CEACAM7 is an Effective Target for CAR T-cell Therapy of Pancreatic Ductal Adenocarcinoma

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ABSTRACT

Purpose: To investigate whether CEACAM7 represents a novel therapeutic target for treating pancreatic ductal adenocarcinoma (PDAC) and to generate CEACAM7-targeting CAR T cells to test this hypothesis.

Experimental Design: We identified CEACAM7 (CGM2), a member of the CEA family of proteins with expression restricted to the colon and pancreas, as a potential CAR T-cell target for PDAC. We probed a panel of PDAC tumor sections as well as patient-derived PDAC cell cultures for CEACAM7 expression. We generated CAR-targeting CEACAM7, and assessed antitumor efficacy of CEACAM7 CAR T cells using in vitro and in vivo models.

Results: We show here that CEACAM7 is expressed in a large subset of PDAC tumors, with low to undetectable expression in all normal tissues tested. CEACAM7 is also expressed in primary PDAC cultures isolated from patient-derived tumors, with high expression within the cancer stem cell-enriched subset. CAR T cells targeting CEACAM7 are capable of targeting antigen-expressing tumor cells, and mediate remission in patient-derived xenograft tumors.

Conclusions: We identify CEACAM7 as a potential therapeutic target in PDAC and describe the development of CEACAM7-targeted CAR T cells with efficacy against PDAC.

Introduction

Pancreatic ductal carcinoma (PDAC) is the fourth most common cause of deaths due to solid tumors and is characterized by a low 5-year survival rate of <5%. It is generally diagnosed at a late stage, with extensive metastases at presentation (1, 2). PDAC is largely refractory to conventional therapies, which at best extend survival by a few months and is therefore a disease of unmet need. At a molecular level, PDAC tumors are characterized by considerable genetic heterogeneity (3, 4) and the presence of a cancer stem cell (CSC) compartment (5, 6), that together contribute to disease progression and resistance to chemotherapy.

CAR T cells have shown remarkable efficacy in CD19-expressing B-cell malignancies (7, 8) but limited efficacy in solid tumors (9, 10). A major challenge in this respect is the lack of CAR T target antigens strongly expressed on malignant cells, but with expression on normal tissues sufficiently low as to preclude on-target off-tumor toxicities. The use of CAR T cells against HER2-, CAIX-, and CEACAM5-expressing tumors has been associated with severe toxicities in clinical trials due to expression of these antigens in lung and bile duct epithelium (11–13). Recent clinical trials using CAR T cells targeting antigens such as HER2 and mesothelin on PDAC have shown limited efficacy, with slight to no improvement in overall survival (14–16).

Identification of target antigens with more restricted expression in normal tissues is likely to increase the margin of safety of CAR T cells against solid tumors, allowing safe administration of higher doses of CAR T cells and potentially increasing antitumor efficacy. CEACAM7 (CGM2) is a little-studied member of the CEA family, a large group of proteins with diverse roles, and which are typically dysregulated in malignancies (17). CEACAM7 is a GPI-anchored protein with no intracellular domain and a relatively small extracellular domain (as compared with several other CEA members such as CEACAM5; ref. 18). Analysis of the N-terminal region of CEACAM7 suggests that it forms a homodimer, with a similar fold to other CEA family members, but much higher affinity within the dimer (19). The physiologic function of CEACAM7 remains unknown.

Unlike several other members of the CEA family that are expressed widely, expression of CEACAM7 is restricted to the apical surface of pancreatic ductal cells and epithelial cells of the adult colon. In fetal colon tissues, CEACAM7 is localized at the base of epithelial cells, and moves to the apical surface shortly after birth. No expression of CEACAM7 has been demonstrated in other areas of the gastrointestinal tract such as the esophagus, stomach, and small intestine, nor was expression detected in tissues such as lung and biliary system that have been previously associated with CAR T-cell toxicity (20, 21). Interestingly, CEACAM7 expression is reported to be downregulated in colorectal carcinoma as well as hyperplastic polyps and tubular adenomas, as compared with normal colon (22–24). CEACAM7 mRNA expression is, however, detectable in PDAC tumor epithelial cells at higher levels than in normal pancreas, and has been described as a potential diagnostic marker for PDAC (25); however, much heterogeneity of expression was observed within the panel of patients with PDAC examined.
**Translational Relevance**

Pancreatic ductal adenocarcinoma (PDAC) is characterized by a dismal prognosis and poor response to conventional therapies. A hurdle to CAR T-cell therapy of solid tumors is that commonly targeted tumor antigens are expressed at low levels in normal tissues, leading to on-target off-tumor toxicity. We here identify CEACAM7 as a potential target antigen for CAR T-cell therapy of PDAC. CEACAM7 has low to undetectable expression in all normal tissues tested, with strong surface expression on a subset of primary human PDAC tumors. Cell surface expression of CEACAM7 was maintained in primary cultures from patient-derived PDAC tumors, as well as xenografts generated from these PDAC cultures. CAR T cells targeting CEACAM7 were generated, and showed significant antitumor activity against patient-derived PDAC tumor cultures both in vitro and in vivo. Our data suggest that CAR T-cell therapy targeting CEACAM7 may have potential for safe and efficacious therapy of PDAC.

We therefore hypothesized that CEACAM7 may be a suitable target for CAR T-cell therapy of PDAC, and describe here the development of CEACAM7-directed CAR T cells that can efficiently target PDAC cultures in vitro and eliminate patient-derived tumor xenografts from early as well as patients with late-stage PDAC.

**Materials and Methods**

**Primary human blood and cancer-derived cells**

Blood and tumors from patients with PDAC were supplied by the Barts Pancreas Tissue Bank (www.bartspancreas.tissuebank.org.uk) and obtained with written consent from all patients. The collection of human tissue was performed under the Barts Pancreas Tissue Bank Protocol, REC reference 13/SC/0592. PDAC tumors were expanded as patient-derived xenografts (PDXs) in immunocompromised mice as described previously (26).

Several primary patient-derived PDAC cell cultures were used in this study. PDAC lines 253, 354, 247, 286, 420, A6L, and 185 were gifts from James Eshleman and Christine Iacobuzio-Donahue (Johns Hopkins University, Baltimore, MD), PDAC lines 12707, 12556, and 12560 were a gift from Aldo Scarpa (University and Hospital Trust of Verona, Verona, Italy). In addition, circulating tumor cells (CTC)-derived PDAC cell cultures c76, c102, and c139 generated in-house from patients with late stage, chemo-naïve PDAC were also used. Short tandem repeat profiling (Public Health England) of all patient-derived PDAC cell lines was performed and confirmed the uniqueness of each of these lines.

To culture spheres enriched in CSCs, cells were resuspended in 1× DMEM/F-12 (Gibco) supplemented with 20 ng/mL FGF-2 (CellGFS), 0.4% amphotericin B, 1% penicillin/streptomycin, 2% B27 supplement (Gibco), and 200 mmol/L of l-glutamine (Gibco). A cell suspension of 10,000 cells/mL was then prepared and distributed into ultralow attachment surface flasks (Corning) for 1 week. Prior to use, spheres were filtered using a 40-μm filter and washed once with cold PBS. Spheres were filtered through 40-μm filter and cell strainers. Purified 2869 antibody isolated from hybridoma supernatants was used to detect CEACAM7 expression, and cells were incubated for 1 hour at 4°C. The cells were washed again with cold PBS and incubated with Alexa Fluor 647–conjugated goat anti-mouse antibody (10337882, Thermo Fisher Scientific) used as a secondary antibody at a 1:200 dilution for 30 minutes at 4°C in the dark. Following one final wash with cold PBS, cells were resuspended in FACS buffer (1× PBS containing 3% FBS, 3-mmol/L EDTA, and 0.5 μg/mL DAPI) and analyzed by flow cytometry on a BD LSR Fortessa (BD Biosciences).

**ELISA**

The IFNγ ELISA was performed on cell-free supernatants from cytotoxicity cocultures using a kit from eBioscience (88-7316-88) according to the manufacturer’s instructions.

**Cytotoxicity assays**

Target cells (primary PDAC cultures) and effector cells (T cells transduced with either 2869 CAR, mutant CAR, or unmodified T cells as a negative control) were plated at defined effector:target (E:T) ratios in 96-well cell culture plates. Lysis of target cells was assessed by microscopy and quantitated by WST-1 viability assays, using a kit from Roche (05015944001).

**In vivo experiments**

All experiments were approved by the Animal Experimental Ethics Committee (Home Office Project License PPL P1EE3ECB4) and performed in accordance with the guidelines for Ethical Conduct in the Care and Use of Animals. Firefly luciferase-expressing PDAC cells were established by transducing cells with a PGK-GFP-IRELS–Luciferase Lentivirus vector from Addgene. Cells were sorted for GFP expression with a FACS BD Aria II instrument (BD) and subsequently expanded in vitro. Then, 50 μL of 1 × 10^5 PDAC-Luc cells were surgically injected either into the pancreas of NSG mice (NOD.Cg–Prkdcscid Il2rgtm1Wj/Lj/SzJ; Charles River) ages 6–8 weeks. To perform surgery, the mice were anesthetized with isoflurane (2%) and an incision was made into the left flank of the skin following sedation and analgesia. Then, a second incision through the peritoneum was carried out and the spleen with adjacent pancreas was visualized for the injection of PDAC cells. After closing the peritoneum with 6/0 sutures (B/Braun; 0022002), the skin was closed by surgical staples. An IVIS-200 system (PerkinElmer) was used for weekly in vivo luciferase imaging. Mice were anesthetized with isoflurane (2%) and injected intraperitoneally with 150 mg/kg of luciferin (Caliper Life Sciences) diluted 15 mg/mL in PBS. Sequential images were obtained after luciferin injection every 30 seconds (maximum light emission, ~20 minutes after luciferin injection). Luciferase activity is in photons per second per square centimeter per steradian (p/s cm^-2 sr^-1).

**IHC**

IHC staining was carried out on 2.5-μm sections of PDAC and normal tissues on a tissue microarray, using the Ventana Discovery XT system (Roche). Sections were deparaffinized, hydrated, and loaded on the Discovery XT. Antigen retrieval was performed using citrate buffer solution. Endogenous peroxidases were quenched using H2O2 reagent (Ventana). The BAC2 mA be has previously been used to specifically
detect CEACAM7 by immunostaining (20). The BAC2 primary antibody (Santa Cruz Biotechnology, sc-59946; 1:1,000) was therefore applied, incubated for 20 minutes, and detected using an anti-mouse secondary antibody and the ChromaMap DAB detection kit (Ventana). Tissues were counterstained with hematoxylin, and image analysis was performed using Panoramic Viewer Software (3DHISTECH).

**Sequences of 2869 constructs**

The protein sequence of the 2869 scFv-Fc is as follows:

MALPVTTALLPLLLHAAARPDKDDDKGGGSGGGGSQ-VQLQKQQLQGPVQLQSLTLICTYSGFSSLGGHVRQSPGKGE-LWGLVWTTGGMNTDFNAAFSDLTTKIDNSKQVFFKMNLEPD-DTIAYCAKHLYNSIHFMDYWGQGTSVISVSSEKGGGSSSGGGGGGGG-SSGGGSEILQSPTLTTLVPGDSVSLRCRASISNNLHNWyQQYK-SHEPSRLLIKYSIQYSGTSPRSFGISGTDIIRLSNVEGMDLYFCQSH- NWPHTFGATKLEKGGGSGGGGSQVLQKQQLQGPGL-YQPSQSLTICTYSFSSLGGHVRQSPGKGE-LWGLVWTTGGMNTDFNAAFSDLTTKIDNSKQVFFKMNLEPD-DTIAYCAKHLYNSIHFMDYWGQGTSVISVSSEKGGGSSSGGGGGGGG-SSGGGSEILQSPTLTTLVPGDSVSLRCRASISNNLHNWyQQYK-SHEPSRLLIKYSIQYSGTSPRSFGISGTDIIRLSNVEGMDLYFCQSH-

The protein sequence of the 2869 (with CD8a hinge) CAR is as follows:

MALPVTTALLPLLLHAAARPDKDDDKGGGSGGGGSQ-VQLQKQQLQGPVQLQSLTLICTYSGFSSLGGHVRQSPGKGE-LWGLVWTTGGMNTDFNAAFSDLTTKIDNSKQVFFKMNLEPD-DTIAYCAKHLYNSIHFMDYWGQGTSVISVSSEKGGGSSSGGGGGGGG-SSGGGSEILQSPTLTTLVPGDSVSLRCRASISNNLHNWyQQYK-SHEPSRLLIKYSIQYSGTSPRSFGISGTDIIRLSNVEGMDLYFCQSH-

**Results**

CEACAM7–directed CAR T-cell Therapy of Pancreatic Cancer

To assess patterns of CEACAM7 protein expression in PDAC, immunostaining was performed on a panel of human PDAC tumor sections. The BAC2 mAb showed specific binding to CEACAM7 and showed no reactivity against other CEA family members (20), and was therefore used for all immunostaining work. CEACAM7 expression was detectable with higher to intermediate expression in 19 of 30 tumor sections, and low to undetectable in 11 of 30 sections. Expression was clearly visualized in the epithelial component of PDAC tumors, with no staining of the stroma or the blood vessels (Fig. 1A; Supplementary Fig. S1A).

Normal human pancreas and colon are the only tissues where CEACAM7 expression has been demonstrated (20, 21). To assess CEACAM7 expression in normal tissues, immunostaining was performed on a panel of normal tissues from the same tissue microarray as the PDAC sections. Expression was undetectable in pancreas or colon under the same staining conditions that had been used for PDAC sections. Expression was therefore used in all normal sections including tonsil, lung, liver, and prostate, consistent with previous reports (Fig. 1B; Supplementary Fig. S1B). These data do not rule out CEACAM7 expression in pancreas and colon, but suggest significantly lower antigen expression as compared with PDAC, suggesting that upregulation of CEACAM7 protein in PDAC may occur at the posttranscriptional level.

Patient-derived PDAC tumor cultures, unlike cell lines, may retain properties of the original tumor such as genetic heterogeneity and antigen expression that is not an artefact of long-term culture (27). We therefore used qRT-PCR to assay expression of CEACAM7 mRNA at 1,000 \( \times \) g for 2 hours at 32°C. The 2869 and mutant CAR expression level was determined by flow cytometry with Alexa Fluor 488–labeled anti-FLAG antibody. CAR T cells were then expanded in culture with the same concentration of IL2, and used for in vitro and in vivo work between 5 and 14 days after T-cell isolation.

### Statistical analysis

Unless stated otherwise, results are expressed as the mean ± SD. Statistical analyses were performed using GraphPad Prism 7, two variables were analyzed using Student's t test, and three or more variables were analyzed using one-way ANOVA with Bonferroni correction. For statistical analyses of tumor progression (Fig. 5C), a log-rank test was used with \( \chi^2 = 4.2 \) on 1 degree of freedom, with data plotted ± SEM (\( n = 5 \)).

### Patient consent

Blood and tumors samples were obtained with written consent under the Barts Pancreas Tissue Bank Protocol, REC reference 13/SC/0952, and RAS project ID 142061.

### Ethics approval

All experiments were approved by the Animal Experimental Ethics Committee (Home Office Project License PPL P1EE3ECB4) and performed in accordance with the guidelines for Ethical Conduct in the Care and Use of Animals.

### Patients and tumor culture

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Figure 1.
Expression of CEACAM7 in PDAC. A panel of PDAC tumor sections (A) and normal human tissues (B) were stained with the CEACAM7-specific BAC2 antibody (scale = 50μm). C, qRT-PCR to determine CEACAM7 mRNA expression in adherent and sphere cultures of primary PDAC, as well as non-PDAC cultures and human pancreas (n = 2). β-actin mRNA expression was used as a control. HPDE, human pancreas-derived epithelial cells. WBC, white blood cells. D, qRT-PCR to determine CEACAM7 mRNA expression in a panel of normal tissues from three different human donors (n = 2). β-actin mRNA expression was used as a control. E, Representative flow cytometry using 2869 hybridoma supernatants on adherent and sphere cultures derived from primary PDAC cell lines from stage I-II patients (left) or patients with late-stage metastatic disease (right). Values are shown as fold increase in median fluorescence intensity (MFI) compared with unstained controls.
transcript in a panel of primary cultures from patients with stage I–II PDAC. CEACAM7 transcript was found to be upregulated in three of seven primary PDAC cultures tested, with low expression in human pancreas, and no detectable expression in blood cells nor in non-PDAC cell types (Fig. 1C). The CSC compartment of PDAC has been shown in several studies to drive chemoresistance, metastasis, disease progression, and relapse and any therapy developed against PDAC must target this subset of cells for long-lasting disease correction (28, 29). We therefore generated nonadherent sphere cultures from all the primary PDAC cell cultures to enrich the CSC component (30). Significantly, CEACAM7 transcript expression was consistently raised in CSC-enriched sphere cultures as compared with differentiated adherent cultures, suggesting potential to target the key CSC compartment (Fig. 1C).

To further investigate levels of CEACAM7 expression in normal tissues, CEACAM7 transcript expression was analyzed by qRT-PCR in a panel of normal tissues from three human donors, including kidney, lung, skeletal and cardiac muscle, visceral and subcutaneous fat, liver, brain, ovary, and breast. No detectable expression was observed in any of these tissues, as compared with primary PDAC cultures (Fig. 1D). Expression patterns of CEACAM7 transcript were therefore found to be consistent with the immunostaining results as well as with previous studies analyzing patterns of CEACAM7 expression.

We then investigated whether CEACAM7 transcript correlated with cell surface expression of CEACAM7 protein in PDAC. Using primary cultures from stage I–II patients with local disease, or blood-derived CTCs from patients with late-stage metastatic disease, adherent populations and CSC-enriched sphere cultures were generated. The 2869 hybridoma was previously raised against CEACAM7, and flow cytometry using 2869 hybridoma supernatants showed high CEACAM7 expression in one of three metastatic and two of five stage I-II PDAC cultures. Consistent with the qRT-PCR data, CEACAM7 expression was consistently raised in spheres as compared with adherent cultures, highlighting potential to target the CSC compartment (Fig. 1E; Supplementary Fig. S2A). 2869 binding was undetectable in non-PDAC cell types such as human PBMCs, human fibroblasts (HFF-1), and embryonic kidney cells (HEK293T; Supplementary Fig. S2B). Like several members of the CEA family, CEACAM7 has no ortholog in the mouse. Consistent with this, no 2869 binding was observed on two mouse PDAC lines tested (Supplementary Fig. S2C).

Generation of 2869-derived CAR T cells

On the basis of these results, we further investigated the specificity of the 2869 hybridoma against CEACAM7. The 354 and c102 PDAC cultures, which showed undetectable CEACAM7 expression when grown as adherent cultures (Fig. 1E), were modified to express GFP and luciferase (354GL and c102GL) to allow tracking in vitro and in vivo. To assess specificity of 2869 antibody to CEACAM7 protein, 354GL and c102GL cells were then further modified to stably express CEACAM7. Western blotting using 2869 hybridoma supernatants showed strong bands of expected size in modified cells, showing successful ectopic expression of CEACAM7, with stronger expression in c102GL than in 354GL (Fig. 2A). Flow cytometry of both cell types showed binding of 2869 hybridoma supernatants specifically in CEACAM7-modified cells, suggesting successful membrane localization of ectopic CEACAM7 (Fig. 2B). Consistent with Western blotting data, stronger expression of CEACAM7 was observed on modified c102GL than on 354GL.

To assess suitability of this hybridoma for CAR development, an scFv was generated on the basis of the variable regions of the heavy and light chains of the 2869 antibody, and expressed in frame with a human IgG1-derived Fc fragment to enable detection with an anti-Fc antibody (Fig. 2C). The 2869 scFv-Fc fusion was transfected into 293T cells, and supernatants containing the secreted protein were used for flow cytometry on modified and unmodified 354GL. As expected, the 2869 scFv-Fc showed a similar binding pattern to 2869 hybridoma supernatants, with specific binding to cells ectopically expressing CEACAM7, demonstrating the potential of the 2869 scFv for CEACAM7-targeted CAR development (Fig. 2D).

Second-generation chimeric antigen receptors were then constructed by fusing the 2869 scFv to a hinge domain and a CD8α transmembrane domains, in frame with the CD137 (4-1BB) costimulatory domain and the CD3ζ activation domain. Several studies have shown that the distance of the epitope-binding domain from the T-cell surface can significantly impact CAR T-cell function (31, 32). We therefore designed two CARs with either a 45 amino acid CD8α-derived hinge or a 12 amino acid IgG4-derived hinge (Fig. 2E). Both CARs could be efficiently transduced into primary human T cells with expression ranging between 40% and 90%, though the smaller CAR (IgG4 hinge) consistently showed higher expression, consistent with previous studies showing that lentiviral transduction efficiency may be enhanced by smaller constructs (ref. 33; Fig. 2F). T-cell proliferation was not affected by expression of CAR, nor was there any detectable change in the phenotypic profile of T cells with either CAR, as compared with unmodified T cells (Supplementary Fig. S3A and S3B). Specific activity of 2869 CAR T cells against CEACAM7-expressing targets

The specificity and efficacy of 2869 CAR against CEACAM7-expressing targets was then investigated. Ectopic CEACAM7-expressing 354GL and c102GL cells were used as CAR T-cell targets, with unmodified cells as a negative control. T cells that were unmodified, or modified to express either one of the 2869 CARs, were overlaid on a monolayer of PDAC target cells at a 5:1 E:T ratio. Viability of the target cells was initially assessed by GFP expression. Lysis of CEACAM7-expressing target PDAC cells, but not unmodified cells, was observed following coculture with either of the 2869 CAR T-cell types (Fig. 3A and B). Quantitation of target cell viability using WST-1 showed that neither of the 2869 CAR T-cell types had any effect on unmodified PDAC cells, but decreased viability of CEACAM7-expressing targets when applied both at 5:1 and 1:1 E:T ratios. Cytotoxic killing appeared to be antigen density dependent, with higher cytotoxicity observed against c102GL than 354GL, consistent with higher CEACAM7 expression on c102GL cells. The smaller 2869 CAR (IgG4 hinge) had a slight but significantly higher cytotoxic efficacy when CAR T cells were applied at the 5:1 E:T ratio on either target cell type (Fig. 3C and D). 2869 CAR T cells can therefore specifically target CEACAM7-expressing cells, with no effect on cells that do not express the target antigen.

Besides enrichment of the CSC compartment, sphere cultures are also characterized by a dense extracellular matrix that may serve as a barrier to effector cells (34). Cytotoxic assays against sphere cultures may therefore be informative in (i) CAR T-cell targeting of the CSC compartment and (ii) ability of T cells to penetrate a barrier that recapitulates the physical features of the tumor microenvironment (TME). Sphere cultures of 354GL and c102GL cells with or without CEACAM7 expression were therefore seeded and allowed to form for 7 days, following which effector T cells were added at a 5:1 E:T ratio. Upon coculture of spheres with T cells expressing either 2869 CAR, no effect was seen on unmodified spheres, but complete lysis of CEACAM7-expressing spheres was observed. As with adherent targets, cytotoxic killing was density dependent, with more efficient clearance...
of CEACAM7-expressing c102GL than 354GL spheres (Fig. 3E and F). 2869 CAR T cells can therefore target CEACAM7-expressing PDAC sphere cultures, under in vitro conditions that enrich CSC and recapitulate a stromal barrier.

To compare CAR T-cell activation broadly across both target cell types under both adherent and sphere conditions, parallel cocultures were set up with CEACAM7-expressing and unmodified c102GL and 354GL target cells cultured as both adherent and spheres, cocultured with effector T cells at E:T ratios of 5:1 and 1:1. Supernatants from each individual coculture were assayed for IFNγ secretion. Significantly higher secretion of IFNγ was observed from T cells expressing either 2869 CAR when expressed with CEACAM7-expressing targets, but
Figure 3.

In vitro efficacy of 2869 CAR T cells. Microscopy analysis of GFP-expressing 354GL (A) and c102GL (B), expressing adherent cultures, either unmodified or ectopically expressing CEACAM7, following 24 hours of culture with untransduced T cells or 2869 CAR T cells at a 5:1 E:T ratio (scale = 40 μm). (Continued on the following page.)
not with unmodified target cells. Similar patterns of IFNγ secretion were observed upon coculture with either sphere or adherent targets, while unmodified T cells showed no IFNγ secretion under any condition (Fig. 3G and H). These data suggest that 2869 CAR T cells are activated specifically and effectively by target antigen in the context of either adherent or sphere culture.

To assess whether CAR T activity was sustained in vivo, we orthotopically implanted 354GL cells (with and without ectopic CEACAM7) to generate a xenograft model of stage I/II PDAC. The tumors engrafted and expanded rapidly, and were treated with two doses of 2869 CAR T cells at days 6 and 13 posttumor implantation (Fig. 4A). Rapid tumor progression continued in the 354GL cohort, with all mice reaching endpoints due to tumor burden by day 23. Significant tumor regression was seen in the 354GL+CEACAM7 cohort, with complete tumor clearance in four of five animals (Fig. 4B and C). Nevertheless, all animals in this cohort succumbed to graft vs. host disease (GvHD), and were euthanized by day 38 (Fig. 4D).

We wished to investigate the efficacy of 2869 CAR T cells against larger, more established tumors. We therefore established a second xenograft model with 354GL+CEACAM7 cells as before, and monitored tumor growth in vivo. When tumors had reached a size approaching the permissible endpoint, we administered two doses of 2869 CAR T cells at days 13 and 20 (Fig. 4E). All animals in the 354GL cohort succumbed rapidly to progressive disease, while tumor stabilization and significantly extended survival were observed in four of five animals in the 354GL+CEACAM7 cohort (Fig. 4F and G). These data demonstrate the antigen-specific in vivo activity of 2869 CAR T cells against xenograft models generated from patient-derived primary PDAC cultures.

**2869 CAR T cells target endogenously expressed CEACAM7**

The patient-derived PDAC lines c76 and 253 cells express moderate levels of CEACAM7 when grown as adherent cultures, though at much lower levels than sphere cultures (Fig. 1E). Adherent cultures of c76 and 253 were therefore used to assess activity of 2869 CAR against endogenously expressed CEACAM7. As the smaller 2869 CAR (lgG4 hinge) showed higher activity against CEACAM7-expressing targets (Fig. 3C), this CAR was used in all subsequent experiments. 2869 CAR T cells mediated lysis of monoclonal cultures of c76 and 253 cells, while unmodified T cells had no effect (Fig. 5A). Quantitation of target cell viability by WST-1 revealed lysis of both target cell types by 2869 CAR T cells in an effector cell dose-dependent manner, with higher cytotoxicity observed at E:T ratios 1:1 and 2:1 as compared with E:T 1:1 (Fig. 5B). Consistent with target cell lysis, raised IFNγ secretion was observed upon coculture of 2869 CAR T cells with target PDAC (Fig. 5C).

The 2869 CAR contains a 4-1BB costimulatory domain, which has been associated with antigen-independent tonic signaling in previous studies (35). We wished to investigate whether cytotoxicity observed against c76 targets was specifically driven by 2869 CAR engagement with target antigen, rather than nonspecific stimulation of T cells driven by antigen-independent signaling. A mutant CAR was therefore constructed with similar intracellular costimulation and activation domains, but lacking the scFv epitope-binding domain (Fig. 5D).

Mutant and 2869 CAR T cells were generated and used in cytotoxicity assays with c76 targets at different E:T ratios. In all instances, coculture with 2869 CAR T cells led to significantly diminished viability of PDAC c76 targets compared with mutant CAR T cells (Fig. 4F). Finally, cytotoxicity using PDAC 253 targets showed significant target lysis by 2869 CAR T cells compared with mutant CAR T cells, demonstrating specific targeting of CEACAM7 by 2869 CAR (Fig. 5F).

We next investigated patterns of CEACAM7 expression in more established cell lines. A panel of six PDAC lines showed 2869 binding to all cell types, at similar levels to PDAC c76 cultures, though at a lower level compared with ectopic CEACAM7 expression on modified 354GL cells (Fig. 6A and B). CEACAM7 is therefore expressed in established PDAC cell lines as well as in primary PDAC cultures, at similar levels on the cell surface.

To further validate the specificity of 2869 CAR, we prepared cocultures of 354GL+CEACAM7 with mutant CAR T cells or 2869 CAR T cells at an E:T ratio of 3:1. Microscopy as well as WST-1 quantitation of target cell viability confirmed specific targeting of CEACAM7-expressing targets by 2869 CAR T cells, while mutant CAR T cells had no effect on antigen-expressing or -nonexpressing cell lines (Fig. 6C). We performed cytotoxicity assays on all six PDAC lines using 2869 CAR T effectors as well as mutant CAR T cells as a control. At E:T ratios of 3:1 and 1:1, significant cytotoxicity was observed on all PDAC lines cocultured with 2869 CAR T cells. Cytotoxicity was comparable with that observed on primary PDAC cultures, but lower than on ectopically expressing 354GL+CEACAM7 cultures (Fig. 6D and E). These data further demonstrate the specific activity of 2869 CAR T cells against CEACAM7-expressing cultures; nevertheless effector activity appears to be dependent on antigen expression, with low levels of endogenously expressed CEACAM7 (BxPC-3, PANC-1) being more refractory to cytotoxicity than higher levels of endogenously (AsPC-1, CFPAC-1) or ectopically expressed antigen (354GL+CEACAM7).

**In vivo activity of 2869 CAR T cells against CEACAM7-expressing xenografts**

PDAC c76GL cells, grown as adherent cultures, were implanted orthotopically into NSG mice. This cell culture, derived from the blood of a patient with PDAC with metastatic late-stage disease, was chosen for its aggressiveness, rapid growth, and extensive metastases (26). The tumors were monitored until they reached a size of >0.5 cm³, and detectable metastases had developed, to accurately model late-stage disease presentation in human patients. At this stage, the animals were administered a single dose of 5 × 10⁶ mutant CAR T cells or 2869 CAR T cells (Fig. 7A). In compliance with the 3Rs (UK Home Office Regulations), the animals were euthanized under any of the following conditions: upon tumor size exceeding 1.4 cm³, development of ascites, loss of >20% body weight, or any physical signs of distress or pain. Continued progression of tumors was observed in five of five animals treated with mutant CAR T cells but only in two of five animals that received 2869 CAR T cells. Complete regression of primary tumors and liver metastases was observed in the responding animals in the 2869 CAR T-cell cohort (P = 0.04; Fig. 7B and C). Of
Figure 4. 

In vivo efficacy of 2869 CAR T cells. 

A, Early treatment model: PDAC 354GL (with or without ectopic CEACAM7) were orthotopically engrafted into NSG mice. A total of $5 \times 10^6$ 2869 CAR T cells were administered via tail vein 6 days and 13 days later. IVIS bioluminescence imaging at various time points (B), IVIS quantitation of tumors across both cohorts (C), and survival curves (D) are shown ($n = 5$; *, $P < 0.05$). 

E, Late treatment model: PDAC 354GL (with or without ectopic CEACAM7) were orthotopically engrafted into NSG mice. A total of $5 \times 10^6$ 2869 CAR T cells were administered via tail vein 13 and 20 days later. IVIS bioluminescence imaging at various time points (F), IVIS quantitation of tumors across both cohorts (G), and survival curves (H) are shown ($n = 5$; *, $P < 0.05$).
note, overall survival of the 2869 CAR T–treated cohort was significantly improved over the mutant CAR T–treated cohort (mean survival 58.6 days vs. 27.4 days; median survival 61 days vs. 29 days; \( P < 0.05 \)).

The three surviving mice in the 2869 CAR cohort began to exhibit GvHD symptoms including weight loss, reduced mobility, and hunched posture, and were therefore euthanized 61 days and 84 days post–T-cell administration (Fig. 7D). Gross morphologic examination confirmed the absence of tumor. These results therefore demonstrate that 2869 CAR T cells are capable of mediating complete regression of large tumors and metastases in an aggressive and metastatic xenograft model with presentation similar to late-stage human disease.

**Discussion**

The most successful and well-understood example of targeting tissue-specific antigens is CD19 CAR T-cell therapy of B-cell–derived hematologic malignancies (36, 37). CAR T-cell therapy in this context generally leads to B-cell aplasia that is generally treated by intravenous immunoglobulin infusions, but is not directly associated with toxicity against any other tissue due to B cell–specific expression of CD19 (38). Other B-cell antigens, such as ROR1, are more widely expressed in tissues throughout the body (39), and targeting these antigens may place an upper limit on the maximally tolerated CAR T-cell dose.

More recently, development of CAR T cells targeting claudin18.2 has been described for preclinical therapy of a gastric carcinoma model. Claudin18.2 is expressed in the stomach epithelium, with low to undetectable expression elsewhere in the body. Gastric carcinoma xenographs were also shown to express high levels of antigen, and CAR-T therapy directed against claudin18.2 successfully mediated gastric carcinoma regression. No toxicity to any normal tissue including, surprisingly, the stomach, was...
Figure 6.
2869 CAR T cells target CEACAM7-expressing established PDAC lines. A, Flow cytometry showing 2869 binding on PDAC c76 as well as six established PDAC lines (B) quantified by mean fluorescence intensity (MFI) normalized to the unstained control. C, PDAC 354GL (with or without ectopic CEACAM7) were coincubated with mutant CAR T cells or 2869 CAR T cells. Microscopy (left) and WST-1 assay (right) show specific targeting of CEACAM7-expressing 354GL by 2869 CAR T cells at E:T::3:1 (n = 3; * P < 0.05). WST-1 quantitation of cytotoxicity of six different established PDAC cell lines following co-culture with either mutant or 2869 CAR T cells at E:T ratio of 3:1 (D) or 1:1 (E; n = 3; *, * P < 0.05; **, * P < 0.01; ***, * P < 0.001).
observed in any of the animals (40). This study highlights the utility of targeting a tumor antigen expressed solely in the tissue of tumor origin.

CEACAM7 is a little-studied protein, not implicated in cancer besides being downregulated in colorectal carcinomas (22–24). Previous studies have shown CEACAM7 to have a very restricted tissue expression as compared with other CEA members, with expression detected only in pancreas and colon (20). In a study that searched for diagnostic markers for PDAC in cellular material from pancreatic juice, CEACAM7 mRNA expression was found to be significantly upregulated as compared with healthy controls, suggesting tumor upregulation of CEACAM7 (25). To our knowledge, CEACAM7 protein expression in PDAC has not previously been studied.

We therefore analyzed CEACAM7 protein expression on a panel of PDAC tumor sections as well as normal tissues on a tissue microarray. We found robust staining of CEACAM7 on the majority of PDAC tumors, with expression restricted to the apical surface of epithelial cells, typical of expression patterns of other CEA proteins (41, 42). CEACAM7 expression was not observed on
any normal tissue tested, including pancreas and colon. It is important to note that while pancreas and colon may express CEACAM7 at basal levels, expression in PDAC is substantially upregulated. CEACAM7 expression was observed on the cell surface of a subset of primary PDAC cultures, and consistently higher expression was observed on CSC-enriched sphere cultures. These data, showing (i) surface expression of CEACAM7 on PDAC, (ii) enhanced expression on CSC-enriched PDAC cultures, and (iii) lack of expression in all normal tissues tested, suggest that CEACAM7 would be an ideal target for CAR T-cell development against PDAC.

We constructed a CAR to target CEACAM7 based on the sequence of a hybridoma raised against this antigen. PDAC cultures ectopically expressing CEACAM7 could be specifically targeted using CAR T cells, with unmodified PDAC cells serving as a negative control. Endogenous CEACAM7 expression in most PDAC cultures were, however, far lower, but CAR T cell-mediated cytotoxicity was none the less observed. The costimulatory domain 4-1BB within a CAR has previously been associated with nonspecific and antigen-independent signaling in CAR T cells (35), and we speculated whether this phenomenon may drive nonspecific target cell killing within our in vitro model. To control for this effect, we generated a mutant CAR with similar intracellular domains as the 2869 CAR (4-1BB costimulation and CD3ζ activation domains), but lacking a tumor antigen-binding domain. Using this negative control, CEACAM7-directed CAR T cells killing of PDAC targets was confirmed both in differentiated PDAC as well as CSC-enriched sphere cells.

For in vivo modelling of CEACAM7 CAR T-cell activity, we chose a model that would recapitulate the most aggressive features of human PDAC disease. We have previously shown that PDAC c76 cells, isolated from a patient with PDAC with late-stage metastatic disease, form extremely aggressive and metastatic tumors in immunodeficient animals (26). We therefore generated xenograft models of PDAC c76 in NSG mice and allowed tumors to progress with extensive metastases; thus, recapitulating the common clinical presentation of late-stage PDAC (43). Following a single dose of 2869 CAR T cells, complete tumor regression was observed in three of five animals. CEACAM7 expression in c76 xenografts was previously observed to be modest, similar to differentiated c76 cells in culture, and considerably lower than CSC-enriched sphere cultures (Supplementary Fig. S4A). This modest expression, as compared with HER2 in similar xenograft models (26), may have precluded a more complete response across the cohort.

As in previous studies, we have observed a strong GvHD effect in a subset of animals following CAR T-cell administration (26, 44). NSG (NOD.Cg-PrkdcscidIl2rgtm1Wjl/Sj) mice are useful models for CAR-T study due to the efficient engraftment of human T cells as well as primary tumors, the latter also retaining their unique characteristics such as rapid invasiveness and propensity for metastases (26). This strain is therefore commonly used as a model for GvHD, which in this system is mediated by human T-cell receptor (TCR) targeting mouse tissues; consequently NSG animals lacking MHC class I and class II are resistant to GvHD following human T-cell administration (45). Xenogenic GvHD following CAR T-cell administration to NSG is therefore likely to be a side effect of TCR signaling rather than CAR activity, and may be an artefact of these animal models, rather than reflect potentially debilitating clinical phenomena such as cytokine release syndrome observed in patients.

The efficacy of CAR T-cell targeting of tumor antigen is determined by a number of factors, including (i) antigen density on tumor cells and (ii) binding affinity of CAR to antigen. It has been observed that increased antigen density is associated with more efficient killing (46), but the relationship of CAR-antigen binding affinity to CAR efficacy is more complex, with extremes of both low- and high-affinity target binding associated with diminished efficacy (47, 48). It is also important to note that xenograft models of human PDAC in immunodeficient animals do not model the immunosuppressive TME. Clinical use of CAR T cells against PDAC may therefore need to be complemented with agents that target the TME.

In summary, we have identified CEACAM7 as a novel target antigen for CAR T-cell therapy of a sizeable fraction of PDAC. We have developed a CAR that can specifically and effectively target CEACAM7-expressing PDAC cells, and we demonstrate that CEACAM7-directed CAR T cells can effectively mediate remission of late-stage patient-derived PDAC xenograft tumors. Because of its restricted tissue expression, CEACAM7-directed CAR T cells may offer a higher safety margin than commonly used targets such as CEACAM5 and HER2 with more broad systemic expression (11, 13).

Authors' Disclosures

H.M. Kocher reports grants from Celgene, Mylan, Medtronic, and Oncosil outside the submitted work. No disclosures were reported by the other authors.

Authors' Contributions

B. Raj: Data curation, formal analysis, investigation, writing-original draft.
X. Duan: Resources. B.J. Nelson: Resources. N.R. Lemoine: Funding acquisition, writing-review and editing. J.F. Marshall: Supervision, funding acquisition, writing-original draft, project administration, writing-review and editing.

Acknowledgments

Research was supported by a Pancreatic Cancer UK Grand Challenge award to Nicholas Lemoine, John F Marshall, John Maher, and Yaohe Wang. Barts Tissue Bank (BTBP) is funded by the Pancreatic Cancer Research Fund. Funding for antibody production was generously provided to Brad H. Nelson by the Canary Foundation of British Columbia Cancer Foundation. Cancer Research UK funded the Barts Cancer Institute Core Facilities (C16420/A18066). We are grateful to patients with pancreatic cancer who donated tissues to the BTBP (www.bartstissuebank.org.uk). We also thank Claude Chelala, Archana Ambily, Thomas Dowey, and Dayem Ullah and the Tissue Access Committee, key members of BTBP who contributed to this study. We are grateful to James Eshleman and Christine Iacobuzio-Donahue (Johns Hopkins University, Baltimore, MD) for the gift of the PDAC lines 253, 354, 247, 286, 420, A6L, and 185, to Aldo Scarpa (University and Hospital Trust of Verona, Verona, Italy) for the gift of PDAC lines 12707, 12556, and 12560, and to Irene Esposito (University of Düsseldorf, Düsseldorf, Germany) for tissue samples. We are grateful to George Elia for expert assistance with IHC, and to Cancer Research UK funded Barts Cancer Institute Core Facilities (C16420/A18066). The project concept was started in C. Heeschen's laboratory when an employee of QMUL.

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Received July 1, 2019; revised November 13, 2020; accepted December 21, 2020; published first January 20, 2021.
References


30. Nollau P, Prall F, Helmchen U, Wagner C, Neumaier M. Dysregulation of carcinoembryonic antigen family members CGM2, CDE6a (biliary glycoprotein), and nonspecific cross-reacting antigen in colorectal carcinomas. Com-...


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Clin Cancer Res  Published OnlineFirst January 21, 2021.

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Access the most recent version of this article at:
doi:10.1158/1078-0432.CCR-19-2163

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