CAR T Cells Equipped With a Fully Human scFv Targeting Trop2 Can Be Used to Treat Pancreatic Cancer

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Abstract

Purpose: Chimeric antigen receptor (CAR) T cell therapy has demonstrated clinical success in treating haematologic malignancies but has not been effective against solid tumours thus far. Trop2 is a tumour-related antigen broadly overexpressed on a variety of tumours and has been reported as a promising target for pancreatic cancers. Our study aimed to determine whether CAR T cells designed with a fully human Trop2-specific single-chain fragment variable (scFv) can be used in the treatment of Trop2-positive pancreatic tumours.

Methods: We designed Trop2-targeted chimeric antigen receptor engineered T cells with a novel human anti-Trop2 scFv (2F11) and then investigated the cytotoxicity, degranulation, and cytokine secretion profiles of the anti-Trop2 CAR T cells when they were exposed to Trop2+ cancer cells in vitro. We also studied the antitumour efficacy and toxicity of Trop2-specific CAR T cells in vivo using a BxPC-3 pancreatic xenograft model.

Results: Trop2-targeted CAR T cells designed with 2F11 effectively killed Trop2-positive pancreatic cancer cells and produced high levels of cytotoxic cytokines in vitro. In addition, Trop2-targeted CAR T cells, which persistently circulate in vivo and efficiently infiltrate into tumour tissues, significantly blocked and even eliminated BxPC-3 pancreatic xenograft tumour growth without obvious deleterious effects observed after intravenous injection into NSG mice. Moreover, disease-free survival was efficiently prolonged.

Conclusion: These results show that Trop2-targeted CAR T cells equipped with a fully human anti-Trop2 scFv could be a potential treatment strategy for pancreatic cancer and could be useful for clinical evaluation.

Introduction

Pancreatic cancer remains a devastating malignancy with the lowest 5-year relative survival rate (10%) despite advances in modern targeting and immune therapies (Siegel et al., 2021). Chimeric antigen receptor (CAR) T cell therapy has been effective in B cell malignancies, although breakthroughs in solid tumours have not yet been achieved. Some antitumour activities have been observed in early phase clinical trials of CAR T cell therapies targeting mesothelin (Haas et al., 2019) and EGFR (Liu et al., 2020), suggesting that CAR T cell therapy has the potential to be used to treat solid tumours (Date and Nair, 2020). CARs consist of a single chain variable fragment (scFv), a hinge, a transmembrane domain, and an endodomain with one (2nd generation) or two (3rd generation) costimulatory signalling domains linked to CD3ζ, in which scFv is involved in targeting tumour antigens (June et al., 2018). However, most single-chain antibodies available to date have been obtained by animal immunization, a bottleneck restricting many applications in immunotherapy. Fully human single chain antibodies should be used to reduce immunogenicity (Frenzel et al., 2016).

Human trophoblastic cell surface antigen 2 (Trop2), also known as tumour-associated calcium signal transducer 2 (TACSTD2), belongs to the TACSTD family (Lenart et al., 2020). Trop2 was first described as a protein highly expressed on the surface of trophoblast cells in 1981 (Lipinski et al., 1981). Previous studies indicated that Trop2 is a transmembrane, surface glycoprotein that is not expressed or is expressed at low levels in normal human tissues but is overexpressed in a variety of epithelial cancers (Trerotola et al., 2013). In 2021, 40 years after the discovery of Trop2, a Trop2-specific antibody drug conjugate, IMMU-132 (sacituzumab govitecan), obtained accelerated approval for the treatment of metastatic triple-negative breast cancer (TNBC), which was shown to be successful (Wahby et al., 2021), indicating that targeting Trop2 for immunotherapy of Trop2-expressing cancers deserves to be studied. In addition, D Fong et al. reported the overexpression of Trop2 in 109 (55%) of 197 pancreatic tumour samples, and this factor was significantly associated with poor prognosis (Fong et al., 2008). Thus, we considered Trop2 to be a promising immunotherapy target for CAR T cells against Trop2-positive pancreatic cancer. However, there are no reports about the Trop2 molecule being used as a target for CAR T therapy of pancreatic cancer thus far.

In this study, we first demonstrated that CAR T cells designed with anti-Trop2 scFv isolated from a naïve human scFv phage library could efficiently kill pancreatic cancer cells in vitro and entirely eliminate pancreatic cancer in vivo in a BxPC-3 xenograft model. The data from our work provide an important scientific basis for the successful development of CAR T immunotherapy for malignant pancreatic cancer.

Materials And Methods

2.1 Cell lines and EMDs of Trop2

Pancreatic cancer cell lines (ASPC-1, CFPAC-1, BxPC-3) were purchased from the Cell Bank of the Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. The initial characterization of the three cell lines was analysed through FACS. All cell lines were cultured in RPMI-1640 medium (Gibco) with 10% foetal bovine serum (Gibco), penicillin (100 U/mL) and streptomycin (100 g/mL). The extracellular domain (EMD) of human Trop2 with poly-his tag was used to screen the phages that specifically bind to the EMD antigen of Trop2, which was purchased from ACRO (TR2-H5223, Acrobiosystems).

2.2 Phage Display

An ELISA plate was coated with Trop2-His Tag protein and incubated overnight at 4°C. After 16 h, both the human naïve scFv phage library obtained from Unicar Therapy, Ltd., and the coated wells were blocked with PBST with 2% milk at room temperature for 1 h. Then, the blocked phages were added to the coated wells and incubated at room temperature for 1 h. After 15 washes with PBST, the Trop2-binding phages were eluted with 1 mg trypsin (Sigma-Aldrich, T1426). All eluted phages were collected and used to infect TG1 cells cultured to the logarithmic growth stage. Single TG1 clones infected by the fourth round of eluted phage were picked and cultured in 2YT broth medium containing 100 mg/mL ampicillin

and 1% glucose. M13KO7 Helper Phage was then added to each vial. Then, TG1 cells infected with helper phage were cultured overnight (220 rpm) in 2YT medium containing 100 mg/mL ampicillin and 50 mg/mL kanamycin at 30°C. One hundred microlitres of supernatant from each vial was added to each well of 96-well plates coated with 100 ng Trop2-His tag, and the supernatant was preblocked with 2% milk for an hour. After incubation and washing, 100 µl of horseradish peroxidase (HRP)-conjugated anti-M13 antibody (NBbiolab; 1:5000 dilution in blocking buffer) was added to each well, followed by incubation with 100 µl of HRP substrate solution (eBioscience™ TMB). The absorbance value at 450 nm was read by a full wavelength reader Multiskan GO (Thermo Scientific).

2.3 Construction, expression, purification of anti-Trop2 scFv-hFc

The Trop2-specific scFv (2F11) sequence was fused to human Fc Tag and cloned into the PUTAM003 vector (obtained from Unicar therapy). With the EcoRV/BspEI double enzyme digestion cloning method, the sequence of anti-Trop2 scFv was cloned into a mammalian cell expression vector (PUTAM003). The recombinant plasmid was identified by sequencing (Tsingke Biological Technology) and then transfected into 293T cells cultured with DMEM. After transfection, the dimerized Trop2 scFv recombinant protein secreted into the medium was collected and purified via protein A chromatography (AKTA Purifier 100, GE). For analysis of protein purity, 10% SDS-polyacrylamide gel electrophoresis (PAGE) (ultrafast preparation kit; Beyotime) was used to characterize the purified 2F11-hFc.

2.4 The affinity of Trop2-specific scFv-hFc was detected by ELISAs

The Trop2 antigen with poly-his tag (TR2-H52H5, Acrobiosystems) was coated on ELISA wells at the concentration of 1 µg/ml at 4 ℃ overnight. The wells were blocked with 200 µl of 2% nonfat milk for 1 h at room temperature. Trop2 scFv-hFc was diluted three times from 5 µg/ml, for a total of 12 concentrations (5 µg/ml~9.89274x10^{-6} µg/ml). After dilution, antibodies were added to the wells and incubated for 1 h. Then, after 3 washes with 0.5% PBST, anti-human IgG-Fc conjugated with HRP (Sino Biological, Inc., 10702-MM01T-H) was added to the wells and incubated at room temperature for 1 h. TMB and H_2SO_4 (2 M) were used to detect the OD450 nm value.

2.5 Generation of an anti-Trop2/CD19 CAR construct

The CAR expression cassette adopted the third-generation format. A Trop2-targeted single-chain variable fragment (2F11) was inserted into a CAR construct (obtained from Unicar-Therapy Biomedicine Technology) containing the EF1a promoter, and the 41BB (CD137) costimulatory domain was fused to CD3 zeta. Except for a CD19-specific single-chain variable fragment (FMC63) used in CD19 CAR, the elements of CD19 CAR are exactly similar to those of Trop2 CAR. The CAR plasmid was cotransfected with three packaging plasmids into HEK 293T cells, and the resulting lentiviruses secreted in the supernatant were harvested and immediately stored at −80°C.

2.6 Generation of CAR T cells

Apheresis samples were obtained from healthy donors, and peripheral blood mononuclear cells (PBMCs) were isolated with gradient centrifugation using Lymphoprep™ (Oriental Hua Hui). CD3-positive T cells were isolated using anti-CD3 positive-selection beads (Miltenyi Biotec) and activated with anti-CD3/CD28 monoclonal antibodies (Miltenyi Biotec) for 24 h. The activated CD3+ T cells were then transduced with lentiviral vectors encoding the CAR for 48 h followed by culture in AIM-V medium (Gibco) supplemented with 10% FBS (Gibco) and 1000 IU/mL recombinant human IL-2 (PeproTech) for 14 days. The manufacturing method of mock T cells was the same as that of Trop2 CAR T cells.

2.7 Flow cytometry

All samples were analysed using an Attune NxT flow cytometer (Thermo Scientific), and data were analysed using FlowJo software (TreeStar). The antibodies used included PE anti-human IgG Fc antibody (BioLegend), APC anti-human CD107a (BioLegend), FITC-Labeled Recombinant Protein L His Tag (RPL-PF141, ACRO), FITC-labelled anti-human CD3 antibody (BioLegend), Alexa Fluor 700 CD45 Monoclonal Antibody (eBioscience), and APC anti-His Tag Antibody (BioLegend). All FACS-related staining procedures were performed at 4°C for 45 min, and then, the cells were washed with PBS before flow cytometric analysis. Peripheral blood from mouse xenografts was treated with red blood cell lysis buffer (Biolegend) for 10 min, and the cells were stained with the corresponding antibodies as described above.

2.8 Cytotoxicity assay, degranulation assay and quantitation of cytokine levels

Target cells and CAR T cells were coincubated at an effector:target (E:T) ratio of 10:1, 5:1 or 2.5:1 in 5% CO2 at 37°C for 6 h. Lactate dehydrogenase (LDH) activity was detected using a cytotoxicity detection kit (Promega), and the absorbance value at 490 nm was read by a full wavelength Multiskan GO reader (Thermo Scientific). CD107a expression on CD8-positive T cells was detected by flow cytometry to show the degranulation of T cells. CAR T cells were coincubated with target cells at a 1:1 ratio in 5% CO2 at 37°C for 6 h in the presence of the Golgi inhibitor Monessen (Invitrogen) and CD107a-APC antibody (BD Bioscience). A protease inhibitor cocktail (Invitrogen) was used to stimulate the high CD107 expression of T cells, acting as a positive control. A Th1/Th2 Cytometric Bead Array Kit II (BD Bioscience) was used to detect cytokine levels. Supernatants collected from the in vitro coincubation system of effector cells and target cells or mouse xenograft blood serum were incubated with fluorophore-labelled antibodies against IL-2, IL-4, IL-6, IL-10, IFN-γ, TNF-α and IL-17A for 3 h. Before analysis by flow cytometry, the samples were washed twice with PBS.

2.9 BxPC-3 pancreatic cancer xenograft tumour model

Animal experiments were performed in the Shanghai Key Laboratory of Brain Functional Genomics, and all animal procedures and protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of East China Normal University. All NOD/ShiLtJGpt-Prkdc^{em26}Il2rg^{em26}/Gpt (NSG) mice were obtained from Gempharmatech, maintained in specific pathogen-free (SPF)-grade cages and provided sterile food and water.

For the first in vivo experiment, 5×10^6 BxPC-3 cells were injected subcutaneously into the right flanks of NSG mice aged 6–8 weeks. When the tumour volume was approximately 100 mm^3, the mice were divided into three groups (untreated, CD19 CAR T, Trop2 CAR T) with 5 mice in each group. Then, the mice in the untreated group were not injected, and 10 million CD19 CAR T cells or Trop2 CAR T cells were infused into mice on Days 28, 32, 36 and 40 by intravenous injection. Tumour sizes and mouse weights were monitored three times per week. Tumour sizes were measured by an electronic caliper and calculated according to the formula 'tumour volume=length × width2/2'. At Day 51, all mice were sacrificed, and tumours, blood and key organs (heart, liver, spleen, lung, kidney and brain) were collected.

The second in vivo experiment was designed to study disease-free survival and CAR T cells in vivo. On D90, mice in the Trop2 CAR T group were randomly selected for orbital blood collection to examine the proportion of CAR-positive cells by FACS.

2.10 Immunohistochemistry analysis

Formalin-fixed, paraffin-embedded tumour tissues and peritumoural tissues were stained by haematoxylin and eosin (H&E) to analyse histopathological changes. Tumour tissues were immunostained using an anti-Trop2 antibody (Abcam) to test the presence of Trop2-positive tumour cells. Tumour tissues were immunostained using an anti-CD3 antibody (Abcam) to test the infiltration of CAR T cells (CD3+) into tumours. The procedures were performed as follows. The primary monoclonal antibody was incubated overnight at 4°C. Sections were then washed with PBS and incubated with peroxidase-conjugated secondary antibodies (Servicebio) for 45 min at RT. The sections were visualized using a diaminobenzidine staining kit (DAKO) and then counterstained with haematoxylin, dehydrated, cleared, mounted, and photographed. DAB-immunostained sections were analysed by bright-field microscopy using an Olympus microscope.

2.11 Statistical analysis

All representative experiments were repeated with three independent biological replicates. All statistical analyses were conducted using GraphPad Prism 8.0. Two-tailed Student's t test of the means, one-way ANOVA and two-way ANOVA were used for statistical analysis, with $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$ defined as significant and $p > 0.05$ defined as not significant.

Results

3.1 The identification of a fully human Trop2-specific scFv (2F11)

We detected the cell surface expression of Trop2 in three pancreatic cancer cell lines (AsPC-1, CFPAC-1, BxPC-3) by flow cytometry. The cell lines showed different levels of Trop2 expression; AsPC-1 cells were negative and the others were positive (Fig. 1a). Compared to CFPAC-1 cells, BxPC-3 cells had a significantly enhanced median fluorescence intensity (MFI) of Trop2 expression.

To obtain a human scFv that specifically bound to Trop2, we utilized phage display technology for screening from the whole human naive scFv phage library. After the fourth round of panning, the phage titre was obviously increased (Fig. S1a). Five sequences (scFv-1, 2, 3, 4, and 5) were finally identified (Fig. S1b). The scFv-3 phage clone (2F11) was selected for further analysis in our study because it had the highest binding activity. The Trop2-specific scFv (2F11) sequence was fused to human Fc Tag and cloned into the PUTAM003 vector. Then, the 2F11-Fc (scFv-Fc format) recombinant plasmid was introduced into 293T cells by calcium chloride transfection. The 2F11-Fc expressed in the supernatant had binding activity to the EMD of Trop2, as shown by ELISAs, indicating the successful expression of 2F11-Fc (Fig. S1c). Then, the supernatant containing 2F11-Fc was purified via protein A chromatography (Fig. 1b). The protein purity was assessed by SDS-PAGE, and a single band showed that 2F11-Fc was pure after purification (Fig. 1c). As expected, the purified scFv appeared as a band of approximately 50 kDa under reduced conditions. Binding activity between purified 2F11-Fc and cell surface Trop2 on BxPC-3 cells was detected by flow cytometry. FACS data showed that anti-Trop2 scFv (2F11) could specifically bind to Trop2 on the cell membrane (Fig. 2d). The protein binding activity was identified by ELISAs (Fig. 2e). ELISAs showed that anti-Trop2 scFv could specifically bind to the extracellular domain of Trop2 (His tag).

3.2 Trop2-specific CAR T cells effectively kill Trop2-positive tumour cells

After the identification of scFv, two lentiviral vectors encoding CARs (Trop2 CAR and CD19 CAR) comprising the anti Trop2 (2F11) or CD19 (FMC63) scFv, intracellular costimulatory domain CD137 (41BB) linked to CD3ζ domain, human CD8 hinge and CD8a transmembrane regions were generated (Fig. 2a). CD19 CAR T cells with CD19-specific scFv were used as non-Trop2 control CAR T cells. T cells cultured for 14 days expanded at least 100 times (Fig. S2a). The transfection efficiency of Trop2-specific CAR T cells from three donors was similar (60.5%, 55.4%, 48%) (Fig. S2b). An in vitro LDH cytotoxicity assay showed that Trop2-specific CAR T cells could efficiently lyse Trop2-positive cells in a dose-dependent manner but not Trop2-negative cells, whereas mock CD19 CAR T cells were unable to lyse any Trop2-positive target cells at any indicated effector to target (E:T) ratio (Fig. 2b). Notably, Trop2-specific CAR T cells had higher cytotoxic activities against BxPC-3 cells than CFPAC-1 cells. Together, the Trop2-specific CAR T cells had target-dependent and dose-dependent cytotoxic activity. Furthermore, IL-17A, IL-2, TNF-a and IFN-r levels were significantly increased after coincubation of Trop2 CAR T cells with BxPC-3 cells compared with mock CAR T cells (Fig. 2c). CD107a expression is a key process responsible for cytotoxicity against target cells and is positively associated with T cell degranulation (Lorenzo-Herrero et al., 2019). As a result, a Trop2-specific CD8+ CAR T cell-associated degranulation assay showed high expression of CD107a (Fig. 2d), indicating that CD8+ Trop2 CAR T cells released a large amount of cytokines, such as granzyme B and perforin, to kill Trop2-positive cells (BxPC-3), while no CD107A expression was observed for Trop2-negative cells (ASPC-1). In summary, human Trop2-targeted CAR T cells specifically killed pancreatic cancer cells, simultaneously expressed CD107a, secreted granzyme B and perforin, and produced a variety of cytokines, such as IL-2, IL-17A, IFN-r and TNF-a, in vitro in the presence of Trop2-positive tumour cells.

3.3 Trop2-CAR T cells effectively decrease pancreatic cancer xenograft tumour growth and entirely eliminate pancreatic tumours.

To examine the in vivo efficacy and safety of Trop2-specific CAR T cells against pancreatic cancer, we utilized NOD/ShiLtJGpt-Prkdc^{em26}Il2rg^{em26}/Gpt immunodeficient mice to establish BxPC-3 xenograft mouse models. The infusion dose was 1x10^{7} per mouse at D28, D32, D36, and D40 when the tumour volume was approximately 100 mm^{3}, 4 times in total, as depicted (Fig. 3a). The tumour growth curves of the mice showed that in the Trop2-targeted CAR T cell treatment group, tumours began to shrink after CAR T cell infusion for 11 days (D39) and then disappeared, while the tumour volume of the mice in the CD19 CAR T cell-treated group increased rapidly, indicating that Trop2-targeted CAR T cells could specifically kill pancreatic cancer tissue with high Trop2 expression and mediate regression of human pancreatic cancer in NSG mice (Fig. 3b). In addition, we measured a higher concentration of IFN-γ in the blood of the mice treated with Trop2 CAR T cells, indicating the indirect killing ability of CAR T cells (Fig. 3c).

After the experiment progressed to D51, mouse tumours in the Trop2 treatment group disappeared, but the tumour volume of the mice in the control and CD19 groups had exceeded 1500 mm3. Then, the mice of all three groups were sacrificed, and the tumours were removed and weighed (Fig. 3d, Fig. 3e and Fig. 3f).

Furthermore, to verify the disappearance of the tumours in the Trop2 CAR T group, we removed the large tumours in the CD19 group and control group, and adjacent tissues were removed from the target cell inoculation site where tumours had been eliminated in the Trop2 CAR T group. Haematoxylin and eosin (H&E) staining and immunohistochemical staining of Trop2 were performed after the tissues were fixed with formalin and made into paraffin sections. The morphology of the tissue removed from the inoculation site of the mice in the Trop2 CAR T group was significantly different from that of the tumour tissue removed from the mice in the mock and control groups, preliminarily showing that there were no tumour cells at the inoculation site of the mice treated with Trop2 CAR T cells (Fig. 3g). The elimination of pancreatic tumour cells detected by Trop2-positive tumour cell immunostaining demonstrated that Trop2-positive tumour cells were thoroughly eliminated following Trop2 CAR T cell infusion, whereas Trop2-positive tumour cells were found in the tumours treated with CD19 T cells and the untreated tumours (Fig. 3h). The histopathological data suggested that Trop2-specific CAR T cells could completely eliminate pancreatic tumours. Collectively, Trop2-CAR T cells effectively inhibited pancreatic cancer xenograft tumour growth and completely eliminated pancreatic tumours.

3.4 Safety and toxicology studies of Trop2-specific CAR T cells

Surprisingly, the mouse weight in the Trop2 CAR T cell treatment group did not decrease significantly, and there was no significant difference in the mouse weight in the control and CD19 groups, indicating that

Trop2-specific CAR T cells have good safety and strong antitumour effects (Fig. 4a). After the mice in the untreated control, CD19 CAR T cell and Trop2 CAR T cell groups were sacrificed, the hearts, livers, spleens, lungs, kidneys and brains were removed and stained with H&E. H&E staining showed that there was no obvious organ damage in the Trop2 CAR T cell treatment group (Fig. 4b), indicating the good safety of Trop2 CAR T cells in the pancreatic xenograft model. Furthermore, blood was collected from all mice to detect serum chemistry, including albumin (ALB), alanine aminotransferase (ALT), aspartate aminotransferase (AST), total protein (TP), creatine (CREA), total bilirubin (TBIL) and blood urea nitrogen (BUN). The Trop2 CAR T-treated groups had increased blood urea nitrogen levels (Table 1), which may be metabolic abnormalities caused by tumour lysis. Thus, no weight loss in the mice treated with CAR T cells was observed, and no obvious damage was observed in the critical organs of the mice treated with Trop2-specific T cells. All these data indicate that Trop2-specific CAR T cells did not induce deleterious side effects in vivo.

Table 1
Toxicological results

Parameters	Mock CAR	Untreated	Trop2 CAR
Alanine aminotransferase(IU/L)	57.2±47.63	23.8±6.83	53.25±10.69
Aspartate aminotransferase(IU/L)	164.8±61.42	103.6±12.34	152±12.83
Total protein (g/L)	46.68±4.45	44.56±1.21	47.82±6.22
Albumin (g/L)	22.1±1.95	20.6±1.34	22.74±2.61
Total bilirubin (umol/L)	1.4±0.29	1.22±0.26	1.14±0.25
Blood urea nitrogen (mmol/L)	7.96±1.27	6.62±1.18	10.5±0.76**
Creatine (umol/L)	12.8±2.39	12±1	11±1.87

Fifty-one days after inoculation, blood was collected, n = 5/group. Values represent the mean ± SD. P** <0.01 compared with the mock CAR group, Student's t test.

3.5 Trop2-specific CAR T cells largely prolonged the disease-free survival of mice with pancreatic tumours

To study whether the mice treated with Trop2-specific CAR T cells relapsed, we conducted in vivo experiments using the same pancreatic xenograft model as previous experiments (Fig. 5a). Consistent with the former model, CAR T cell infusion was performed intravenously from the tail vein. In line with previous results, pancreatic tumours disappeared in the mice treated with Trop2 CAR T cells (tumour volume and weight data not shown). The survival curve of the BxPC-3 subcutaneously injected mice in the three groups showed that Trop2 CAR T cells could significantly prolong the disease-free survival of the mice, and there was no relapse or death in any of the mice treated with Trop2-specific CAR T cells after infusion (Fig. 5b). Moreover, Trop2-specific CAR T cells persistently circulated in vivo for 2 months after CAR T cell infusion (Fig. 5c). Altogether, Trop2-specific CAR T cells displayed profound antitumour

responses, induced pancreatic cancer regression and prolonged the survival of these mice compared with CD19 CAR T cells in a BxPC-3 xenograft mouse model.

Discussion

Pancreatic cancer is the seventh leading cause of cancer-associated mortality in both sexes and has an extremely poor prognosis, with a 5-year overall survival (OS) rate below 10% (Schizas et al., 2020). The poor prognosis is mainly due to the untimely diagnosis, limited efficacy of available drugs and rapid tumour progression (Mizrahi et al., 2020). Traditional treatment modalities for pancreatic cancer, such as surgery, chemotherapy, radiotherapy and other locoregional treatments, have poor therapeutic effects (Manji et al., 2017). Tumour immunotherapy, such as immune checkpoint inhibitors, has shown encouraging results for pancreatic cancer (Torphy et al., 2018), but the overall response rate is still low (Timmer et al., 2021). Due to the major challenges above, CAR T cell therapy may offer a new approach for the treatment of pancreatic cancer.

The effective treatment of CAR T cells depends on the specific expression of the recognized target antigens on the surface of tumour cells, while solid tumours usually express tumour-associated antigens rather than tumour-specific antigens. Moreover, the expression of antigens in tumour tissues is highly heterogeneous. Therefore, it is extremely important to find a tumour-related antigen with an excellent expression profile, which will enhance the antitumour activity of CAR T cell therapy against solid tumours.

Trop2, also known as tumour-associated calcium signal transducer 2, is overexpressed in epithelial cancer (Bignotti et al., 2011) and plays a critical role in tumour growth. Specifically, Trop2 can increase the intracellular calcium concentration, acts as a calcium signal transducer (Ripani et al., 1998), and is closely related to the JAK/STAT (Hou et al., 2019), MAPK/ERK (Cubas et al., 2010), PI3K/AKT (Guerra et al., 2016) and EPK/JNK (Guan et al., 2017) signalling pathways. As reported, knockdown or knockout of Trop2 on the cell surface significantly inhibits the proliferation of tumour cells (Zhang et al., 2018). Since Trop2 is indispensable in the proliferation and migration of tumour cells, it may be a potential and attractive target for epithelial carcinomas (Cubas et al., 2009), and Trop2-specific chimeric antigen T cells may offer a new therapeutic approach for pancreatic cancer.

Despite the efficacy of Trop2 CAR T cells in gastric cancer (Zhao et al., 2019) and triple-negative breast cancer (Chen et al., 2021) in vitro and in vivo, the efficacy in pancreatic cancer has not been reported thus far. Herein, we employed the human scFv phage library to isolate a human single-chain fragment variable that specifically recognizes EMD of Trop2. Then, we identified its binding specificity and affinity to native Trop2 as well as Trop2-positive cancer cells. Furthermore, we designed Trop2-targeted CAR T cells with this novel scFv and evaluated the antitumour effects of CAR T cells in vitro and in vivo.

Surprisingly, Trop2-specific CAR T cells displayed strong antitumour effects in vivo and completely eliminated BxPC-3 pancreatic cancer xenograft tumours 3 weeks after infusion. Compared with several ADC drugs targeting Trop2 (Cardillo et al., 2015; Mao et al., 2016; Strop et al., 2016), which could effectively inhibit tumour growth but not eliminate tumours in a similar BxPC-3 xenograft model, Trop2-

specific CAR T cells exhibited a potent tumour elimination capacity. The functional difference between Trop2-targeted ADC drugs and CAR T cell therapy may be ascribed to CAR T cells being “living drugs” with the capacity of proliferation. Interestingly, we observed that Trop2-specific CAR T cells proliferated when co-cultured with Trop2-positive cells but not with the negative cells (Fig. S3). In addition, CAR T cells targeting Trop2 failed to eliminate tumours in previous studies of gastric cancer (Zhao et al., 2019) and triple-negative breast cancer (Chen et al., 2021), which was probably related to the affinity of scFv for the CAR structure. Our 2F11-scFv bound to Trop2 with high affinity (Kd=0.17 nM), which can be one of the reasons for the thorough tumour clearance.

One of the main hurdles of CAR T cell therapy for solid tumours is the poor infiltration of CAR T cells into tumour tissue (Martinez and Moon, 2019). At present, two kinds of infusion methods, intravenous or intratumoural injection, are mainly used in solid tumour-related in vivo studies. Compared with intratumoural injection, intravenous infusion increases the difficulty of CAR T cells infiltrating solid tumours because of the abundant deposition of extracellular matrix. CAR T cells were injected intratumourally in several CAR T cell-related studies to enhance infiltration (Golubovskaya et al., 2017; Zhao et al., 2019). It has been reported that CD47 CAR T cells injected intratumourally could inhibit pancreatic tumour growth in the same BxPC-3 xenograft model but did not completely remove tumours (Golubovskaya et al., 2017). Thus, intratumoural injection may have better short-term infiltration but not long-term maintenance to eliminate tumours. In the in vivo study, Trop2-targeted CAR T cells were injected only intravenously and then eliminated pancreatic tumours in 3 weeks. We performed immunohistochemical staining with an anti-CD3 antibody on the removed tumours and the tissues at the inoculation site. Unexpectedly, T cell infiltration in the BxPC-3 tumours treated with Trop2 CAR T cells was more abundant than that in the tumours treated with CD19 CAR T cells (Fig. S4), which should partially be ascribed to 2F11 having a high binding capacity for the Trop2 antigen.

The poor persistence of CAR T cells in vivo is another difficulty of CAR T cell treatment. Even though the existing CAR T therapy has greatly improved the complete remission rate of leukaemia and lymphoma, up to one-third of patients eventually relapse (Park et al., 2018), which may be related to the persistence of CAR T cells in vivo (Heng et al., 2020). Surprisingly, no relapse was observed in the pancreatic tumour-bearing mice treated with Trop2 CAR T cells after tumour disappearance, and all mice remained disease-free. We have three hypotheses that may explain this surprising disease-free survival. First, the tumour was completely cleared. Second, no deleterious toxicities were observed. Third, CAR T cells persistently circulated in vivo for 50 days after infusion (Fig. 5c).

Furthermore, one of the factors affecting the persistence of CAR T cells in the clinic is T cell-mediated anti-transgene rejection responses, which will lead to a reduction in CAR-positive T cells. CAR-positive T cells could be detected in the blood of two patients with vesicular lymphoma 24 hours after the first infusion of CD19 CAR T cells but were not detected after 1 week. Even if higher doses of CAR T cells were infused, they could still not survive in vivo. It is speculated that the antitransgene rejection responses may be caused by the use of mouse anti-CD19 scFv on CAR (Jensen et al., 2010). Additionally, Jennifer N et al. reported that the human scFv segment can eliminate antitransgene rejection responses and reduce the

risk for off-tumour toxicity (Song et al., 2015) and neurological toxicity (Brudno et al., 2020). Therefore, Trop2 CAR T cells equipped with the fully human 2F11 scFv may have superior persistence and safety in future clinical applications.

Nevertheless, the cell line xenograft model cannot accurately simulate the complex tumour microenvironment of solid tumours in humans. One limitation of our study was the absence of a Trop2-positive pancreatic tumour patient-derived xenograft (PDX) model in the Trop2 CAR T cell efficacy studies. It is unknown whether Trop2 CAR T cells exert the same antitumour activity and persistence against patient-derived pancreatic tumours. In a future study, we intend to use patient-derived tumour xenograft (PDX) models to evaluate the in vivo antitumour activities of CAR T cells.

Conclusions

We report here the first demonstration of an in vivo antitumour response against pancreatic cancer xenografts using fully human Trop2-redirected CAR T cell therapy. We therefore conclude that patients with pancreatic cancer that express a high level of Trop2 protein may benefit from Trop2-targeted CAR T cell therapy. Moreover, our preclinical studies are conducive to further accelerating the translation of Trop2 CAR T cell therapy to the clinic for pancreatic cancer.

Abbreviations

CAR: Chimeric antigen receptor; Trop2: Tumour-associated calcium signal transducer 2; scFv: Single chain variable fragment; SDS-PAGE, Sodium dodecyl sulphate–polyacrylamide gel electrophoresis; ELISA, Enzyme-linked immune absorbance assay; CFSE, Carboxyfluorescein diacetate succinimidyl ester.

Declarations

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Authors’ contributions

LY, YTW, ZQY and HJZ designed this research and controlled the project schedule. HJZ, JWT and YJJ conducted the experiments and obtained data. HJZ analysed the data, generated the images and wrote the article. IJT, MAM and others were responsible for revision of the manuscript. YJ, XYF and others collected relevant materials, and all the authors read and approved the final manuscript.

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Data availability

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

Ethics approval and consent to participate

All mice were obtained from GemPharmatech and treated under the protocol approved by the Ethics Committee of the Animal Core Facility of East China Normal University.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

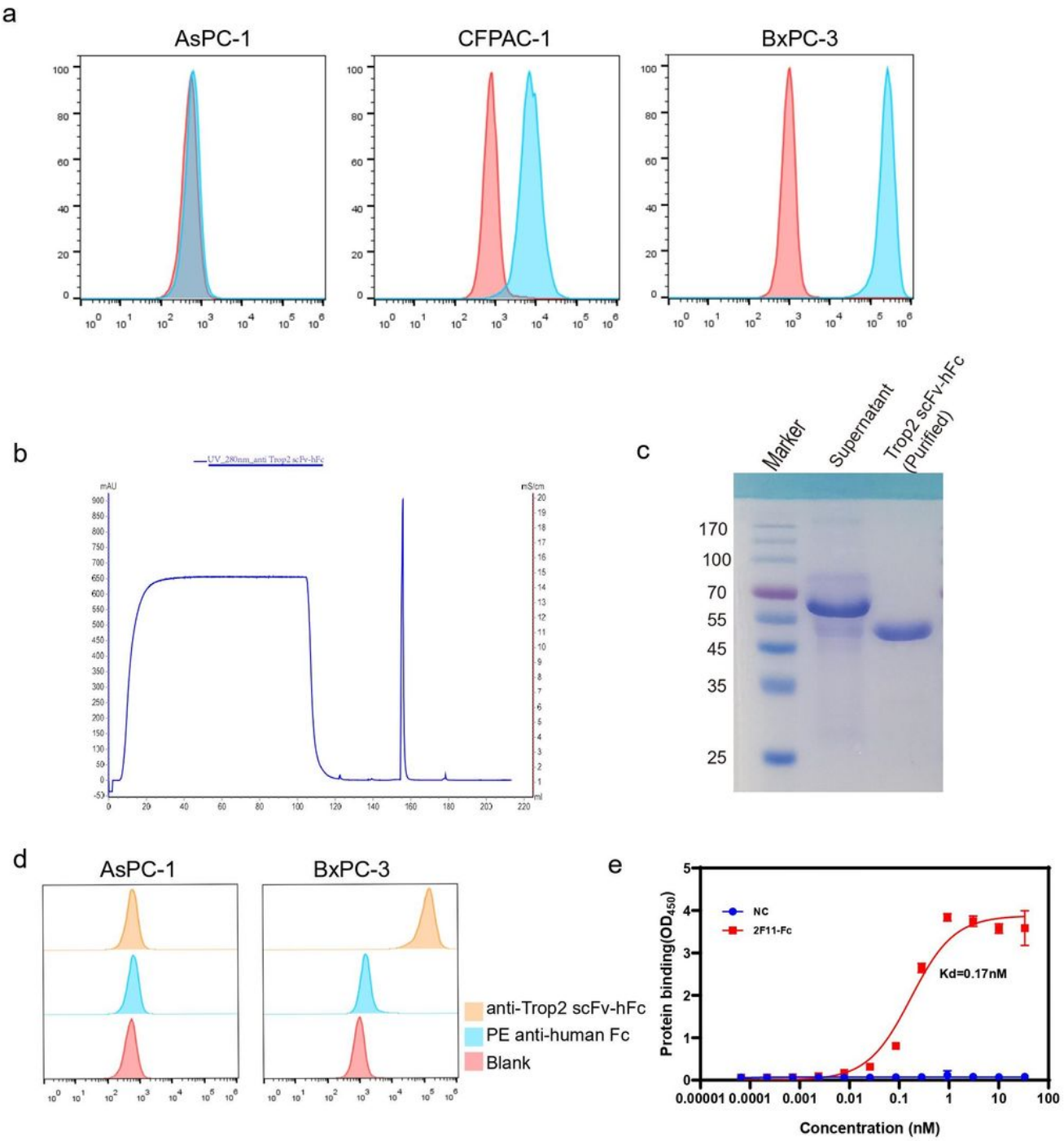

Figure 1

The identification of a fully human Trop2-specific scFv

(a) TROP2 expression in three pancreatic cancer cell lines was determined by flow cytometry using an anti-Trop2 antibody (Biolegend).

(b) UV spectrum of the purified anti Trop2 scFv-Fc.

(c) SDS-PAGE confirmed the purification of anti-Trop2 scFv-hFc.

(d) Flow cytometric analysis of anti-Trop2 scFv-hFc binding to pancreatic cancer cells. A microgram of the anti-Trop2 scFv-hFc was coincubated with one million cells. Antibody binding was detected by phycoerythrin-conjugated anti-human IgG Fc. Blank: only target cells without antibody staining; PE anti-human Fc, only secondary antibody staining.

(e) ELISAs showed the binding properties between anti-Trop2 scFv-hFc and Trop2 EMD.

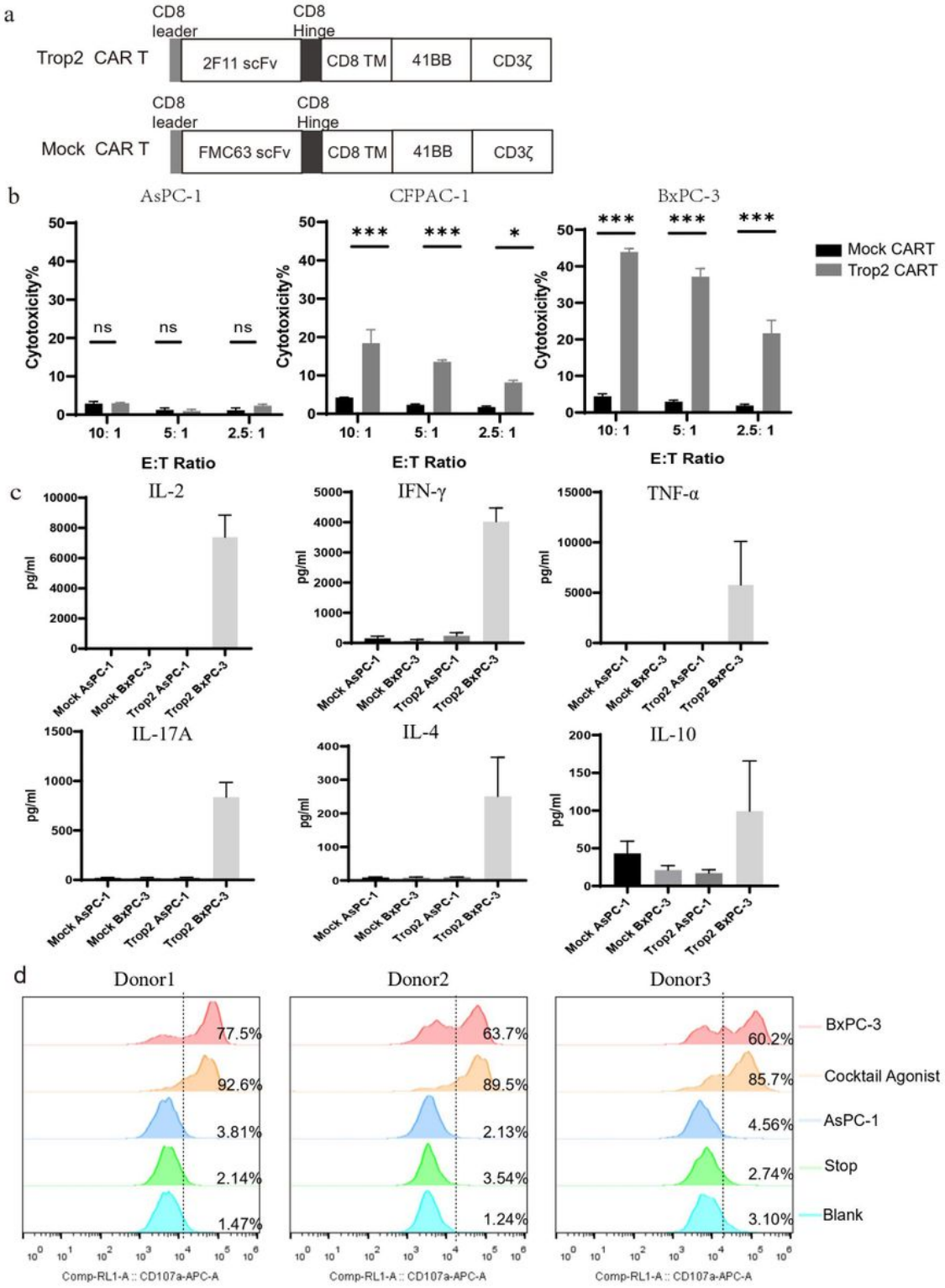

Figure 2

Trop2-specific CAR T cells effectively kill Trop2-positive tumour cells

(a) The structure of Trop2 CAR and CD19 CAR. Second-generation CARs with a 41 BB costimulatory domain were used.

(b) Mock- or Trop2-targeted CAR T cells were coincubated with target cells for 6 h at E:T ratios of 10:1, 5:1 or 2.5:1. Specific cytotoxicity was detected using a standard LDH cytotoxicity assay. Data represented the mean ± SEM. Experiments were repeated three times, and P values were calculated using two-way ANOVA.

(c) CD19- or Trop2-targeted CAR T cells were coincubated with target cells (ASPC-1 and BxPC-3) at a 10:1 E:T ratio; after 6 h, the production of IL-17A, IL-2, IL-4, IL-10, IFN-γ and TNF-α by T cells was determined by flow cytometry (n=3 donors).

(d) CD107a expression in Trop2-targeted CD8+ CAR T cells after induction with target cells (AsPC-1 and BxPC-3) was detected by flow cytometry (n=3 donors, CD19 CAR T data not shown). Blank: only Trop2 CAR T cells. Stop: CAR T cells were coincubated with Golgi inhibitor. Cocktail agonist. CAR T cells were coincubated with stimulation cocktail.

Figure 3

Trop2-targeted CAR T cells effectively inhibit pancreatic cancer xenograft tumour growth and entirely eliminate pancreatic tumours.

(a) Schematic representation of the experiments. BxPC-3 cells were implanted subcutaneously into 6-week-old female NSG mice. Twenty-eight days post-tumour cell implantation, mice were infused intravenously with PBS, Trop2-CAR T or Mock-CAR T cells ($1x10^7$ cells per mouse).

(b) Tumour growth curves during the experiment (n=5). The results are expressed as the mean tumour volume (mm3 ± SD) for the three groups (N=5 per group). The standard deviation (SD) is represented by error bars, ***P < 0.001.

(c) The release of IL-2, IL-4, IL-6, IL-10, IFN-γ, TNF-α and IL-17A in mouse blood was detected at D40.

(d) Mouse image (n=5)

(e) Tumour image (n=5), un, undetectable.

(f) Tumour weight (n=5) in different groups. Tumour weights were analysed by one-way ANOVA, and data represent the mean ± SEM. ***P < 0.001.

(g) Histopathological analysis of murine tumour tissues by haematoxylin and eosin (H&E) staining. At D51, the tumours were collected in the mock and CD19 groups, and the tumours were eliminated in the Trop2 CAR T cell treatment group, so adjacent tissues were collected for staining in the Trop2 group.

(h) Tumour tissues were immunostained using an anti-Trop2 antibody to test the presence of Trop2-positive tumour cells. All tumours in the Trop2 CAR T group were cleared, so we used adjacent tissues for

immunohistochemical staining. Representative photomicrographs are shown. Scale bar is 50 μm.

Figure 4

Safety and toxicology studies of Trop2-specific CAR T cells

(a) Mouse weight curves during the experiment (n=5). CAR T cells did not affect mouse weight in the mock CAR T cell, Trop2-CAR T cell and untreated control groups. Mouse weight was measured in grams three times a week. ns, not significant.

(b) Histopathological analysis of murine organ tissues by haematoxylin and eosin (H&E) staining. NSGs were inoculated with $5x10^6$ BxPC-3 cells. Fifty-one days after inoculation, different organs were harvested, formalin-fixed, paraffin-embedded, and stained with H&E. Representative photomicrographs are shown. Scale bar is 500 μm.

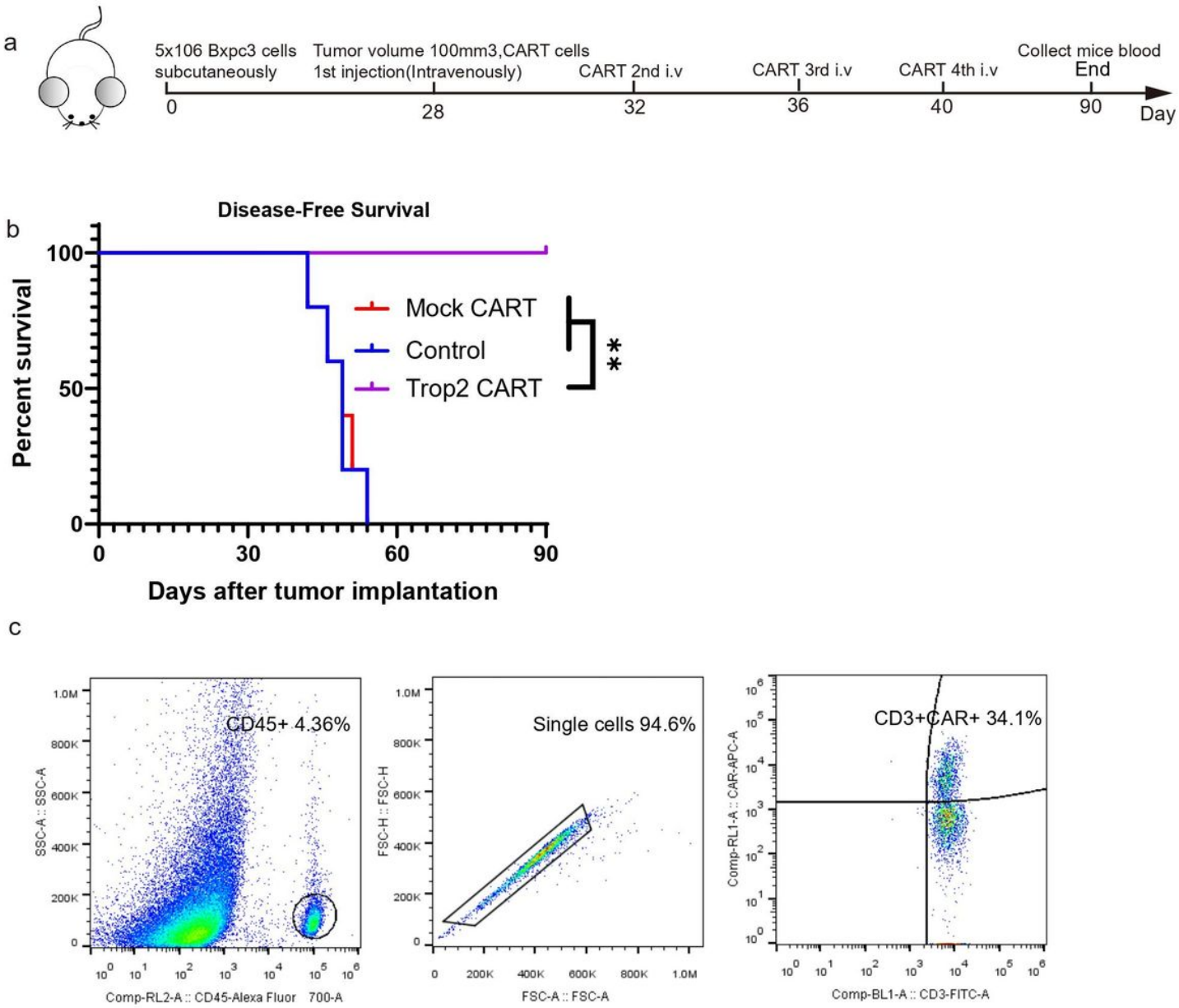

Figure 5

Trop2-specific CAR T cells largely prolongs the disease-free survival of mice with pancreatic tumours.

(a) Schematic representation of the experiments in vivo.

(b) Survival curve of BxPC-3 subcutaneously inoculated mice (n=5). No relapse was observed in the mice treated with Trop2-specific CAR T cells. The results were analysed with the log-rank (Mantel-Cox) test. P value<0.01.

(c) At D90, the proportion of Trop2 CAR-positive cells in the mouse blood were detected by flow cytometry. CD3+CAR+ positive T cells were gated from CD45-positive leukocytes.

Supplementary Files

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