cells. The CEA-positive cells were purified and confirmed to have over 99% purity by FACS (Figure 1B). The CEA CAR-T cells were then added to either the DLD-1-CEA-Luc-GFP cells or the control DLD1-Luc-GFP cells at different E:T ratios to evaluate tumor cytotoxicity. CEA CAR-T cells displayed high lytic action toward CEA-positive DLD1-CEA-Luc-GFP cells compared to the untransduced control T cells (Control T) at all E:T ratios tested (Figure 1C). In contrast, the CEA CAR-T cells displayed limited lysis of DLD1-Luc-GFP, which was indistinguishable from that of the control T cells (Figure 1C). CEA CAR-T cells also produced higher IFN-γ and IL-2 against DLD1-CEA-Luc-GFP cells than control T cells, but not against DLD1-Luc-GFP cells (Figure 1D).

**Intraperitoneal administration of CAR-T cells exhibits superior antitumor activity compared with systemic i.v. infusion.**
To evaluate the efficacy of CAR-T cells in targeting CEA in mice with PC from CRC, we first established a PC xenograft model by i.p. injection of DLD1-CEA-Luc-GFP colon cells into NCG mice 7 days before treatment (-7 days). On the day before CEA CAR-T cell treatment (-1d), tumor burden was checked by bioluminescence to verify the success of the tumor model, after which the mice were randomly allocated to different groups based on the bioluminescence values. Thereafter, CEA CAR-T cells were either administered intraperitoneally or systemically through the TV at a high dose of 3 $\times$ 10$^7$ copies/mouse or a low dose of 1 $\times$ 10$^7$ copies/mouse, and the mice underwent weekly bioluminescence imaging to monitor variations in tumor burden after treatments. The entire process is depicted schematically in Figure 2A.

Firstly, we observed that either a single dose of i.p. or i.v. CAR-T cells was capable of eliminating the tumor cells. Importantly, i.p. injection exhibited more effective antitumor activity (P<0.01) (Figure 2B). Mice receiving high i.p. or i.v. doses of CAR-T cells experienced greater tumor reduction compared to low doses. Additionally, high doses of i.p. CAR-T cells were related with a more powerful response (P <0.01), which resulted in no detectable tumor by bioluminescence on day 13. This response was evident not only on first evaluation (day 13), but also 76 days after treatment. Tumor relapse was present in 25% of the high-i.v.-dose group on day 27 but in none of the mice receiving a high dose intraperitoneally. All control group mice died on day 34, but 75% of mice receiving either a low i.p. dose, a high i.p. dose, or a high i.v. dose survived with no tumor burden (Figure 2C and 2D).

**Intraperitoneal administration confers a durable effect and protection against tumor recurrence**

After evaluating i.p. and i.v. delivery and having demonstrated the superiority of local delivery, we next studied the duration of the CAR-T cell antitumor effect when administered intraperitoneally and the activity of this treatment route against tumor rechallenge. Animals of the previous experiment persisted with complete tumor regression until 76 days after i.p. and i.v. CEA CAR-T cells treatment. At this time point, tumor-free animals were rechallenged with an i.p. tumor injection of 1 $\times$ 10$^6$ DLD-1-CEA-Luc-GFP cells. Tumor regression was detected after rechallenge and a complete response was observed in all mice on day 91 (14 days following rechallenge) (Figure S1). Bioluminescence imaging detected no presence of tumor until day 105, at which point we rechallenged each mouse again using a stronger tumor burden by i.p. administration of 5 $\times$ 10$^6$ DLD-1-CEA-Luc-GFP cells and SC 1 $\times$ 10$^6$ DLD-1-CEA-Luc-GFP cells. Tumor growth was higher after this second rechallenge: on this occasion, the mice also showed tumor regression, though to a lesser degree, and on day 127, residual disease was detected in both treated groups (Figure S1). However, more effective antitumor activity was again found in the animals that received a high i.p. dose of CAR-T cells.

**Locally administered CAR-T cells exhibit effective elimination of peritoneal carcinomatosis and intraabdominal metastasis**

To confirm that i.p. administration of CAR-T cells exerted an effect in other intraabdominal tumor locations, we generated an orthotopic xenograft model of liver/spleen tumors with PC. Spleen and liver tumors were developed by direct injection of tumor cells into the corresponding organs. In each group, mice were classified into groups receiving control T cells, i.p. administration of CEA CAR-T cells, or i.v. CEA CAR-T cells (Figure 3A). In mice with PC and orthotopic tumors of the spleen (Figure 3B), i.p. delivery showed significant tumor reduction compared to the systemic administration and control T cell groups. Complete tumor regression was observed only in the tumor-bearing animals treated by i.p. CEA CAR-T cells (Figure 3C and 3D). This reduction
was visible on day 15, and bioluminescence imaging evidenced no tumors in the i.p. group beginning on day 22 after infusion and lasting until day 64. CEA CAR-T cell expansion in blood was significantly higher with i.p. infusion than systemic administration (Figure 3E). To detect the distribution of CAR-T cells, mice were sacrificed on day 68, and tissues/organs were collected for CAR gene copy number testing. Liver, spleen, lung, ascites, and blood showed significantly higher copies of the CAR gene in the i.p.-delivery group. Meanwhile, the number of CAR gene copies could be detected in the remaining tumors from the i.v. administration group due to ongoing stimulation of CAR-T cells in this group. No tumors were observed in the i.p.-administration group (Figure 3F). Likewise, mice with PC and liver tumor that received i.p. infusion of CEA CAR-T cells exhibited greater tumor reduction, and no visible tumor was detected by bioluminescence on day 15; furthermore, these animals remained tumor-free until the final evaluation on day 43. i.p. administration showed a greater number of CAR-T cell copies in peripheral blood (data not shown). Taken together, these data indicate that i.p. administration of CEA CAR-T cells not only demonstrated potent antitumor activity against PC, but also against PC with metastasis in peritoneal organs.

**Regional infusion confers protection against extraperitoneal tumors**

Since patients can be diagnosed with both PC and extraperitoneal metastasis, we set out to evaluate the impact of i.p. CAR-T cell infusion and its effect on extraperitoneal tumors. We thus created a mouse model of PC associated with subcutaneous flank tumor. For the s.c. and i.p. xenograft model, DLD-1-CEA-Luc-GFP cells ($1 \times 10^6$) were injected subcutaneously and intraperitoneally. Afterward, the mice were treated with CEA CAR-T cells (Figure 4A). CAR-T cell therapy showed an antitumor effect, while the tumors continued to grow in the control group. Once again, a comparison of i.v. administration and i.p. regional infusion revealed that the latter was associated with increased tumor reduction, not only in peritoneal xenograft tumors (Figure 4B and 4C), but also in s.c. tumor implants (Figure 4D and 4E). Similarly, the volume of the s.c. tumors was significantly reduced in the i.p. group compared to animals receiving i.v. administration (Figure 4F). This result therefore indicates that regional i.p. infusion contributes potent protection against distal extraperitoneal tumors.

**Dynamic kinetics of CAR-T cell expansion and distribution in tumor-bearing animals after i.p. treatment administration**

To explore whether CAR-T cells could be expanded by i.p. administration, we designed a tumor model of PC and extraperitoneal metastasis and treated tumor-bearing animals using i.p. or i.v. CAR-T cell administration. Our data evidenced a rapid and substantial expansion of CAR-T cells in peripheral blood resulting from i.p. administration. The peak level of CAR-T cells was found at day 22 and persistence of CAR-T cells at moderate levels could be detected as of day 42 (the end of experiment) (Figure 5A). In contrast, we observed a delayed and much more modest CAR-T cell expansion in tumor-bearing animals receiving i.v. administration (Figure 5A). We next investigated the organ distribution of CAR-T cells in tumor-bearing animals receiving i.p. and i.v. CAR-T cell administration. Low levels of CAR-T cells were observed in ascites as well as the mesentery and stomach at day 1 after i.p. treatment. The highest level of cells was detected in ascites, peripheral blood, mesentery, spleen, lung, liver, and stomach at day 7 and gradually decreased at day 14 and 42. However, the peak level of CAR-T cells was observed in s.c. tumors at day 14 and 42. In contrast, low levels of CAR-T cells were observed in peripheral blood, lung, spleen, and liver at day 1 and day 7 after i.v. treatment. A gradual
increase in CAR-T cells was seen in peripheral blood, lung, spleen, liver, ascites, mesentery, and stomach at day 14 and day 42 after i.v. treatment (Figure 5B).

In this experiment, we also measured the levels of IFN-γ, an indicator of CAR-T cell activity, in ascites and in blood. Initially, i.p. administration of CAR-T cells showed higher levels of INF-γ in ascites compared to systemic administration. On day 14, however, i.p. administration was associated with lower levels of INF-γ in ascites as compared to systemic administration, which coincided with a low peritoneal tumor burden. On the other hand, i.p. administration was associated with higher levels of IFN-γ in blood at all time points, showing a significant rise on day 14, when we observed a 7-fold increase coinciding with antitumor activity (Figure 5C and 5D).

**Intraperitoneal CAR-T cell administration provides greater extraperitoneal antitumor activity in a non-PC model**

All data reported above demonstrate that i.p. delivery of CAR-T cells in tumor models with PC and intra- or extraperitoneal metastasis achieved better antitumor efficacy than systemic i.v. delivery. This raised the question as to whether this could be the result of instant activation of CAR-T cells by direct contact with target tumor cells. Therefore, we compared the performance of regional i.p. vs. systemic i.v. delivery of CAR-T cells against a distal non-PC tumor. A mouse model of SC tumors with no intraabdominal malignancy was then established by injecting $1.0 \times 10^6$ DLD-1-CEA-Luc-GFP tumor cells subcutaneously followed by i.p. or systemic CAR-T cell infusion ($2.81 \times 10^7$ copies/mouse) of CEA CAR-T cells as treatment (Figure 6A). Interestingly, i.p. delivery significantly outperformed systemic administration once again. After CAR-T cell administration, the i.p. group first showed progressive tumor reduction on day 13 until the final evaluation on day 41, when no macroscopic tumor was detected by bioluminescence imaging (Figure 6B and 6C); this reduction was consistent with the tumor growth curve (Figure 6D). In contrast, macroscopic tumor masses were identified in 5 of 6 mice in the systemic therapy group (Figure 6B to 6D). Meanwhile, we measured CAR-T cell expansion and distribution in this same experiment. Our data showed that the number of CAR gene copies detected in the i.p. delivery group was significantly higher in peripheral blood, spleen, lung, ascites, liver, and mesentery than the i.v. group at the end point of the experiment (Figure 6E), which coincided with the higher degree of antitumor efficacy. This study indicated that i.p. administration of CAR-T cells may provide a better route of CAR-T cell delivery for treatment of extraperitoneal as well as peritoneal tumors.

**Intraperitoneal delivery is superior in different types of CAR-T cells compared to IV administration**

Having demonstrated that i.p. CAR-T cell delivery targeting CEA achieves greater tumor reduction, we proceeded to investigate whether this was also the case for other CAR-T cells targeting different antigens as well as different kinds of tumors. To vary the type of malignancy, we chose pancreatic carcinoma, widely known to be insensitive to immunotherapy and the most difficult tumor to treat. The alternate cell type was prostate stem cell antigen (PSCA) CAR-T cells, which have the same structure as CEA CAR-T cells (see Figure S2A). To test the in vitro cytotoxicity of these cells, we generated a PSCA-positive human pancreatic acinar cell carcinoma cell line labeled with the luciferase and GFP reporter gene (HPAC-Luc-GFP), expressing 99.5% of PSCA and 98.93% of GFP (Figure S2B). As expected, PSCA CAR-T cells elicited antigen-dependent cytokine production and tumor cell killing in vitro (Figure S2C and 2D).

To evaluate the efficacy of CAR-T cells in targeting CEA in mice with PC from pancreatic carcinoma, we established a xenograft model of PC by injecting HPAC-Luc-GFP cells intraperitoneally into NCG mice 7 days
before treatment (-7 days). On day 0, PSCA CAR-T cells were administered either systemically or regionally at high (3 × 10^7 copies/mouse) or low doses (1 × 10^7 copies/mouse), and the mice underwent weekly bioluminescence imaging to monitor variations in tumor burden after treatments (Figure 7A). Our data showed that i.p. PSCA CAR-T cell administration remained more efficacious than systemic administration in terms of its capacity to eliminate tumor cells. Moreover, i.p. administration exhibited an earlier onset of antitumor activity (Figure 7B to 7D). This study indicates that i.p. administration of CAR-T cells may have different therapeutic indications.

**Preclinical safety evaluations show that intraperitoneal administration is a safe route for treatment of cancer with CAR-T cells**

The most common toxicity associated with the use of CAR-T cells in clinical contexts is cytokine release syndrome (CRS). As shown earlier in the present section, i.p. administration outperformed i.v. delivery in antitumor efficacy. However, mice in the i.p. group also presented much greater cell amplification and cytokine release (Figure 5). To confirm the safety of i.p. administration, we relied on third-party preclinical safety studies for both i.v. and i.p implants. For the i.v. group, a SC xenograft NOG mouse model was used by implanting DLD1-CEA tumor cells, and 7 days after that CAR-T cells were administered intraperitoneally at either a low dose (1.33 × 10^8 total T cells/kg or 4.75 × 10^8 copies/kg) or a high dose (6.67 × 10^8 total T cells/kg or 2.38 × 10^9 copies/kg). Given the enhanced antitumor efficacy of the i.p. tumor implant, we reduced the cell doses to 5.04 × 10^7 total T cells/kg or 1.5 × 10^8 copies/kg for low doses, and 5.04 × 10^8 total T cells/kg or 1.5 × 10^9 copies/kg for high dose levels, respectively. CAR-T cells were implanted by i.p. injection into an i.p./s.c. xenograft NOG mice model containing DLD1-CEA tumor cells, which resulted in changes related to tumor growth and spread as well as antitumor effects (e.g., increased IFN-γ production); independently of the implant procedure used, we observed mononuclear cell infiltration of multiple organs and increased granulocyte cellularity in bone marrow (femur and sternum), which was primarily caused by graft-versus-host-disease (GVHD). There were no test article-related toxicities or body weight changes in either study (data not shown). In contrast with earlier findings, there were no differences in IFN-γ secretion in blood between i.p. and systemic i.v. administration, which may have been the result of the cell-dose reduction in the i.p. group (Figure 8A).

Importantly, despite the lower cell dose implanted, we detected a higher number of CD3^+ cells and CAR gene copies in the blood of animals in the i.p. group as determined by FACS and RT-PCR (Figure 8B and 8C). There were no differences observed in the amount of other cytokines (e.g., IL-2, IL-4) secreted in blood between i.p. and i.v. administration. We found significantly increased percentages of lymphocytes (Lymph) and monocytes (Mono) in blood, and an equivalent reduction in the percentage of neutrophils (Neut), eosinophils (Eos), and basophils (Baso) with i.p. administration compared to systemic i.v. administration, thereby suggesting superior antitumor effects when the i.p. route is used (Figure 8D).

**Discussion**

The peritoneum is a frequent site of colorectal tumor dissemination. Forty percent of patients with CRC develop PC over the course of the disease (4,5). Research suggests that PC is an end stage of CRC, and the survival rate among patients with this condition did not reach 6 months without treatment, whereas systemic chemotherapy was associated with an overall survival of 12-17 months (7). The most widely accepted treatment for PC from CRC, cytoreductive surgery + HIPEC, leads to improved prognosis as evidenced by an overall survival rate of 22-
However, many patients are not candidates for surgery due to systemic metastases or inoperable peritoneal metastases. Therefore, new therapeutic approaches must be developed for treating PC.

Current advances in immunotherapy have expanded the application of CAR-T cell therapy as a treatment approach, particularly in hematologic malignancies, where patients with refractory or relapsed disease may achieve complete remission (18). In light of the surprising clinical success of CAR-T cells in targeting CD19 in liquid tumors, investigations have been conducted to explore the effect of these cells in solid tumors. Multiple targets are currently being investigated for CAR-T cell therapy in solid tumors, such as human epidermal growth factor receptor 2 (HER-2), carcinoembryonic antigen (CEA), disialoganglioside 2 (GD2), inerleukin-13 receptor alpha 2 (IL-13Ra2), epidermal growth factor receptor, carbonic-anhydrase-IX (CAIX), MUC, mesothelin, and prostate-specific membrane antigen, and CD133, among others (25,26,30–36).

However, the use of CAR-T cells in solid tumors has not lived up to expectations (20,21) due to the presence of certain barriers that liquid tumors do not encounter. One of the factors limiting their applicability is the low capacity of CAR-T cells to migrate to and penetrate the tumor. The other obstacle is related to the tumor microenvironment, which consists of an immunosuppressive barrier and a physical barrier, which enables the tumor to escape the effects of the immune system and other medical therapies (19–22).

Local or regional delivery of CAR-T cell immunotherapy can overcome difficulties related to migration by enhancing localized antitumor activity and reducing the risk of adverse effects such as systemic CRS and on-target, off-tumor response. Brown et al. described intracranial delivery of anti-IL13Ra2 CAR-T cells into the resection cavity of patients with glioblastoma as feasible and well-tolerated (37). Subsequently, the same authors published a case report in which a patient with multifocal glioblastoma received CAR-T cells infused into the resected tumor cavity and the ventricular system, showing regression of all intracranial and spinal malignancies when administered intraventricularly, although disease progression was observed in distant sites when administration was intracavitary (30). Also, preclinical studies have proven that intrapleural CAR-T cell administration leads to enhanced antitumor activity with prolonged T cell persistence. This finding was later confirmed in a phase I clinical trial evidencing that intrapleural CAR-T cell infusion is safe and feasible (38,39).

The promise of CEA-directed CAR-T cell therapy stems from the expression of the antigen on the entire tumor cell surface as well as the potential to deliver the therapy via local instillation. For these reasons, we believe i.p. infusion will bolster the efficacy of CAR-T cell therapy in peritoneal metastases and will do so with fewer adverse effects (23,40).

In this study, we evaluated 2 routes (i.p. and i.v.) of CAR-T cell administration for treatment of PC alone and when associated with intraabdominal and extra-abdominal tumor implants. Using second-generation CAR-T cells in a murine model of PC, we found that CEA-directed CAR-T cells are more effective in reducing tumor burden than a control group, and that i.p. administration outperformed i.v. infusion. Administration of high doses of CAR-T cells was associated with a more potent antitumor response in both the i.p. and i.v. administration groups.

This study further demonstrates that i.p. CAR-T cell infusion confers protection against peritoneal disease recurrence. Mice treated with either i.v. or i.p. CAR-T cells exhibited stabilization of peritoneal disease, particularly in the i.p. therapy group. In addition, all treated mice achieved complete remission of peritoneal
disease on first peritoneal rechallenge at day 76. Even after a second rechallenge in the peritoneum and s.c. flank, all treated mice showed tumor reduction, though residual tumor was still visible. These data reveal that patients at high risk of recurrence may benefit from this therapy even if the tumor recurs in extra-abdominal sites. Despite inducing notable tumor regression after a second rechallenge, the efficacy of CAR-T cells was lower. This could be related to extra-abdominal disease and the time of rechallenge (105 days after treatment) in addition to factors related to CAR-T cell migration and persistence. These results indicate that even after optimal treatment (cytoreductive surgery + HIPEC) and no residual tumor, patients with CRC and PC can benefit from this therapy, as 46% to 73% can recur (41).

Compared to i.v. delivery, i.p. administration exhibits a better antitumor effect on spleen/liver in situ tumor-bearing models and models of liver metastases. The approach can not only eliminate peritoneal implants, but also clears abdominal solid tumors. Furthermore, i.p. administration has a higher amplification factor in various organs compared to TV administration. These findings support the use of i.p. delivery of CAR-T cells in patients diagnosed with PC who present metastases in additional abdominal organs.

When injected into mice peritoneum and s.c. flank tumor cells, the CAR-T cells used in this experiment exhibited higher tumor reduction in both sites, rendering peritoneal and s.c. flank tumors almost undetectable by bioluminescence after treatment. In contrast, residual tumor was observed following i.v. administration. In our i.p. + SC tumor-bearing model, i.p. administration expanded and cleared tumors more rapidly than TV administration, with higher IFN-ϒ secretion found in ascites and blood as well as more widespread CAR-T cell distribution in each organ.

This distant phenomenon resembles the abscopal effect seen in radiotherapy, as pointed out by other authors (27,38,42). In CAR-T cell studies such as this one, the effect is produced by tumor-antigen release by cancer cells upon elimination by CAR-T cells, allowing cross-presentation by dendritic cells and generating an immune response against tumor antigens not originally targeted by the CAR-T cells. Additionally, activated CAR-T cells secrete cytokines that stimulate innate immunity, triggering killing of tumor cells that are invisible to CAR-T cells (40,43,44). These two processes explain why i.p. administration has a systemic effect, but offers no indication of why the systemic effect in i.p. therapy is superior to i.v. administration. We hypothesize that the physiology of the peritoneum plays an important role in the systemic effect. Peritoneal absorption of CAR-T cells into the bloodstream favors systemic activity, though the peritoneal surface also supports the immune response. This organ is characterized by a complex immunological environment, which serves as a natural barrier against peritoneal infections and can respond rapidly and recruit T cells. The immune cells in the peritoneum are primarily composed of macrophages and T cells as well as natural killer and dendritic cells in low quantities. Furthermore, mesenchymal cells in the peritoneal cavity can secrete pro-inflammatory factors such as IL-1, IL-6, monocyte colony stimulating factor (MCSF), granulocyte colony stimulating factor (GCSF), granulocyte macrophage colony stimulating factor (GM-CSF), and epidermal growth factor (EGF), all of which promote the immune response and improve antigen presentation (45,46). Our data revealed higher levels of i.p. IFN-ϒ with i.p. administration as compared to i.v. administration followed by a late decrease coinciding with a low i.p. tumor burden. In contrast, blood levels of IFN-ϒ in i.p. administration were slightly lower than with i.v. administration. Though also low, IFN-ϒ exhibited a 7-fold increase when CAR-T cells were delivered intraperitoneally, coinciding with antitumor activity. Moreover, an increased number of copies of CAR-T cells was found on D7 following i.p. administration in all tissues including blood. This supports our theory of the
high capacity of the peritoneum to absorb CAR-T cells into the bloodstream, enabling proper distribution to different organs. This result further indicates that the peritoneum is equipped to mount an effective local immune response as seen by higher levels of IFN-ϒ in the peritoneum and blood.

Surprisingly, i.p. administration exhibits a stronger antitumor effect on distal SC tumors, even in the absence of PC. When abdominal tumors are stimulated, however, the antitumor effect on distal SC tumors takes effect earlier. i.p. administration produces higher CAR-T cell concentrations in peripheral blood and complete distant tumor remission, even in the absence of PC. These findings suggest that the extraperitoneal effect seen with i.p. infusion is not entirely due to an abscopal effect and that local peritoneal immunologic stimulation has a greater impact on CAR-T cell function than expected, thereby confirming that the peritoneal surface allows adequate systemic absorption.

Indeed, the CAR-T cell target is still crucial to the efficacy of CAR-T cell therapy in spite of the proven superiority of i.p. administration over systemic delivery. Thus, we set out to investigate whether this conclusion could be applied in other CAR-T cells directed at different targets as well as different tumor types. We chose pancreatic carcinoma, a malignancy well-known for its insensitivity to immunotherapy and for being the most difficult tumor to be treated, and used PSCA CAR-T cells for immunotherapy. The results of this experiment also revealed the advantage of i.p. infusion, which indicates that the approach does not need to be restricted to CRC tumor, but rather can be applied to other types of PC.

Finally, we used a safety evaluation system for both i.v. and i.p. routes. i.p. administration achieved superior antitumor efficacy, even at lower doses. Importantly, no test article-related toxicities or body weight changes were found in either study. Therefore, these data further support the clinical application of i.p. administration.

**Conclusion**

Our study demonstrates superior antitumor activity in i.p. of second-generation CAR-T cell delivery targeting CEA. CEA-directed CAR-T cells are efficacious in eliminating peritoneal implants. Moreover, i.p. delivery provides enhanced antitumor activity compared to i.v. when PC is associated with intraabdominal tumors and also in extra-abdominal sites, conferring prolonged protection against potential tumor recurrence, even in distant locations. i.p. CAR-T cells are not only effective in destroying tumor cells in a PC model, but also when administered intraperitoneally in a non-PC model of extra-abdominal tumors, yielding higher tumor reduction compared to systemic administration. Thus, i.p. CAR-T cells represent a curative treatment option for PC from CRC, and can be used as preventive therapy for patients with high risk of developing PC, conferring protection against recurrence and possibly benefiting patients with extra-abdominal metastatic disease. Our findings further demonstrate the superiority of regional i.p. delivery when using CAR-T cells that target antigens other than CEA.

**Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>CAR-T cell</td>
<td>Chimeric antigen receptor-T cell</td>
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<tr>
<td>CEA</td>
<td>Carcinoembryonic antigen</td>
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CRC  Colorectal cancer
CRS  Cytokine release syndrome
CTL  Cytotoxic T lymphocytes
EGF  Epidermal growth factor
GCSF  Granulocyte colony stimulating factor
GM-CSF  Granulocyte macrophage colony stimulating factor
GVHD  Graft-versus-host-disease
HIPEC  Hyperthermic intraperitoneal chemotherapy
HPAC  Human pancreatic acinar carcinoma
IFN-ϒ  Interferon-ϒ
i.p.  Intraperitoneal
i.v.  Intravenous
MCSF  Monocyte colony stimulating factor
PBMC  Peripheral blood mononuclear cells
PC  Peritoneal carcinomatosis
PSCA  Prostate stem cell antigen
s.c.  Subcutaneous
TV  Tail vein

Declarations

Ethics approval and consent to participate

All experiments conducted on animals were approved by the Committee of Chongqing Precision Biotechnology Co. Ltd.

Consent for publication

Not applicable

Availability of data and materials
The data sets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

**Competing interests**

J. C., Y. Z., X. Z., D. D., L. Q., J. H., Y. X., Z. Y., Y. L., J. S., C. Q. are employees of Chongqing Precision Biotechnology Co. Ltd and the rest of authors declare no competing financial interests.

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**Authors’ contributions**

S.Q, J.S., P.V., D.G-O., and C.Q initiated and designed the experiments; S.Q., J.C., Y.Z., and D.D., L.Q., J.H., X.Z., and Y.L., performed the experiments; Y.X., Z.Y., and J.S. analyzed and interpreted the data; S.Q. and J.C., drafted the manuscript; S.Q., J.S., P.V., I.G., S.J., H.G., M.G-A., D.G-O., and C.Q revised the manuscript; J.S., P.V., and C.Q obtained funding and supervised the study. All authors approve the final version.

**Acknowledgments**

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**References**


Figures
Figure 1

CEA CAR-T-cells show antigen-dependent cytokine production and tumor killing \textit{in vitro}.

(A) Diagram of the lentiviral expression cassette with CEA CAR-T-cells containing the anti-CEA single-chain variable fragment (ScFv), the CD8 alpha hinge region, 4-1BB co-stimulatory domain, and the CD3zeta T cell activation domain. (B) GFP and CEA expression of DLD-1-CEA-Luc-GFP cells detected by FACS. (C) \textit{In vitro} killing assay in which CEA CAR-T-cells or activated untransduced T cells (Control T) were co-cultured with DLD1-Luc-GFP cells or DLD1-CEA-Luc-GFP cells at various effector:target (E:T) ratios. (D) IFN-\gamma and IL-2 levels...
in supernatant quantified by ELISA from activated untransduced T cells (Control T) or CEA CAR-T-cells following a 24h co-culture with DLD1-Luc-GFP cells or DLD1-CEA-Luc-GFP cells at an E:T ratio of 1:1.

Figure 2

Regional intraperitoneal delivery of CEA CAR-T-cells significantly reduces tumor burden and extends overall survival of DLD-1-CEA-Luc-GFP tumor-bearing mice when compared to systemic TV infusion.

(A) Schematic representation of i.p. engraftment of $1.0 \times 10^6$ DLD-1-CEA-Luc-GFP tumor cells in NCG mice on day 7, followed by i.p. delivery of $9.6 \times 10^6$ cells/mouse of activated untransduced T cells (Control T) and either i.v. or i.p. delivery of $1.0 \times 10^7$ copies/mouse (low dose, $3.2 \times 10^6$ cells/mouse) or $3.0 \times 10^7$ copies/mouse (high dose, $9.6 \times 10^6$ cells/mouse) of CEA CAR-T cells on day 0 (7 days after tumor-cell infusion). (B) Quantification of flux (total flux for each mouse) from DLD-1-CEA-Luc-GFP tumor-bearing mice treated intravenously or intraperitoneally with Control T or CEA CAR-T-cells. (C) Kaplan–Meier survival curve of mice receiving i.v. or i.p.
treatment with Control T or CEA CAR-T-cells (low dose and high dose). (D) Bioluminescent flux imaging of mice with i.v. or i.p. treatment consisting of Control T or CEA CAR-T-cells (low dose and high dose).

Figure 3

Regional intraperitoneal delivery of CEA CAR-T-cells significantly reduces tumor burden and exerts longer anti-tumor activity over systemic TV infusion in a liver/spleen orthotopic and i.p. xenograft model.

(A) Schematic representation of a liver/spleen orthotopic and i.p xenograft model: orthotopic engraftment of $1.0 \times 10^6$ DLD-1-CEA-Luc-GFP tumor cells in NCG mice 17 days before CAR-T infusion; subsequently, $1.0 \times 10^6$
DLD-1-CEA-Luc-GFP tumor cells were engrafted intraperitoneally 10 days after (day -7), followed by either i.v. or i.p. delivery of $2.0 \times 10^7$ copies/mouse ($5.89 \times 10^6$ cells/mouse) of CEA CAR-T cells on day 0; $5.89 \times 10^6$ cells/mouse of activated untransduced T cells (Control T) were intraperitoneally infused in the control group.

(B) Orthotopic tumor of the spleen (Right) and metastatic liver tumor (Left) indicated by a red circle in an orthotopic xenograft model treated intraperitoneally on day 0. (C-D) Bioluminescent flux imaging and quantification of flux in an orthotopic xenograft model of the spleen with i.v. or i.p. infusion of CEA CAR-T-cells. (E) CEA CAR-T cell gene copies in blood on different days after treatment measured by qPCR. (F) qPCR detection of CEA CAR-T cell distribution in different tissues/organs including the heart, liver, spleen, lung, kidney, stomach, small intestine, large intestine, mesentery, gonad, brain, skeletal muscle, tumor (on the mesentery), bone marrow, ascites, and blood 68 days after treatment.
Figure 4

Regional intraperitoneal delivery of CEA CAR-T-cells significantly reduces tumor burden compared to systemic TV infusion in a subcutaneous intraperitoneal xenograft model.

(A) Schematic representation of a subcutaneous (SC) i.p. xenograft model: subcutaneous engraftment of $1.0 \times 10^6$ DLD-1-CEA-Luc-GFP tumor cells in NCG mice on day -12, i.p. engraftment of $1.0 \times 10^6$ DLD-1-CEA-Luc-GFP tumor cells on day -7, followed by i.p. delivery of $9.85 \times 10^6$ cells/mouse of activated untransduced T cells (Control T) and either i.v. or i.p. delivery of $3.26 \times 10^7$ copies/mouse ($9.85 \times 10^6$ cells/mouse) of CEA CAR-T
cells on day 0. (B, C) Bioluminescent flux imaging and quantification of flux in the abdomen of an SC intraperitoneal xenograft model with either i.v. or i.p. delivery of control T/CEA CAR-T cells. (D, E) Bioluminescent flux imaging and quantification of flux in the backside of an SC i.p. xenograft model with either i.v. or i.p. delivery of control T/CEA CAR-T cells. (F) Tumor volume of SC flank tumors.

Figure 5

Regional intraperitoneal delivery of CEA CAR-T cells shows significantly longer and higher IFN-γ secretion than systemic TV infusion in a subcutaneous intraperitoneal xenograft model.

(A) CEA CAR-T cell expansion in blood detected by qPCR. (B-C) IFN-γ levels in ascites and blood detected by ELISA at different time points after treatment. (D) Distribution of CEA CAR-T-cells in different tissues/organs including blood, heart, liver, spleen, lung, kidney, stomach, small intestine, large intestine, mesentery, skeletal muscle, bone marrow, gonad, brain, ascites, and tumor (SC flank tumor) on day 1, day 7, day 14, and day 42 in an SC i.p. xenograft model with either i.v. or i.p. delivery of CEA CAR-T-cells.
Figure 6

Regional intraperitoneal delivery of CEA CAR-T cells significantly reduces tumor burden and confers extended anti-tumor activity than systemic TV infusion in a subcutaneous xenograft model.

(A) Schematic representation of an SC xenograft model: subcutaneous engraftment of $1.0 \times 10^6$ DLD-1-CEA-Luc-GFP tumor cells in NCG mice on day -12, followed by either i.v. or i.p. delivery of $2.81 \times 10^7$ copies/mouse ($7.37 \times 10^6$ cells/mouse) CEA CAR-T cells on day 0, and i.p. injection of $7.37 \times 10^6$ cells/mouse of activated untransduced T cells (Control T) as controls. (B-C) Bioluminescent flux imaging and quantification of flux. (D) Tumor volume of SC flank tumors. (E) Distribution of CEA CAR-T cells in different tissues/organs on day 50.
Figure 7

Regional intraperitoneal delivery of PSCA CAR-T-cells shows a significantly greater anti-tumor effect than systemic TV infusion.

(A) Schematic representation of an i.p. xenograft model: intraperitoneal engraftment of $1.0 \times 10^6$ HPAC-Luc-GFP tumor cells in NCG mice 7 days before CAR-T cell treatment, followed by either i.v. or i.p. delivery of $1 \times 10^6$ copies/mouse ($2.23 \times 10^5$ cells/mouse)(Low dose) or $3 \times 10^6$ copies/mouse ($6.70 \times 10^5$ cells/mouse)(High dose) PSCA CAR-T cells on day 0, and $6.70 \times 10^5$ cells/mouse of activated untransduced T cells (Control T) were delivered intraperitoneally as controls. (B-C) Bioluminescent flux imaging and quantification of flux. (D) PSCA CAR-T-cells expanding in blood as detected by qPCR.
Regional intraperitoneal delivery of CEA CAR-T-cells also shows no obvious test article-related toxicity compared to systemic TV infusion.

(A) IFN-γ secretion in blood 7/21 days after CEA CAR-T cell infusion in an SC i.p. xenograft model as detected by ELISA in preclinical safety studies. (B) Percentage of CD3+ cells in blood detected by FACS. (C) CAR-T cell distribution in blood as detected by qPCR. (D) Hematologic analysis of blood 7/21 days after CEA CAR-T cell infusion in an SC i.p. xenograft model detected by Sysmex®XN Series automated hematology system. WBC: White blood cells. Neut: Neutrophils. Lymph: Lymphocytes. Mono: Mononuclear cells. Eos: Eosinophils. Baso: Basophils. Intraperitoneal delivery of CEA CAR-T-cells (i.p.) and systemic TV delivery of CEA CAR-T-cells (i.v.) were conducted in independent experiments. Student t test was used to compare data for i.p. and i.v. delivery of CEA CAR-T-cells.
Figure 9

No test article-related toxicity was in evidence in regional intraperitoneal delivery of CEA CAR-T-cells.

dose group. Low-dose group: i.p. delivery of $5.04 \times 10^7$ total cells/kg of CEA CAR-T-cells. High-dose group: i.p. delivery of $5.04 \times 10^8$ total cells/kg of CEA CAR-T-cells.

**Supplementary Files**

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