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Original Research

Cell surface GRP78-directed CAR-T cells are effective at treating human pancreatic cancer in preclinical models

Yuncang Yuan [a], Jiawei Fan [a], Dandan Liang [a], Shijie Wang [a], Xu Luo [b], Yongjie Zhu [a], Nan Liu [a], Tingxiu Xiang [c,*], Xudong Zhao [a,*]

[a] *Laboratory of Animal Tumor Models, Frontiers Science Center for Disease-Related Molecular Network, National Clinical Research Center for Geriatrics, West China Hospital, Sichuan University, Chengdu 610041, Sichuan, China*

[b] *Development and Application of Human Major Disease Monkey Model Key Laboratory of Sichuan Province, Sichuan Hengshu Bio-Technology Co., Ltd., Yibin 644600, China*

[c] *Chongqing Key Laboratory of Translational Research for Cancer Metastasis and Individualized Treatment, Chongqing University Cancer Hospital, Chongqing 400030, China*

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ABSTRACT

Pancreatic cancer is a highly lethal solid malignancy with limited treatment options. Chimeric antigen receptor T (CAR-T) cell therapy has been successfully applied to treat hematological malignancies, but faces many challenges in solid tumors. One major challenge is the shortage of tumor-selective targets. Cell surface GRP78 (csGRP78) is highly expressed on various solid cancer cells including pancreatic cancer, but not normal cells, providing a potential target for CAR-T cell therapy in pancreatic cancer. Here, we demonstrated that csGRP78-directed CAR-T (GRP78-CAR-T) cells effectively killed the human pancreatic cancer cell lines Bxpc-3-luc, Aspc-1-luc and MIA PaCa-2-luc, and pancreatic cancer stem-like cells derived from Aspc-1-luc cells and MIA PaCa-2-luc cells *in vitro* by a luciferase-based cytotoxicity assay. Importantly, we showed that GRP78-CAR-T cells efficiently homed to and infiltrated Aspc-1-luc cell-derived xenografts and significantly inhibited pancreatic tumor growth *in vivo* by performing mouse xenograft experiments. Interestingly, we found that gemcitabine treatment increased csGRP78 expression in gemcitabine-resistant MIA PaCa-2-luc cells, and the coapplication of gemcitabine with GRP78-CAR-T cells led to a robust cytotoxic effect on these cells *in vitro*. Taken together, our study demonstrates that csGRP78-directed CAR-T cells, alone or in combination with chemotherapy, selectively and efficiently target csGRP78-expressing pancreatic cancer cells to suppress pancreatic tumor growth.

Introduction

Pancreatic cancer is the seventh leading cause of cancer death worldwide in males and females [1] and is known as "the king of cancers" because of its extremely poor survival rate. The 5-year survival rate for pancreatic cancer in the United States is reported to be concerningly low, at 11 % [2]. Pancreatic ductal adenocarcinoma (PDAC) is the most common type of pancreatic cancer and accounts for more than 90 % of such cases [3]. In terms of the clinical management of pancreatic cancer, postoperative adjuvant chemotherapy or preoperative neoadjuvant therapy has been recommended and can prolong postoperative survival among PDAC patients; however, less than 20 % of such patients have resectable tumors [4,5]. For over 80 % of PDAC patients with locally advanced or metastatic disease, the combination of palliative chemotherapy and supportive care remains the main therapeutic approach to clinical management, yet overall response rates are limited [5]. Immunotherapy with immune checkpoint inhibitors (ICIs) was recently reported to be effective for various cancers. While, only patients with high microsatellite instability or mismatch repair deficiency (MSI-H/dMMR), accounting for less than 3 % of all PDAC patients, can benefit from this therapy [6], most patients often develop drug resistance due to the immunosuppressive tumor microenvironment (TME), which is

Abbreviations: GRP78, Glucose-regulated protein 78 KD; csGRP78, cell surface GRP78; CAR-T, chimeric antigen receptor T cell; PDAC, pancreatic ductal adenocarcinoma; PASC, pancreatic adenosquamous carcinoma; PSCC, pancreatic squamous cell carcinoma; PMC, pancreatic mucinous carcinoma; FDA, US Food and Drug Administration; TMA, tissue microarray; CSCs, cancer stem-like cells; PCSCs, pancreatic cancer stem-like cells.

* Corresponding authors.
E-mail addresses: xiangtx@cqmu.edu.cn (T. Xiang), zhaoxudong@wchscu.cn (X. Zhao).

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characterized by the lack of high-quality effector intratumoral T cells [7, 8]. Malnutrition has a detrimental impact on the outcomes and survival of pancreatic cancer patients [9,10]. It has been shown that nutrition support provided within 3 months from diagnosis may improve the survival of PDAC patients [9]. However, more clinical investigation is needed to assess this potential. Other therapies named irreversible electroporation (IRE) or anti-stromal therapy have yielded serious complications or limited success in patients with PDAC [11,12]. Therefore, improved treatments are urgently needed to increase survival rates for PDAC patients, especially those with advanced PDAC.

Chimeric antigen receptor T (CAR-T) cell therapy is emerging as an effective cancer treatment, with several such treatments approved by the US Food and Drug Administration (FDA) to treat hematological malignancies, such as the most representative B cell malignancies [13]. Currently, CAR-T cell therapy is being explored for the treatment of solid tumors, including pancreatic cancer, with mixed results. The reasons are multifaceted; however, one significant hurdle is the shortage of selective antigenic targets strongly expressed on cancer cells but with sufficiently low expression on normal cells to eliminate on-target off-tumor toxicities. Recent clinical trials for CAR-T cell therapy based on targeting antigens such as CD133 (also referred to as prominin-1), epidermal growth factor receptor (EGFR), human epidermal growth factor receptor 2 (HER2), mesothelin (MSLN) and Claudin18.2 on PDAC have shown limited efficacy, with no improvement to slight improvement in overall survival [14,15].

Glucose-regulated protein 78 KD (GRP78, also referred to as Bip) is encoded by the *HSPA5* gene and is a member of the heat shock protein 70 (HSP70) family. Typically, GRP78 is localized within the endoplasmic reticulum lumen; however, when a cell is subject to stress or insult, GRP78 is partially transferred to the cytoplasm, nucleus, mitochondria, cell membrane surface or even secreted outside the cell [16]. Indeed, cell surface GRP78 (csGRP78) is widely studied, especially as a marker for cancer. The cell surface localization of GRP78 was first reported in malignant T lymphocytes [17], and since its discovery in 1997, it has been recognized that csGRP78 is expressed on various cancer cell types, including pancreatic cancer cell lines [18]. A preliminary study also suggested csGRP78 expression in pancreatic cancer tissues [19]. Notably, researchers have described preferential expression of GRP78 on the surface of cancer cells but not normal cells *in vivo* [20]. Altogether, these reports suggest that csGRP78 may be a potential target for pancreatic cancer treatments such as CAR-T therapy with minimal side effects on noncancer cells.

In this study, we conducted an analysis of csGRP78 expression in a large sample size of pancreatic cancer tissues to investigate its selective expression in tumor specimens. Also, we evaluated the antitumor activity of csGRP78-directed CAR-T (GRP78-CAR-T) cells against pancreatic cancer cells *in vitro* and *in vivo*. We found that csGRP78 expression was upregulated in pancreatic cancer tissues, and GRP78-CAR-T cells showed potent cytotoxic potential for pancreatic cancer cells with moderate to high csGRP78 expression, as well as for pancreatic cancer stem-like cells expressing csGRP78. In addition, given that gemcitabine could induce csGRP78 expression in pancreatic cancer cells, which in turn resulted in resistance [21], we further explored the possibility of GRP78-CAR-T cells in tackling the problem of gemcitabine resistance. We found that GRP78-CAR-T cells could kill gemcitabine-resistant pancreatic cancer cells, and this cytotoxicity was more pronounced when target cells were pretreated with gemcitabine. Our findings suggest that csGRP78 is an attractive target antigen for CAR-T cell therapy of pancreatic cancer, and GRP78-CAR-T cells alone or in combination with the chemotherapeutic drug gemcitabine provide potent and effective therapeutic options for gemcitabine-resistant pancreatic cancer.

Materials and methods

Human subjects, tissue collection and immunofluorescence histochemistry studies

A tissue microarray (TMA) comprising 37 pancreatic cancer tissues and 23 para-cancerous tissues, was obtained from Shanghai Outdo Biotech Co., Ltd. (Shanghai, China). Samples from the thirty-seven patients were subdivided into four subtypes, including 32 pancreatic ductal adenocarcinomas (PDACs), 1 pancreatic adenosquamous carcinoma (PASC), 1 pancreatic squamous cell carcinoma (PSCC), and 3 mixed PDAC and pancreatic mucinous carcinoma (PMC). Dual immunofluorescence histochemistry staining was performed using the TSA-Kit (Perkin Elmer, USA). Briefly, the TMA chip was deparaffinized with xylene and rehydrated through a graded ethanol series and distilled water. Antigen retrieval was carried out using citrate buffer (1:100, Maxim, China) at high pressure for 15 min. Endogenous peroxidase was neutralized with 3 % H_2O_2 diluted in PBS for 15 min. The chip was then incubated with blocking solution (0.25 % BSA, 10 % goat serum and 0.3 % Triton-100 in PBS) for 1 h, followed by incubation with a rabbit-specific anti-GRP78 antibody (1:200, PA1–014A, Thermo Fisher, USA) overnight at 4 °C. The unbound anti-GRP78 antibody was washed off with PBST (0.1 % Tween-20 in PBS), followed by incubation with HRP-conjugated goat-anti-rabbit secondary antibody (1:1000, Abcam, USA) for 2 h. The samples were then washed in PBST and incubated with Cy3-conjugated tyramine in amplification diluents (1:50) provided in the TSA kit for 10 min, after which unbound TSA was removed by washing with PBST. Next, the anti-GRP78 antibody was stripped using citrate buffer at high pressure for 15 min. The cells were incubated with rabbit anti-β-catenin antibody (1:200, ER0805, HUABIO, China) overnight at 4 °C. After washing with PBST, Alexa Fluor® 488-conjugated goat anti-rabbit secondary antibody (1:1000, Thermo Fisher, USA) was added to the tissues and incubated for 2 h. Following final washes in PBST, DAPI stain (1:500) was applied for visualizing cell nuclei. Representative pictures (five visual fields per tissue) were obtained with an inverted Nikon confocal microscope A1 under 60× magnification. For colocalization analysis, Manders' colocalization coefficients were calculated using the JACoP plugin in Fiji software (version 2.0.0). The value of the substrate background in the β-catenin channel was calibrated to minimize interference of cytoplasmic and nuclear β-Catenin signals while preserving the membrane immunofluorescence signal as much as possible. Manders' colocalization coefficients determine the rate of the fraction of β-catenin overlap with GRP78 in all fractions of β-catenin. Five randomly selected fields from individual tissue were included to calculate an average.

Cell lines and culture

The human pancreatic cancer cell lines Aspc-1, Bxpc-3, and Panc-1 were purchased from the Kunming Cell Bank, Kunming Institute of Zoology, Chinese Academy of Sciences. The human pancreatic cancer cell line MIA PaCa-2 was purchased from Shanghai Cell Bank, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. Human embryonic kidney 293T cells were a gift from A. Lasorella (The Institute for Cancer Genetics, Columbia University Medical Center, New York). All cell lines used in this study were validated by short tandem repeat (STR) profiling and testing with a Mycoplasma Detection Kit to avoid such contamination. All cell lines except Aspc-1 were cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, USA) supplemented with 10 % fetal bovine serum (FBS) (Gibco, USA) and 2 % penicillin–streptomycin (P/S). Aspc-1 cells were grown in RPMI-1640 medium supplemented with 10 % FBS and 2 % P/S. All cancer cell lines were used to derive stable, luciferase-expressing lines that were maintained in similar culture conditions as their reference cells.

Screening and identification of pancreatic cancer stem-like cells

Aspc-1-luc cells or MIA PaCa-2-luc cells were cultured in serum-free DMEM/F12 (1:1) supplemented with EGF (20 ng/ml), bFGF (20 ng/ml), and B27 (1:50) growth factors. After three to four weeks in culture, stem-like spheroid cells which were suspended in culture medium formed and were collected and expanded for at least three passages. The stemness of spherical cells was confirmed by detecting the expression levels of the pancreatic cancer stem-like cell markers CD133, CD24 and CXCR4 using qPCR. 18S ribosomal RNA (18S rRNA) was used as an endogenous reference gene. The primer sequences were as follows: CD133 forward primer, 5′-AGT CGG AAA CTG GCA GAT AGC-3′, and reverse primer, 5′-GGT AGT GTT GTA CTG GGC CAA T-3′; CD24 forward primer, 5′-CTC CTA CCC ACG CAG ATT TAT TC-3′, and reverse primer, 5′- AGA GTG AGA CCA CGA AGA GAC-3′; CXCR4 forward primer, 5′- AAA CTG AGA AGC ATG ACG GAC AA-3′, and reverse primer, 5′- GCC AAC ATA GAC CAC CTT TTC AG-3′; 18S rRNA forward primer, 5′-GTA ACC CGT TGA ACC CCA TT-3′, and reverse primer, 5′-CCA TCC AAT CGG TAG TAG CG-3′.

RNA isolation and qPCR

Total RNA was isolated from cells using TRIzol reagent (Sigma, USA). cDNA synthesis was performed using a cDNA synthesis kit (Invitrogen, Thermo Fisher, USA). qPCR was run using SYBR Green qPCR Mix (Applied Biosystems, Thermo Fisher, USA) on a QuantStudio 3 (Applied Biosystems, Thermo Fisher, USA). All steps followed the manufacturer's protocols.

Generation of GRP78-CAR

A Pep42 peptide-based second-generation GRP78-CAR expression vector has been constructed in our laboratory [22]. Briefly, Pep42 (CTVALPGGYVRVC) was reverse-translated to cDNA sequence and codon optimized by GeneOptimizer. Internal restriction sites for AgeI and NHeI were removed by site-directed mutagenesis. A DNA fragment encoding the CD8 signaling peptide with an AgeI restriction site was added upstream of the target sequence, while a DNA fragment encoding the CD8 hinge as well as an NHeI site was added downstream of the target sequence. The whole DNA fragment was synthesized by BGI, China, and cloned into a pTomo-CAR backbone reconstructed in our laboratory by replacing the fragment between XbaI and SalI in the pTomo empty backbone (Addgene) with the synthesized DNA fragment XbaI-AgeI-CD8 signaling peptide (SP)-CD8 hinge containing the NheI-CD8 transmembrane domain (TM)-4-1BB costimulatory domain (4-1BB)-CD3ζ activation domain (CD3ζ)-Thosea asigna virus-derived 2A self-cleaving peptide (T2A)-far-red fluorescence protein mKate2 (mKate2)-SalI. An Fmc63 scFv-based pTomo-CD19-CAR expression plasmid previously cloned using a similar approach in our laboratory was used as a negative control.

Generation of human CAR-expressing primary T cells

Lentiviral particles were generated by transfecting HEK293T cells with pTomo-CAR-mKate2, pMD2.G (Addgene) and pCMV8.9 (Addgene). Culture supernatants were collected at 48 h and 72 h, and lentivirus particles were concentrated by ultracentrifugation at 25,000 rpm and 4 °C for 2.5 h. Lentiviral particles were suspended in 60 μl PBS supplemented with 0.1 % BSA and stored frozen at −80 °C. Human T cells were enriched by negative selection with a RosetteSep Human T Cell Enrichment Cocktail (StemCell Technologies, Canada) from peripheral blood mononuclear cells of healthy volunteers. The purity of the isolated cells was examined by flow cytometry with CD3 staining using a BD LSRFortessa, USA. CD3+ T cells were activated with Dynabeads coated with anti-CD3 and anti-CD28 antibodies (Life Technologies, USA) and expanded in advanced RPMI 1640 medium (Thermo Fisher, USA) supplemented with 10 % FBS, 2 % P/S, 1% L-glutamine and 200 U/ml IL-2 (PeproTech, USA) for three days. T cells were then transduced with CD19-CAR lentivirus at a multiplicity of infection (MOI) of 20 or at an MOI of 100 for GRP78-CAR lentivirus in the presence of 1:100 LentiBOOST solution (Sirion Biotech, Germany). Four days post transduction, total T cells were collected for *in vitro* or *in vivo* experiments. The CAR expression ratio was determined by counting mKate2-positive T cells using a BD LSRFortessa.

Immunofluorescence staining assays

Prior to immunostaining, Bxpc-3-luc cells (0.2×10^5), Aspc-1-luc cells (1×10^5), MIA PaCa-2-luc cells (1×10^5), or Panc-1-luc cells (0.5×10^5) were seeded onto glass coverslips and incubated in media for 24 h, while pancreatic cancer stem-like cells (1×10^5) were plated on laminin-coated coverslips and cultured for 24 h. Live cells were directly incubated with anti-GRP78 antibody (1:200, PA1–014A, Thermo Fisher, USA) diluted in PBS containing 2 % FBS for 1 h. After washing with PBS, the cells were fixed with 4 % paraformaldehyde for 15 min. Following fixation, an antigen blocking step was performed with a solution of 10 % goat serum, 2.5 % BSA, and 0.1 % Tween-20 in PBS (1 hour). Thereafter, the cells on coverslips were incubated with goat anti-rabbit Cy3 secondary antibody (1:1000, Thermo Fisher, USA). Nuclei were stained with DAPI for visualization. Antibody incubation and blocking steps were performed using an orbital shaker set at a modest speed. All steps were processed at room temperature. Images (five visual fields per cell line) were captured using an inverted Nikon confocal microscope A1 under ×120 magnification. The mean cellular fluorescence was calculated using Fiji software.

Luciferase-based cytotoxicity assay in vitro

To assess the cytotoxicity of effector T cells to target cells, nontransduced T cells, CD19-CAR-T cells or GRP78-CAR-T cells, each of these T-cell preparations was cocultured with 2000 luciferase-expressing target cells in a 96-well black plate at the desired effector-to-target ratio (E:T) for 24 h. Three duplicate wells for each combination were set up. Supernatants were harvested and frozen at −80 °C for cytokine release detection. Cytotoxicity was quantified using a luciferase assay kit (Promega, USA). The lysis ratio of each treatment was calculated using the following formula: % lysis=100–100×(single luciferase value $_{(\text{NTD, CD19-CAR-T or GRP78-CAR-T})}$/average $_{(\text{NTD})}$).

Cytokine release assay

IFNγ release by nontransduced T cells, CD19-CAR-T cells or GRP78-CAR-T cells was measured by assaying the supernatant of cocultures using a commercial ELISA kit (Invitrogen) according to the manufacturer's protocol.

Gemcitabine treatment

Gemcitabine (10 mM) in DMSO (LY 188011) was purchased from MedChemExpress (Shanghai, China). To assess whether gemcitabine could induce csGRP78 expression in pancreatic cancer cells, MIA PaCa-2-luc cells (1×10^5) and Panc-1-luc cells (0.5×10^5) were seeded onto coverslips for 24 h followed by 0.25 μM or 1 μM gemcitabine treatment for another 24 h before immunofluorescence staining was performed as described above. To assess the potential for gemcitabine to enhance the cytotoxic effects of nontransduced T cells, CD19-CAR-T cells or GRP78-CAR-T cells on pancreatic cancer cells, MIA PaCa-2-luc cells (5×10^5) were first seeded onto a 6-well plate and cultured for 24 h before media with 0.5 μM gemcitabine was added. Cultures were incubated for another 24 h before a luciferase-based cytotoxicity assay was performed as described above.

Xenograft assay

Six-week-old female NOD-Prkdcem26Cd52Il2rgem26Cd22/Nju (NCG, T001475) mice with severe immunodeficiency were purchased from GemPharmatech Co., Ltd. of Nanjing (China) and housed in a specific pathogen-free environment at the Laboratory Animal Center of West China Hospital. To establish a pancreatic cancer cell line xenograft model, a preparation of 5×10^5 Aspc-1-luc cells suspended in PBS containing 20 % Matrigel (Corning, CA, USA) was injected subcutaneously into the left flank of recipient mice. Seven days after injection, the mice were randomly divided into three groups (*i.e.*, the nontransduced T cell group, CD19-CAR-T-cell group, and GRP78-CAR-T-cell group) by flipping a coin and then intravenously injected with a single dose of 5×10^6 nontransduced T cells, CD19-CAR-T cells, or GRP78-CAR-T cells. The sample size per group was referenced from previous studies regarding CAR-T cells therapy in mice [23]. No specific blinding method was used during mouse experiments. For long-term experiments, tumor burden was monitored by IVIS bioluminescence imaging every 6∼7 days, and animal weights were recorded. For short-term experiments that focused on detecting the capacity for CAR-T cells to infiltrate tumors *in vivo*, mice were euthanized at day 4 post CAR-T-cell injection. Tumors and main organs, including the heart, liver, spleen, and lung, were collected and processed for human CD3 immunofluorescence staining (1:200, ET1607-29, HUABIO) and H&E staining. The number of human $CD3^+$ cells was counted by Fiji software.

Statistical analysis

Data are presented as the mean values ± standard deviations (SD) from at least two independent experiments. Before statistical analysis, the homogeneity of variance between groups was tested using Levene's test. Statistical significance between two independent groups was tested using a two-tailed independent Student's *t* test. For multiple comparisons, one-way ANOVA was used followed by Tukey's test corrected for multiple comparisons, and the homogeneity of variance test was performed before analysis. All statistical analyses and graphic generation were completed in GraphPad Prism 8.0. $p \geq 0.05$ was considered not significant (ns); $p<0.05$ was considered significant (*); and $p < 0.01$ was considered highly significant (**).

Results

Cell surface GRP78 expression is significantly elevated in pancreatic cancer tissues

Previous work has indicated that csGRP78 is expressed in pancreatic cancer tissues [19], but the evidence remains anecdotal. We thus performed a study to investigate the expression of csGRP78 in pancreatic

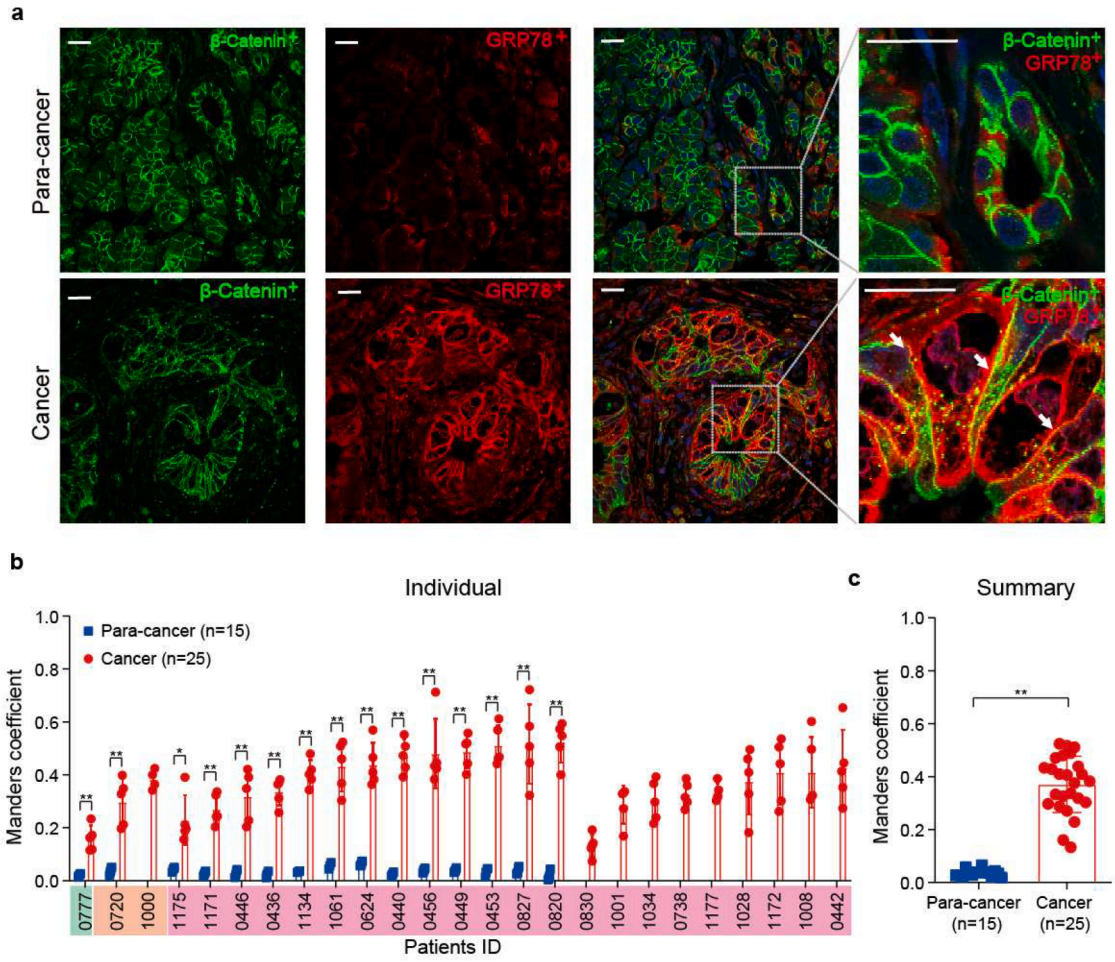

Fig. 1. Expression of csGRP78 in pancreatic cancer tissues. A pancreatic cancer tissue microarray was double-stained with anti-GRP78 (red) and anti-β-catenin (green) antibodies. a Representative confocal image of para-cancer and cancer tissues. White arrows indicate csGRP78 expression colocalized with β-catenin (scale bar, 20 μm). b Manders' colocalization analysis representing csGRP78 expression for individuals (five visual fields per tissue). A sample from a pancreatic squamous cell carcinoma (PSCC) patient is labeled with a green box, mixed pancreatic ductal adenocarcinoma (PDAC) and pancreatic mucinous carcinoma (PMC) patients are labeled with an orange box, and PDAC patients are labeled with a pink box. **c** Summary of average csGRP78 expression in all pare-cancer tissues and cancer tissues. Data are represented as the mean ± SD. Statistical significance for individual or all samples was determined by a two-sided unpaired *t* test. *$p < 0.05$; **$p < 0.01$.

cancer tissues using a pancreatic cancer tissue microarray (TMA) that collected samples from 37 patients. In 23 of the 37 patients, both cancer tissues and adjacent noncancer tissues (which we describe in this study as "para-cancer tissue") were collected. To quantify csGRP78 expression, we performed fluorescence immunostaining assays followed by Manders' colocalization analysis with β-catenin, an epithelial cell membrane marker for multiple tissues, including the pancreas [24,25]. One caveat is that membrane-associated β-catenin can partially translocate to the nucleus; however, the majority of β-catenin signals remain conspicuously visible on the external membrane of cells [26,27], which justifies its selection as a cell membrane marker of pancreatic cancer tissues for the quantitative analysis of csGRP78 expression on cell membranes. Following exclusion of 12 tissue samples for criteria including staining, tissue pathology, and methodological issues (*e.g.*, tissues falling off glass slides), 25 tissue samples were eventually evaluated for csGRP78 expression. Representative micrographs of para-cancer tissue and cancer tissue are presented in Fig. 1a. Staining of para-cancer tissue showed low GRP78 expression and weak colocalization of the GRP78 immunofluorescence signal with the membrane β-catenin signal. In contrast, GRP78 expression was significantly increased in cancer tissue, and colocalization of the GRP78 immunofluorescence signal with the membrane β-catenin signal was significant. We performed Manders' colocalization analysis for samples from 15 patients with cancer tissues and noncancer tissues and found that csGRP78 expression was significantly increased in each cancer tissue compared with their corresponding paired para-cancer tissue samples (Fig. 1b). We also performed an integrated analysis of all samples and observed significantly elevated csGRP78 expression in the cancer tissue group compared to the para-cancer tissue group (0.035 *vs* 0.371; $p < 0.01$) (Fig. 1c). Representative micrographs of the tissues used in these

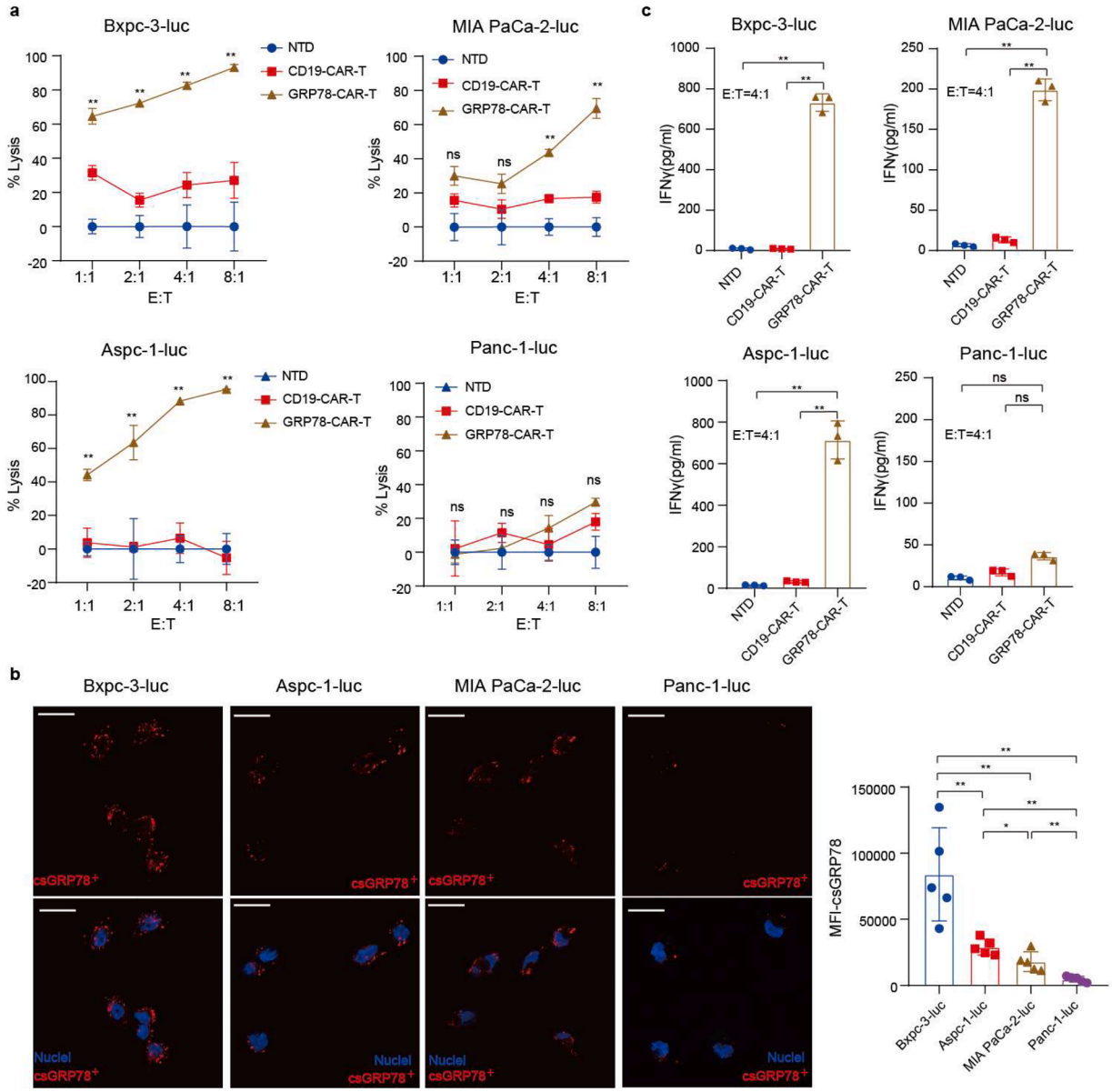

Fig. 2. GRP78-CAR-T cells efficiently lysed pancreatic cancer cells with moderate or high csGRP78 expression *in vitro*. a Cytotoxicity of nontransduced T cells, CD19-CAR-T cells and GRP78-CAR-T cells against luciferase-expressing pancreatic cancer cell lines Bxpc-3-luc, Aspc-1-luc, MIA PaCa-2-luc, and Panc-1-luc at various effector-to-target ratios (1:1, 2:1, 4:1, and 8:1). (b, left) Representative confocal fluorescence microscopy images of four luciferase-expressing pancreatic cancer cell lines expressing csGRP78 (scale bar, 20 μm). (b, right) The mean fluorescence intensity (MFI) of csGRP78 staining per cell. c IFNγ secretion in the supernatants of cocultures obtained from the cytotoxicity assay (E:T, 4:1). Data are shown as the mean ± SD. Statistical significance was determined by one-way ANOVA followed by Tukey's test. $^{**}p < 0.01$; ns, not significant.

quantification studies are shown in Fig. S1a and b. Thus, our results provide strong evidence that csGRP78 expression is elevated in pancreatic cancer tissues compared with para-cancer tissues.

GRP78-CAR-T cells efficiently lyse human pancreatic cancer cells expressing csGRP78

Schematic diagrams of the CAR expression plasmids used in this study are shown in Fig. S2. To construct GRP78-CAR-T cells or CD19-CAR-T cells, we isolated $CD3^+$ T cells from healthy donor peripheral blood mononuclear cells and activated $CD3^+$ T cells with magnetic beads coated with anti-CD3 and anti-CD28 antibodies for three days, after which the cells were transduced with lentiviruses expressing GRP78-CAR or CD19-CAR. Next, we tested the potential dose-dependent effects of GRP78-CAR-T cells on four conventional human pancreatic cancer cell lines, namely, Bxpc-3-luc cells, Aspc-1-luc cells, MIA PaCa-2-luc cells, and Panc-1-luc cells, at effector-to-target ratios of 1:1, 2:1, 4:1, and 8:1. As shown in Fig. 2a, with increasing effect-to-target ratios, GRP78-CAR-T cells showed significant dose-dependent killing effects on Bxpc-3-luc cells, Aspc-1-luc cells, and MIA PaCa-2-luc cells, but this

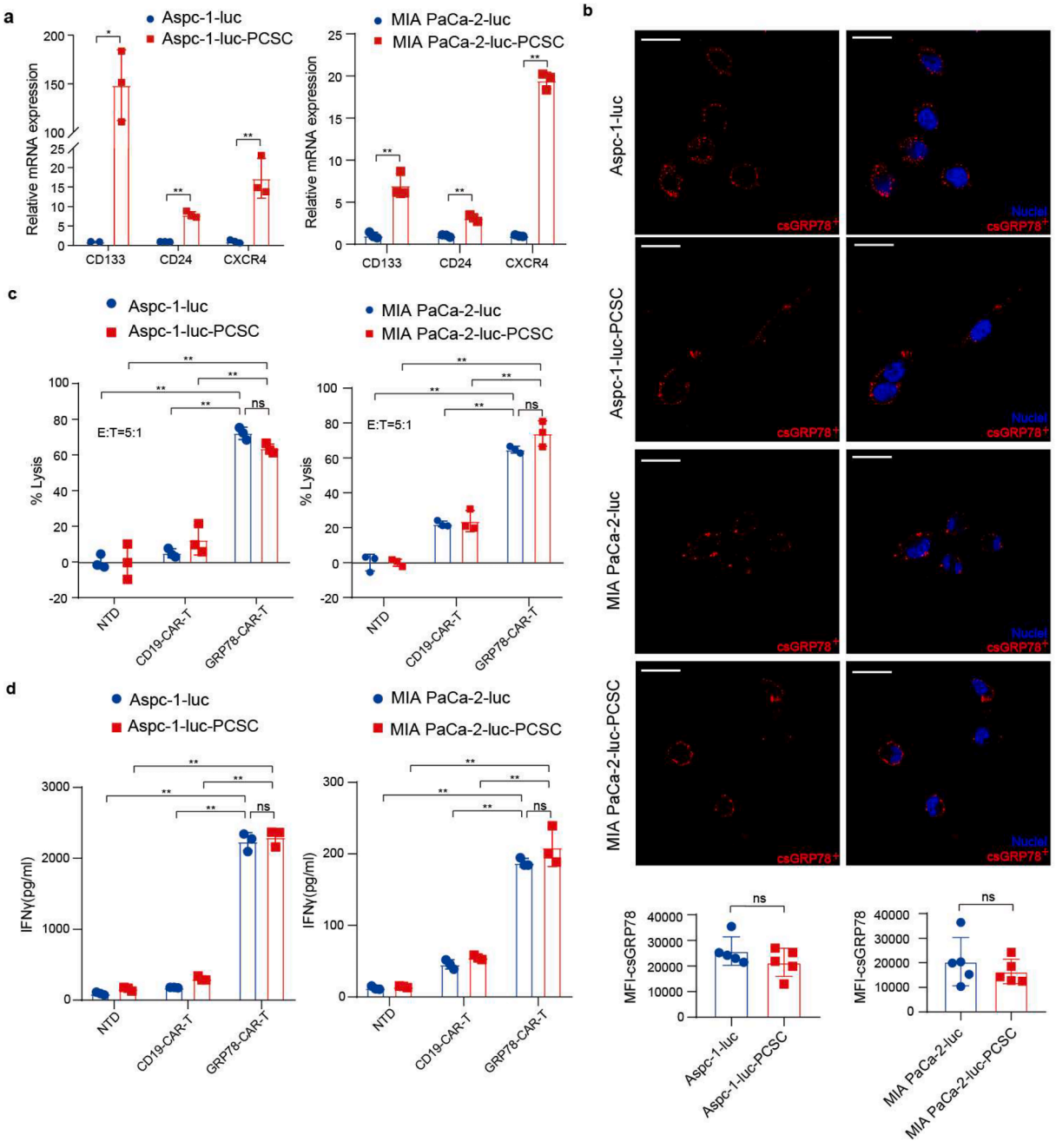

Fig. 3. GRP78-CAR-T cells show potent cytotoxicity against pancreatic cancer stem-like cells. a Characterization of markers for stemness in Aspc-1-luc cell-derived stem-like cells and MIA PaCa-2-luc cell-derived stem-like cells by qPCR analysis of CD133, CD24 and CXCR4. (b, top) Representative confocal images of Aspc-1-luc cell-derived stem-like cells, MIA PaCa-2-luc cell-derived stem-like cells, and their primary cells illustrating csGRP78 immunostaining (scale bar, 20 μm). (b, bottom) The mean fluorescence intensity (MFI) of csGRP78 staining per cell. c Cytotoxicity of nontransduced T cells, CD19-CAR-T cells or GRP78-CAR-T cells against Aspc-1-luc cell-derived pancreatic cancer stem-like cells, MIA PaCa-2-luc cell-derived pancreatic cancer stem-like cells, and their primary cells at 24 h of coculture (E:T, 5:1). **d** Detection of IFNγ release in the coculture supernatant obtained from the cytotoxicity assay. Data are shown as the mean ± SD. Statistical significance was determined by two-sided unpaired *t*-test (a) and one-way ANOVA followed by Tukey's test (c, d). $^{**}p < 0.01$; ns, not significant.

killing effect on Panc-1-luc cells was limited. Notably, we performed immunofluorescence staining and found that the killing efficiency of GRP78-CAR-T cells on individual cancer cell lines was correlated with the level of immunostained csGRP78 expression. As shown in Fig. 2b, Bxpc-3-luc cells and Aspc-1-luc cells expressed high levels of csGRP78, MIA PaCa-2-luc cells expressed moderate levels of csGRP78, and Panc-1-luc cells expressed low levels of csGRP78. Correspondingly, ELISA detection of IFNγ secretion in the coculture supernatant with an effect-to-target ratio of 4:1 showed a significantly higher concentration of IFNγ secretion by GRP78-CAR-T cells than by nontransduced T cells or CD19-CAR-T cells after coculture with Bxpc-3-luc cells, Aspc-1-luc cells or MIA PaCa-2-luc cells. In contrast, no significant IFNγ secretion was detected by GRP78-CAR-T cells compared to nontransduced T cells or CD19-CAR-T cells following coculture with Panc-1-luc cells (Fig. 2c). These results suggest a csGRP78-specific dose-dependent cytotoxic effect of GRP78-CAR-T cells on human pancreatic cancer cells.

GRP78-CAR-T cells display cytotoxicity against cancer stem-like cells derived from pancreatic cancer cell lines

Cancer stem-like cells (CSCs) represent a distinct subpopulation of cancer cells that drive tumor formation, tumor heterogeneity, metastasis, and relapse [28,29]. Currently, an *in vitro* culture protocol utilizing serum-free DMEM/F12 supplemented with EGF, bFGF, and B27 has been developed to promote the enrichment of CSCs [30], which are observed as spheroids that are capable of self-renewal and differentiation. Therefore, we used this CSC culture approach to assess the cytotoxicity of GRP78-CAR-T cells against pancreatic cancer stem-like cells (PCSCs) derived from Aspc-1-luc cells and MIA PaCa-2-luc cells. To confirm the successful derivation of PCSCs, we analyzed the expression of CD133, CD24 and CXCR4 by qPCR [31,32]. When we studied samples of Aspc-1-luc cells and MIA PaCa-2-luc cells maintained with or without CSC culture conditions, we found that for these respective cell lines, CD133 expression was upregulated 148.6-fold and 6.7-fold, CD24 was upregulated 7.9-fold and 3.1-fold, and CXCR4 was upregulated 16.4-fold and 19.4-fold, as shown in Fig. 3a. Then, we examined csGRP78 expression in these putative PCSCs compared to that in non-PCSCs by immunofluorescence. Interestingly, we found no significant differences in csGRP78 signaling properties between PCSCs and non-PCSCs for either the Aspc-1-luc or MIA PaCa-2-luc cell line (Fig. 3b). Next, we evaluated the cytotoxicity of GRP78-CAR-T cells against PCSCs using a cytotoxicity assay. We found that GRP78-CAR-T cells but not nontransduced T cells or CD19-CAR-T cells had significant killing efficiency against both Aspc-1-luc cell-derived PCSCs and MIA PaCa-2-luc cell-derived PCSCs (Fig. 3c). Furthermore, we showed that for both the Aspc-1 and MIA PaCa-2 cell lines, the killing rates of GRP78-CAR-T cells on PCSCs derived from both cell lines were comparable to their cytotoxic effects on non-PCSCs from each corresponding cell line (Fig. 3c). Concomitantly, we observed that cytokine IFNγ release by GRP78-CAR-T cells was significantly increased compared with that by nontransduced T cells or CD19-CAR-T cells after coculture with Aspc-1-luc cell-derived PCSCs or MIA PaCa-2-luc cell-derived PCSCs, while IFNγ secretion levels by GRP78-CAR-T cells were not significantly different upon exposure to PCSC cultures *versus* non-PCSC cultures for both cell lines (Fig. 3d). Therefore, our results indicate that GRP78-CAR-T cells possess potent cytotoxic activity against pancreatic cancer cell lines as well as PCSCs.

Targeting csGRP78 with GRP78-CAR-T cells overcomes gemcitabine resistance in pancreatic cancer

Clinically, gemcitabine is used to treat pancreatic cancer patients. However, acquired resistance to gemcitabine remains a challenge in pancreatic cancer patients [33]. It has been reported that prolonged gemcitabine exposure can induce csGRP78 expression in pancreatic cancer cells, which in turn leads to gemcitabine resistance [21]. Thus, we reasoned that a combination of gemcitabine exposure with GRP78-CAR-T cell treatment may result in an enhanced cytotoxic effect on pancreatic cancer cells with induced csGRP78 expression. To test this possibility, we first exposed human pancreatic cancer cell lines, which were reported to be insensitive to gemcitabine treatment [34], to gemcitabine to observe its effects on csGRP78 expression by performing immunofluorescence staining and found that the csGRP78 immunofluorescence signal was not significantly different in Panc-1-luc cells treated with gemcitabine (data not shown). In contrast, we observed a significant increase in the csGRP78 immunofluorescence signal in MIA PaCa-2-luc cells exposed to gemcitabine (Fig. 4a). Next, we investigated the killing efficiency of GRP78-CAR-T cells against MIA PaCa-2-luc cells treated with gemcitabine at an effector-to-target ratio of 1:1 using a cytotoxicity assay. We observed that the cytotoxic effect of GRP78-CAR-T cells on MIA PaCa-2-luc cells preexposed to gemcitabine was significantly enhanced, while no such effects were observed for cells treated with nontransduced T cells or CD19-CAR-T cells, irrespective of gemcitabine preexposure (Fig. 4b). Correspondingly, we found that cytokine IFNγ release by GRP78-CAR-T cells cultured with MIA PaCa-2-luc cells preexposed to gemcitabine was significantly increased compared with that of nonpreexposed cells, but not in parallel experiments of nontransduced T cells or CD19-CAR-T cells cultured with MIA PaCa-2-luc cells, with or without gemcitabine preexposure (Fig. 4c). Combining the dose-dependent killing results of MIA PaCa-2-luc cells described in part II of the results with the findings in this section highlighted that GRP78-CAR-T cells could kill gemcitabine-resistant pancreatic cancer cells, and this killing efficacy could be enhanced when target cells were preexposed to gemcitabine.

GRP78-CAR-T cells exert a robust antitumor effect without significant toxicity in vivo

To test the tumor cytotoxic effects of GRP78-CAR-T cells against human pancreatic cancer cells *in vivo*, we established an Aspc-1-luc cell-derived xenograft mouse model. As shown in Fig. 5a, immunodeficient NCG mice housed in specific pathogen-free conditions were injected subcutaneously with Aspc-1-luc cells for seven days, and xenograft tumors developed. Next, a single tail vein injection of nontransduced T cells, CD19-CAR-T cells, or GRP78-CAR-T cells was performed, following which tumor growth was monitored for four weeks via bioluminescence imaging. With this approach, we found that tumor progression in mice treated with injections of a nontransduced T cell preparation or a CD19-CAR-T cell preparation was significant and rapid. In contrast, significant tumor regression was observed in five mice treated with injection of GRP78-CAR-T cell preparation, with the exception of one animal that displayed regression and subsequent relapse on day 20 (Fig. 5b and c). When mouse body weights were measured as a safety index, we found that the weights of mice treated with a single GRP78-CAR-T cell injection exhibited little fluctuation compared to those of mice treated with single injections of nontransduced T cells or CD19-CAR-T cells (Fig. S3). In addition to mouse body weights, we also explored the capacity for CAR-T cells to localize and infiltrate tumors, properties critical to this form of treatment for solid tumors [35]. To do this, we harvested tumor tissues from mice injected with GRP78-CAR-T cells or CD19-CAR-T cells over four days and processed these tissues for immunofluorescence staining to detect hCD3, a marker of human T cells. As shown, we found that, compared to immunostained tissues from mice injected with CD19-CAR-T cells, a significant population of GRP78-CAR-T cells infiltrated into the tumor site (Fig. 5d and e, Fig. S4a and b), indicative of tumor-targeted trafficking and enhanced intratumor antigen-dependent expansion ability of GRP78-CAR-T cells. Next, we investigated the distribution of CAR-T cells across the main organs of the mouse body and found that the blood-rich lung and spleen showed prominent CAR-T cell distribution compared to the liver and heart, but the signals were markedly lower than the levels detected in tumors (Fig. S4c and d). Furthermore, we

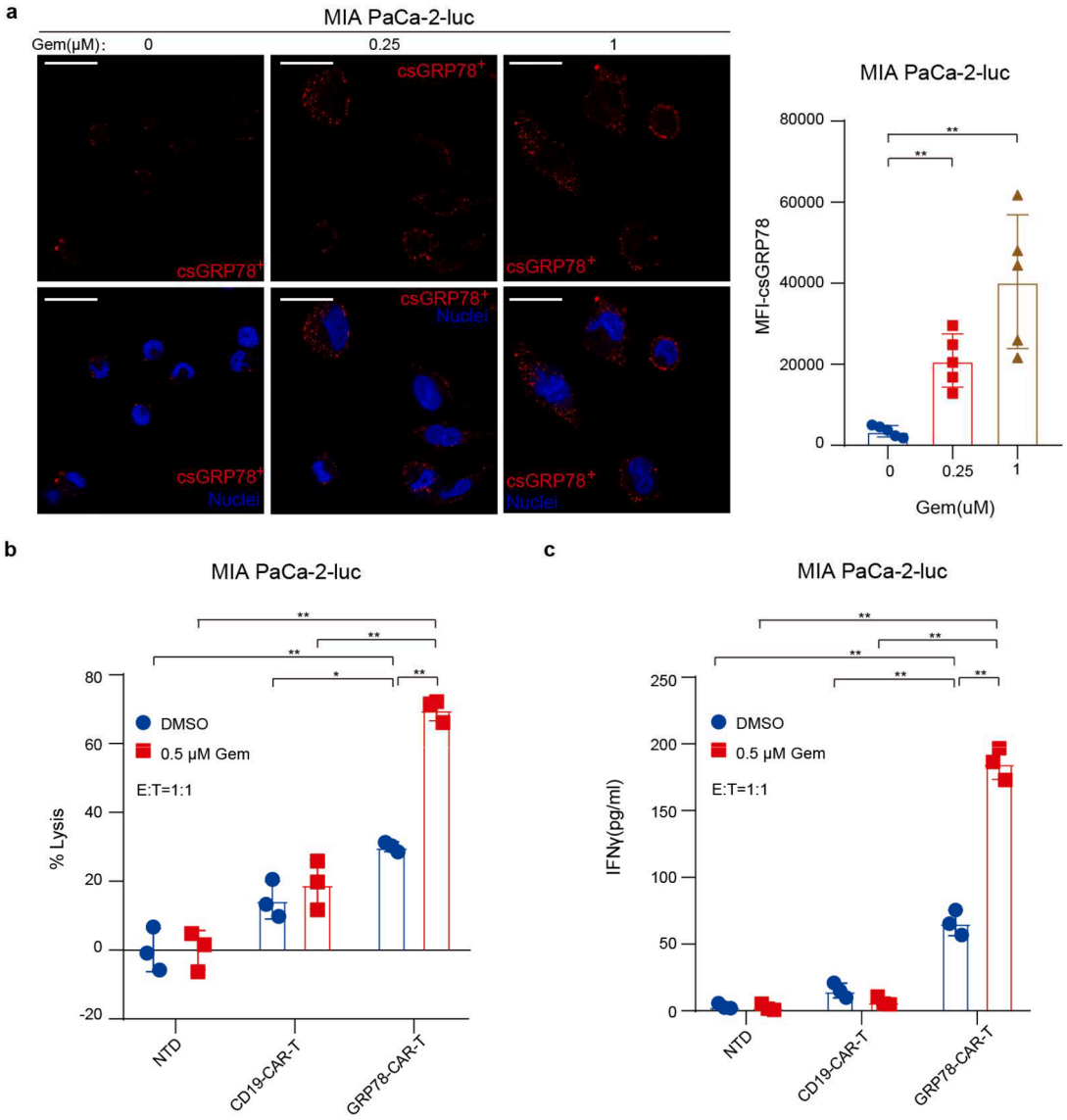

Fig. 4. Targeting csGRP78 with GRP78-CAR-T cells overcomes gemcitabine resistance in pancreatic cancer. a Representative confocal image of csGRP78 immunofluorescence signals in untreated MIA PaCa-2-luc cells and MIA PaCa-2-luc cells treated with 0.25 μM or 1 μM gemcitabine (scale bar, 20 μm) (left). The mean fluorescence intensity (MFI) of csGRP78 staining per cell for the left panel (right). b Killing efficiency of nontransduced T cells, CD19-CAR-T cells or GRP78-CAR-T cells against nontreated MIA PaCa-2-luc cells or MIA PaCa-2-luc cells treated with 0.5 μM gemcitabine at 24 h of coculture (E:T, 1:1). c Detection of IFNγ secreted in the coculture supernatant collected from the cytotoxicity assay. Data are shown as the mean ± SD. Statistical significance was determined by one-way ANOVA followed by Tukey's test. $^{**}p < 0.01$; ns, not significant.

performed imaging analysis of H&E-stained sections of tissues and found no discernible damage to the main organs of mice (Fig. S4e). Taken together, these results demonstrate that GRP78-CAR-T cells have strong antitumor efficacy against pancreatic cancer cells without significant toxicity *in vivo*.

Discussion

Pancreatic cancer is a common and devastating solid malignant tumor with limited therapeutic options. Therapy with genetically modified T cells that express chimeric antigen receptors provides a promising option for pancreatic cancer treatment. However, CAR-T cell therapy for pancreatic cancer presents various challenges, including the risk of clinically serious on-target/off-tumor toxicity owing to CAR-T cell-mediated cytotoxicity against nonmalignant tissues expressing the target antigen [36]. By choosing tumor-restricted antigens, the risk of on-target/off-tumor toxicity can be minimized. In this study, we explored whether csGRP78 could serve as a new therapeutic target for pancreatic cancer in CAR-T cell therapy.

A previous study suggested that csGRP78 expression was relevant to pancreatic cancer tissues [19]. Here, we utilized a pancreatic tumor tissue microarray to determine csGRP78 by immunofluorescence staining and demonstrated that the expression of csGRP78 is significantly increased in pancreatic cancer tissues compared with adjacent noncancerous tissues. The selective expression of csGRP78 in pancreatic tumor tissues suggests the possibility of targeting pancreatic cancer cell surface GRP78 with CAR-T cell therapy. Consistent with this speculation, significant antitumor activities of csGRP78-directed CAR-T cells against csGRP78-expressing pancreatic cancer cells were observed in this study.

Pancreatic cancer stem-like cells (PCSCs) are considered to be a major factor that underlies chemoradiotherapy failure, tumor recurrence and metastasis [37]. Therefore, targeting PCSCs may be central to an effective approach to treat pancreatic cancer. Of note, several PCSC epitopes, such as PSCA, CD276, CD133, c-Met, and CD47, have been

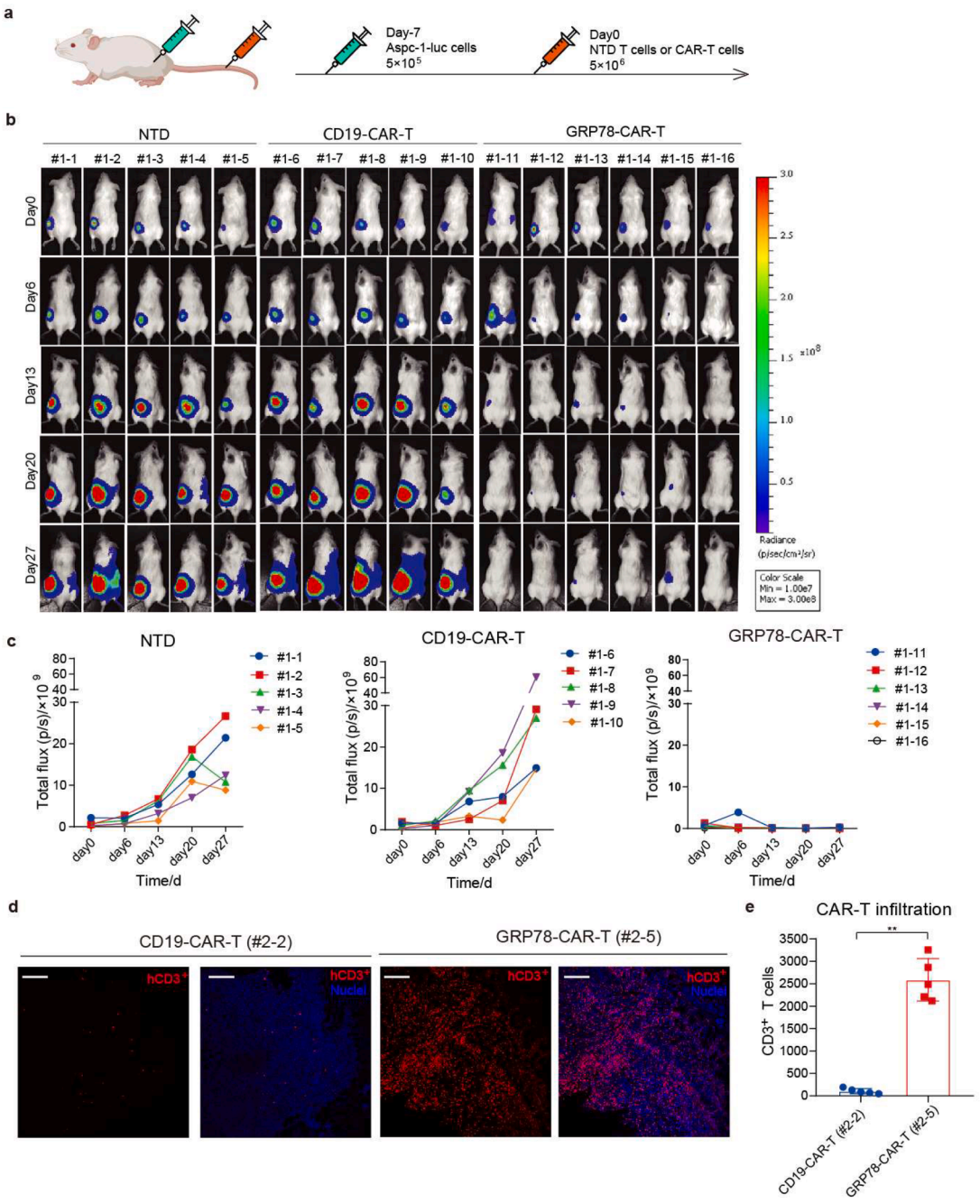

Fig. 5. GRP78-CAR-T cells block pancreatic tumor growth in an NCG xenograft model. **a** A timeline of Aspc-1-luc cell-derived xenograft model construction and CAR-T cell adoptive transfer. A preparation comprising 5×10^5 Aspc-1-luc cells was subcutaneously injected into recipient mice. After seven days, a preparation comprising 5×10^6 nontransduced T cells, CD19-CAR-T cells or GRP78-CAR-T cells was administered via the tail vein, and the experiment was designated Day 0 ($n =$ 5–6 per group). **b** Bioluminescence images of mice on the indicated days after T-cell injection. Color scale, $1 \times 10^7 \sim 3 \times 10^8$ photons/s. **c** Quantification of bioluminescence imaging for individual mice. **d** Representative images of human CD3 immunofluorescence histochemistry staining in tumors from mice treated with CD19-CAR-T cells or GRP78-CAR-T cells that exhibited T-cell infiltration into tumor sites (scale bar, 100 μm). Aspc-1-luc cell tumor-bearing mice were sacrificed 4 days after CAR-T-cell adoptive transfer. #2–2 indicates the CD19-CAR-T-cell group, and #2–5 indicates the GRP78-CAR-T-cell group. **e** The number of $hCD3^+$T cells infiltrating tumor sites (five visual fields per mouse). Data are shown as the mean ± SD. Statistical significance was determined by a two-sided independent Student's *t* test. $^{**}p < 0.01$.

used for the design of CAR-T cell therapy to target PCSCs [38]. csGRP78 expression is widely reported in various CSCs [39], yet its expression in PCSCs is unclear. Here, we demonstrated that csGRP78 is abundantly expressed in both Aspc-1-luc cell-derived PCSCs and MIA PaCa-2-luc cell-derived PCSCs. Functionally, GRP78-CAR-T cells exerted a significant cytotoxic effect on both Aspc-1-luc cell-derived PCSCs and MIA PaCa-2-luc cell-derived PCSCs. In previous reports, we and others have demonstrated that csGRP78 expression is negligible on normal hematopoietic stem cells and that GRP78-CAR-T cells have no cytotoxicity against normal hematopoietic stem cells [22,40]. Combining previous findings and our present results suggest that csGRP78 is a potentially safe antigen for CAR-T cell therapy that can be utilized to kill PCSCs.

Gemcitabine is the first-line chemotherapeutic drug used to treat pancreatic cancer patients. However, patients with pancreatic cancer frequently develop resistance to gemcitabine, and the underlying

mechanism of resistance to gemcitabine is largely unknown [33]. Curiously, abnormal csGRP78 expression is believed to be linked to the development of chemotherapy resistance in tumors [41,42], and gemcitabine induces an upregulation of csGRP78 expression in pancreatic cancer cells that leads to a reduction in its effect as a drug treatment [21]. By using the reported gemcitabine-insensitive cell lines [34], in this study, we demonstrated that csGRP78 expression in gemcitabine-treated MIA PaCa-2-luc cells was significantly increased compared with that in nontreated cells, although cellular heterogeneity was observed in gemcitabine-treated Panc-1-luc cells. Furthermore, the cytotoxic effect of GRP78-CAR-T cells was enhanced when MIA PaCa-2-luc cells were preexposed to gemcitabine. Given that GRP78-CAR-T cells themselves had a certain cytotoxicity against MIA PaCa-2-luc cells, our findings collectively suggest that GRP78-CAR-T cells alone or in combination with gemcitabine may be effective in treating gemcitabine-resistant pancreatic cancer expressing csGRP78.

GRP78 is an important modulator of ER stress, and loss of GRP78 is typically lethal to cells [40,43]. This feature of GRP78 poses a challenge for researchers to confirm the specificity of GRP78-CAR-T cells to target csGRP78 in knockout studies [40]. Therefore, we considered an alternative strategy to confirm the antigen specificity of GRP78-CAR-T cells using HEK293T cells or pancreatic cancer cells transduced with GRP78. However, using this strategy, we could not detect significantly increased csGRP78 expression, and the cytotoxic effects of GRP78-CAR-T cells on transduced cells overexpressing exogenously derived GRP78 were not significantly different from those on empty control vector-transfected cells for both HEK293T cells and pancreatic cancer cells (data not shown). As such, it remains difficult for us to validate the target specificity of GRP78-CAR-T cells by knockout or overexpression strategies, as we described above. Regardless, given that we found that GRP78-CAR-T cells are cytotoxic to pancreatic cancer cells through a mechanism that correlates with csGRP78 levels in cells and that GRP78-CAR-T cells are cytotoxic to AML cells through a mechanism involving csGRP78 expression [40], these observations together support the hypothesis that the cytotoxic actions of GRP78-CAR-T cells are linked to csGRP78 expression in tumor cells.

It is critical to understand the regulatory mechanisms of GRP78 cell surface translocation in pancreatic cancer cells for csGRP78-targeting therapies, including GRP78-CAR-T-cell therapy. However, to date, the regulatory mechanisms of GRP78 cell surface translocation are highly complex, diverse and cell-type specific, involving multiple factors, such as the capacity of the KDEL receptor of the KDEL retrieval system [44], ER stress-induced SRC activity [45], Rab4, 11 and 15 GTPases [46], and the interaction between GRP78 and its client proteins Par-4 [47] or DNAJC3 [46,48]. However, no evidence has indicated that these regulatory mechanisms apply to pancreatic cancer cells, and more work is needed to reveal the regulatory mechanisms of GRP78 cell surface translocation in pancreatic cancer cells.

Nevertheless, this study has two noteworthy limitations that should be addressed in future research. First, there is a lack of comprehensive preclinical testing in patient-derived xenograft (PDX) models due to the current unavailability of fresh pancreatic cancer tissues with different genomic backgrounds or varying sensitivity to gemcitabine. Second, the safety assessment of GRP78-CAR-T cells in rhesus macaques, whose GRP78 amino acid sequence is identical to that of humans, has not been conducted due to the inability of suitable animal subjects. Both aspects are helpful for the further clinical translation of GRP78-CAR-T cells therapy in the treatment of pancreatic cancer.

Tumor associated antigens (TAAs), are prerequisites for the application of CAR-T cells to the treatment of solid tumors. These antigens are characterized by their high expression in tumor tissue and low or absent in normal tissue [49]. In this study, we demonstrated that csGRP78 is a promising TAA for pancreatic cancer CAR-T cell therapy, which is meaningful. Tumor specific antigens (TSAs) or neoantigens, which are tumor-specific antigens derived from somatic mutations that are expressed only on tumor cells and are more immunogenic and have less off-tumor toxicity than TAAs, may represent a new approach for CAR-T cell therapy in the future [49]. With the rapid development and application of multiomics, many strategies including DNA/RNA sequencing of exome, and MHC-loaded peptide techniques, have been developed to identify tumor specific antigens (TSAs) or neoantigens [49,50]. For example, Balachandran et al. used whole-exome sequencing and in silico neoantigen prediction to identify neoantigens in pancreatic ductal adenocarcinoma and found MUC16 (also known as CA125) [51]. Researchers worldwide are trying to identify novel antigens of CAR-T cell therapy, and we believe that it will be a big help toward current cancer CAR-T cell therapy.

In conclusion, we have demonstrated that GRP78-CAR-T cells show strong cytotoxic activity against pancreatic cancer cells with high or moderate csGRP78 expression. Furthermore, GRP78-CAR-T cells alone or in combination with chemotherapy may be effective therapeutic options for gemcitabine-resistant pancreatic cancer. Given the expression of csGRP78 in various solid tumors, we speculate that GRP78-CAR-T cells could be explored as a selective and effective treatment for a broad collection of human solid cancers expressing csGRP78.

Ethics approval

All animal experiments performed in this study were approved by the Animal Ethics Committee of West China Hospital of Sichuan University (approval 20211482A) and conformed with the ethical standards of the 1975 Helsinki Declaration.

Date availability

The original data of the study are available from the corresponding authors upon reasonable request.

CRediT authorship contribution statement

Yuncang Yuan: Validation, Writing – original draft. **Jiawei Fan:** Validation. **Dandan Liang:** Validation. **Shijie Wang:** Data curation, Formal analysis. **Xu Luo:** Data curation, Formal analysis. **Yongjie Zhu:** Methodology. **Nan Liu:** Methodology. **Tingxiu Xiang:** Writing – review & editing, Conceptualization. **Xudong Zhao:** Writing – review & editing, Conceptualization, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.tranon.2023.101803.

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