SUPPLEMENTARY FILE

Proteomics

Chemicals and reagents

Unless otherwise stated, all materials were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Sample preparation

Sub-confluent CFPAC-1 cells were washed twice in PBS, pelleted and solubilized in a solution containing 9 M urea, 4% CHAPS, 1 mM Na_3VO_4, 80 mM DTT, protease inhibitors and nuclease. Samples were incubated overnight at 4°C, and then centrifuged at 13,800 x g for 10 min at 4°C. Clear supernatants were removed, quantified with DC protein assay kit and stored at -20°C until analysis.

2-Dimensional Electrophoresis

Proteins (200 µg per sample) were loaded on ready-made IPG strips (7-cm IPG strips, pH 3-10NL) (Bio-Rad, Hercules, CA, USA). Focusing was started at 250 V, and the voltage was progressively increased to 4000 V, until a maximum of 25000 V-h was reached. The second dimension was run on 10% acrylamide gels (Bio-Rad), and preparative gels were stained with colloidal Coomassie (18% v/v ethanol, 15% w/v ammonium sulfate, 2% v/v phosphoric acid, 0.2% w/v Coomassie G-250) for 48 h.

SERological Proteome Analysis (SERPA).

2DE gels were transferred to nitrocellulose membranes (GE Healthcare, Milan, Italy). Membranes were blocked with 5% w/v nonfat dry milk in PBST (PBS supplemented with polyoxyethylene sorbitan monolaurate [Tween-20] 0.1% v/v) for 1 h, and then probed

with a 1:1000 dilution of human serum overnight at 4°C. Membranes were washed with PBST, incubated for 1 h with a 1:1000 dilution of anti-human IgG horseradish peroxidase (HRP) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and immunoreactivity was detected with an enhanced chemiluminescence kit (Pierce, Rockford, IL, USA).

Image analysis

Images were acquired using ChemiDoc (Biorad, Hercules, CA, USA). Densitometric analyses were performed using ImageJ software and PD-Quest software (version 7.2, Bio-Rad) according to the manufacturer's instructions.

Protein identification by mass spectrometry and database searches

Coomassie G-stained spot digestion, MALDI-TOF analysis and database searches were performed as previously described (Mandili G et al, J Hepatol, 2015).

SERPA Computational analysis

Densitometric values from SERPA experiments were used to quantify the recognition level of antigens in patients before and after CT. The ratios between SERPA values measured in each CT round, and values calculated before CT, were graphically represented as heat maps. The ratios were $\log_{10}$ transformed and used in a hierarchical clustering analysis to identify clusters of patients sharing an antigen recognition profile.

Computational analysis of gene-set enrichment

The gene locus coding for the antigens analyzed was defined using bioDBnet (Kuleshov MV et al, Nucleic Acid Res, 2016). Gene-set enrichment analysis was performed using the Enrichr web tool (Kuleshov MV et al, Nucleic Acid Res, 2016). Only the first ten ontological terms associated with a p-value < 0.001 were considered. Gene

expression analysis was performed by downloading TCGA expression data from the Genomic Data Commons (GDC) Data Portal. RNA-Seq data (FPKM) of 177 PDA samples from the TCGA-PAAD cohort were log_{10}-transformed and represented as a heat map. All the heat maps presented in this article were generated using the heatmap.2 R function, and all the clustering analyses were performed using the Ward.2 method.

ELISA protocols

Human recombinant proteins of TAA were coated onto flat-bottomed plates and incubated overnight at 4°C, with the following concentration FUBP1: 0.5 µg/ml, G3P: 1 µg/ml, K2C8: 3 µg/ml, ENO1 2 µg/ml, all in 0.1 mol/l Na_2CO_3 (ENO1 from Sigma, Merck Life Science, Milan, Italy, FUBP1, G3P, K2C8 from Tema Ricerca). Sera were diluted at 1:1000 in PBS containing 1% bovine serum albumin (BSA) and 0.05% Tween-20. Anti-human HRP (GE Healthcare) diluted at 1:3000 was then added followed by tetramethylbenzidine (TMB) (Tebu Bio, Magenta, Italy) incubation. Appropriate positive controls were included for each protein. Mouse serum samples were diluted at 1:50 in PBS followed by incubation with anti-mouse HRP (GE Healthcare) diluted at 1:2000 and TMB (Tebu Bio). The DELTA values of both human and mouse sera were calculated by subtracting the optical density of coated wells from the uncoated wells to account for background signal.

T cell proliferation 3H-TdR incorporation

At day 4, 1 µCi of 3H-TdR 20 Ci/mMole (PerkinElmer, Milan, Italy) was added to each well and incubated for 18 h. Cells were collected with a cell harvester (Packard Instruments, Meriden, CT) in UniFilter plates (PerkinElmer). After adding MicroScint fluid (PerkinElmer), the 3H-TdR uptake was quantitated (TopCount microplates scintillation counter; Packard).

Flow Cytometer analysis

CFPAC-1 and CAPAN-2 cell lines were surface stained with anti-CD55 antibody (Biolegend, Campoverde, Milan, Italy) or mouse IgG1 matched control antibody (Biolegend). T cell surface was stained with anti CD4-PercP and CD8-APC. After fixation and permeabilization with Fix/Perm Buffer Set; cells were stained intracellularly with anti-FoxP3-FITC. All antibodies and FoxP3 Fix/Perm Buffer Set were from BioLegend. Acquisition of samples was carried out by Accuri C6 BD (Milan, Italy) and data was analyzed by FCS express (De Novo software, CA, USA).

ENO1 DNA vaccination, gemcitabine treatment and schedule for mice depletion

Mice

Mice carrying single-mutated KrasG12D (C57BL/6;129SvJae H-2b) under the endogenous promoter and flanked by Lox-STOP-Lox cassettes were obtained from Dr. David Tuveson (Cancer Research UK, Cambridge Research Institute). C57BL/6 mice expressing Cre recombinase under the promoter of a specific pancreatic transcriptional factor, namely pancreatic and duodenum homeobox 1 (Pdx-1) were obtained from Dr. Andrew Lowy (University of San Diego, San Diego, CA). Mice were bred and maintained under saprophytic and pathogen-free conditions at the animal facilities of the Molecular Biotechnology Center (Turin) and treated in accordance with EU and institutional guidelines. Mice were screened by the polymerase chain reaction (PCR) using tail DNA amplified by specific primers to the Lox-P cassette flanking Kras and wild-type Kras genes. PCR products were separated on 1.5% agarose gels with GelRed (Biotum by SIC, Rome, Italy) and recorded as .tiff images.

DNA vaccination

KC mice anesthetized with Zoletil (Rompun) and Xylazine were injected into the femoral muscle with 50 µg of ENO1 plasmid in 40 µl of sterile water with 0.9% NaCl, and immediately followed by two 25-ms pulses of 375 V/cm applied with a Cliniporator and linear needle electrodes (IGEA, Carpi, Italy). Human ENO1 complementary DNA was obtained as previously described (Cappello P et al, Gastroenterology, 2013).

Tumor lesion evaluation and immunohistochemistry staining

Pancreases of KC mice were formalin-fixed and paraffin-embedded, and 4 µ-thick slices were stained with hematoxylin and eosin; tissues were scanned and the mean diameter and the number of all neoplastic lesions from PanIN to invasive PDA were analyzed using NDP view2 (Hamamatsu, Shizuoka, Japan). For immunohistochemistry staining, peroxidase activity was inhibited by a 3% hydrogen peroxide aqueous solution for 10 min. Samples were pre-treated by microwave antigen retrieval using EDTA buffer (required for CD4 and CD8), citrate pH 6 (Dako, Milan, Italy) (for ENO1) or protease (Ventana, Segrate, Italy) at 37°C, and incubated with CD4 (Abcam, Cambridge, UK) (diluted at 1:1000), CD8 (Affimetrix eBioscience, Milan, Italy) (diluted at 1:3000), ENO1 (Sigma-Aldrich) (diluted at 1:100) or F4/80 (AbD Serotec, Milan, Italy) (diluted at 1:100) antibodies for 30 to 48 min at room temperature. The rabbit EnVision system (Dako), anti-mouse-HRP (GE Healthcare) or biotinylated goat anti-rat (Bio-Rad) followed by HRP-conjugated streptavidin (Millipore, Milan, Italy), were used before diaminobenzidine tetrahydrochloride (Dako) incubation. Negative and positive controls were included to set up the staining protocol. All slides were stained for the same antigen, together with the same antigen-retrieval buffer and antibody dilution. Tissues were examined in a double-blind fashion, and digital images of representative areas were taken. The presence of positive cells in the tumor area of the pancreatic tissues sections (n=6) was counted and classified as absent (score 0.2, 0 positive cells), scarce (score 0.4, 1-10 positive cells),

moderate (score 0.6, 10-50 positive cells), strong (score 0.8, 50-100 positive cells), or huge (score 1, >100 positive cells) on sections stained with the different antibodies.

Enzyme-Linked Immunosorbent Spot Assay (ELISpot)

IFN-γ production from stimulated splenocytes was evaluated with a mouse IFN-γ ELISPOT kit (BD Becton Dickinson, Milan, Italy), and the substrate 3-Amino-9-ethylcarbazole (Sigma, Merck Life Science, Milan, Italy), following the manufacturer's instructions. Images of the wells were acquired and spots were quantified with the microplate reader, along with a computer-assisted image analysis system (Immunospot; CTL Europe, Bonn, Germany).

Statistical Analysis

Data from densitometry analyses were used as values of protein differential expression or recognition in SERPA experiments. Differential expression experiments of untreated CFPAC-1 cells, or treated with GEM were performed in triplicate; to verify statistical significance, the two-sided Student's t test was used. Proteins were classified as differentially expressed or recognized if the ratio in spot intensity between treated cells and control cells (in the expression analysis) or between patient's sera before and after CT (in the SERPA analysis) was greater than 1.5-fold (over-expressed or over-recognized protein), or lower than 0.5-fold (under-expressed or under-recognized protein). Overall survival (OS) was calculated starting from the date of diagnosis to the date of death from any cause, or the date of the last follow-up. OS was estimated by the Kaplan–Meier product-limit method and compared between groups using the log-rank test (STATA version 13.1, StataCorp LP, TX, USA). Differences in LDH release were evaluated by Wilcoxon matched-pairs signed rank test. The Student's t test was used to evaluate statistical differences in ELISA, FACS analysis, immunohistochemical staining and

ELISpot; correlation between the infiltration of immune cells was performed using the Spearman test (GraphPad Prism 7, San Diego CA).